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ARTICLES

Platelet-Derived Growth Factor-BB Phosphorylates Heat Shock Protein 27 in Cardiac Myocytes

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Abstract It is recognized that heat shock protein 27 (HSP27) is highly expressed in heart. In the present study, we investigated whether platelet-derived growth factor (PDGF) phosphorylates HSP27 in mouse myocytes, and the mechanism underlying the HSP27 phosphorylation. Administration of PDGF-BB induced the phosphorylation of HSP27 at Ser-15 and -85 in mouse cardiac muscle in vivo. In primary cultured myocytes, PDGF-BB time dependently phosphorylated HSP27 at Ser-15 and -85. PDGF-BB stimulated the phosphorylation of p44/p42 mitogen-activated protein (MAP) kinase, p38 MAP kinase, and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) among the MAP kinase superfamily. SB203580, a specific inhibitor of p38 MAP kinase, reduced the PDGF-BB-stimulated phosphorylation of HSP27 at both Ser-15 and -85, and phosphorylation of p38 MAP kinase. However, PD98059, a specific inhibitor of MEK, or SP600125, a specific inhibitor of SAPK/JNK, failed to affect the HSP27 phosphorylation. These results strongly suggest that PDGF-BB phosphorylates HSP27 at Ser-15 and -85 via p38 MAP kinase in cardiac myocytes. J. Cell. Biochem. 91: 316–324, 2004.

Key words: HSP27; PDGF-BB; phosphorylation; MAP kinase; cardiac myocytes

It is generally known that heat shock proteins (HSPs) are produced by cells when exposed to the biological stress such as heat and chemical stress [Benjamin and McMillan, 1998]. HSPs are classified into high-molecular-weight HSPs and low-molecular-weight HSPs with molecular masses from 10 kDa to 30 kDa based on apparent molecular sizes. It is well known that the high-molecular-weight HSPs such as HSP70 and HSP90 act as molecular chaperones in protein folding, oligomerization, and translocation [Benjamin and McMillan, 1998]. Though the functions of the low-molecular-weight HSPs such as HSP27 and αB-crystallin 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]. Low-molecular-weight HSPs have significant similarities in terms of amino acid sequences. In a previous study [Inaguma et al., 1993], we have shown that HSP27 is present in various tissues and cells, especially in skeletal muscle and smooth muscle cells. HSP27 is constitutively expressed in several unstressed cells including myocytes where it may have essential functions [Inaguma et al., 1993]. In addition, it is well known that HSP27 is regulated by post-translational modification such as phosphorylation [Welch, 1985; Benjamin and McMillan, 1998]. It has been reported that mouse HSP27 is phosphorylated at two sites (Ser-15 and -85) of serine [Benjamin and McMillan, 1998]. However, the exact role and regulatory mechanism of HSP27 in heart remain to be clarified.

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Platelet-derived growth factor (PDGF) is a potent mitogen for connective tissue cells and certain other cells [Heldin and Westermark, 1999]. In cardiovascular system, it has been shown that PDGF-BB but not PDGF-AA or PDGF-AB among isoforms of PDGF decreases systolic blood pressure in rats [Ikeda et al., 1997]. PDGF-BB reportedly increases myocardial production of vascular endothelial growth factor [Affleck et al., 2002]. In addition, it has recently been reported that the rate of myocardial development of embryonic heart is increased by PDGF-BB [Price et al., 2003]. However, the role of PDGF in heart has not been precisely elucidated.

In the present study, we investigated whether PDGF-BB stimulates the phosphorylation of HSP27 in cardiac myocytes and the mechanism behind the phosphorylation. We here report that PDGF-BB stimulates the phosphorylation of HSP27 at two serine sites via p38 mitogen-activated protein (MAP) kinase-dependent manner among the MAP kinase superfamily in these cells.

MATERIALS AND METHODS

Materials

PDGF-BB was obtained from Invitrogen Co. (Carlsbad, CA). PD98059, SB203580, and SP600125 were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). Phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) antibodies, and SAPK/JNK antibodies were obtained from Cell Signaling Technology (Beverly, MA). An ECL Western blotting detection system was obtained from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. PD98059, SB203580, and SP600125 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. In brief, apical halves of cardiac ventricles from 1- to 2-day-old mice were separated, minced, and dispersed with 80 U/ml collagenase IV and 0.6 mg/ml pancreatin. The myocytes were then incubated on uncoated 90-mm-diameter dishes for 30 min to remove any nonmyocytes, and the nonattached viable cells were seeded into gelatin-coated 60-mm-diameter dishes (2×10^6) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. 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.

Infusion of PDGF-BB In Vivo and Immunohistochemical Observation

Mice were divided into two groups, a control group ($n = 3$) and a group treated with PDGF-BB ($n = 3$) at a dose of 0.1 U/kg. Mice were placed in a supine position under anesthesia with an intraperitoneal injection of pentobarbital at a dose of 50 mg/kg. Catheters (Natume Co. Ltd., Tokyo, Japan) were connected to the left femoral vein for the injection of either PDGF-BB or saline and to the right femoral artery for monitoring blood pressure and pulse rate using a pressure transducer (AP601G Nihon Koden, Tokyo, Japan), respectively. PDGF-BB at a dose of 50 ng/ml was intravenously injected as a bolus via a right jugular vein. After the injection of PDGF-BB or saline, animals were sacrificed by over dose of pentobarbital and then heart of each mouse was collected. Samples were excised in saline and frozen. These sections were first incubated with preimmune for 60 min. HSP27, phospho-HSP27 (15S), and phospho-HSP27 (85S) were immunostained with each affinity-purified antibody and goat antibodies against rabbit IgG conjugated to peroxidase. Sections were examined after development of color with diaminobenzidine in 10 mM Tris/Cl, pH 7.6. The background was stained with hematoxylin.

Western Blot Analysis

Cultured cells were stimulated by PDGF-BB 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 each MAP kinase by Western blotting. SDS–PAGE was performed by the method of Laemmli in 10% polyacrylamide gel [Laemmli, 1970]. Western blot analysis was performed as described previously [Kato et al., 1996], using HSP27 antibodies, phospho-specific HSP27 antibodies, p38 MAP kinase antibodies, and phospho-specific p38 MAP kinase antibodies, with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second 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 PD98059, SB203580, and SP600125 for 60 min prior to stimulation by PDGF-BB. The densitometric analysis was performed using Molecular Analyst for Macintosh (Bio-Rad, Hercules, CA). The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between pairs, a $P < 0.05$ was considered significant.

Immunochemical Reagents

Antibodies specifically recognizing phosphorylated serine residues at Ser-15 and -85 in HSP27 were prepared as previously described [Kato et al., 2002]. HSP27 was purified fromskeletal muscle as previously described [Inaguma et al., 1993].

RESULTS

Effect of PDGF-BB on the Phosphorylation of HSP27 In Vivo

It has been shown that HSP27 is highly expressed in unstressed myocytes [Van de Klundert et al., 1998]. We found that levels of total HSP27 did not change at each time point in mouse heart stimulated by PDGF-BB in vivo (Fig. 1). PDGF-BB (50 ng/ml) markedly stimulated the phosphorylations of HSP27 at Ser-15 (15S) and Ser-85 (85S) at 30 min after the PDGF-BB administration in mouse heart in vivo (Fig. 1). Ninety minutes after the administration, the levels of the phosphorylation of HSP27 at Ser-15 (15S) or Ser-85 (85S) decreased to the control levels (Fig. 1).

Effect of PDGF-BB on the Phosphorylation of HSP27 in Cardiac Myocytes

It has been shown that mouse HSP27 is phosphorylated at two sites (Ser-15 and -85) of serine [Gaestel et al., 1991]. PDGF-BB (50 ng/ml) time dependently stimulated the phosphorylations of HSP27 at Ser-15 (15S) and Ser-85 (85S) in HSP27 in cardiac myocytes (Fig. 2). The maximum effect of PDGF-BB on the HSP27 phosphorylation at Ser-15 was observed at 20 min after the stimulation. In addition, the phosphorylation at Ser-85 in HSP27 reached the maximum at 20 min.

Effects of PDGF-BB on Phosphorylation of p44/p42 MAP Kinase, p38 MAP Kinase, and SAPK/JNK in Cardiac Myocytes

In order to clarify whether or not PDGF-BB activates the MAP kinase superfamily in cardiac myocytes, we examined the effects of PDGF-BB on the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, or SAPK/JNK. PDGF-BB induced the significant phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK (Fig. 3). The phosphorylations of p44/p42 MAP kinase and p38 MAP kinase reached the peak at 10 min after the stimulation of PDGF-BB (Fig. 3). The maximum effect of PDGF-BB on SAPK/JNK was observed at 20 min.

Effects of PD98059 on the PDGF-BB-Induced Phosphorylation of HSP27 and p44/p42 MAP Kinase in Cardiac Myocytes

It has been shown that HSP27 phosphorylation is catalyzed by the MAP kinase superfamily [Benjamin and McMillan, 1998]. We examined the effect of PD98059, a specific inhibitor of the upstream kinase that activates p44/p42 MAP kinase [Alesso et al., 1995], on the PDGF-induced phosphorylation of HSP27. However, PD98059 failed to suppress the PDGF-stimulated phosphorylations of HSP27 at both Ser-15 and -85 (Fig. 4).

In order to clarify whether PD98059 inhibited the PDGF-BB-stimulated activation of p44/p42 MAP kinase in cardiac myocytes, we examined the effect of PD98059 on the PDGF-BB-induced phosphorylation of p44/p42 MAP kinase. The PDGF-BB-induced phosphorylation of p44/p42 MAP kinase was truly attenuated by PD98059 in myocytes (Fig. 5).

Effects of SB203580 on the PDGF-BB-Induced Phosphorylation of HSP27 and p38 MAP Kinase in Cardiac Myocytes

We next examined the effect of SB203580, a specific inhibitor of p38 MAP kinase [Cuenda et al., 1995], on the PDGF-BB-induced phosphorylation of HSP27. SB203580 markedly

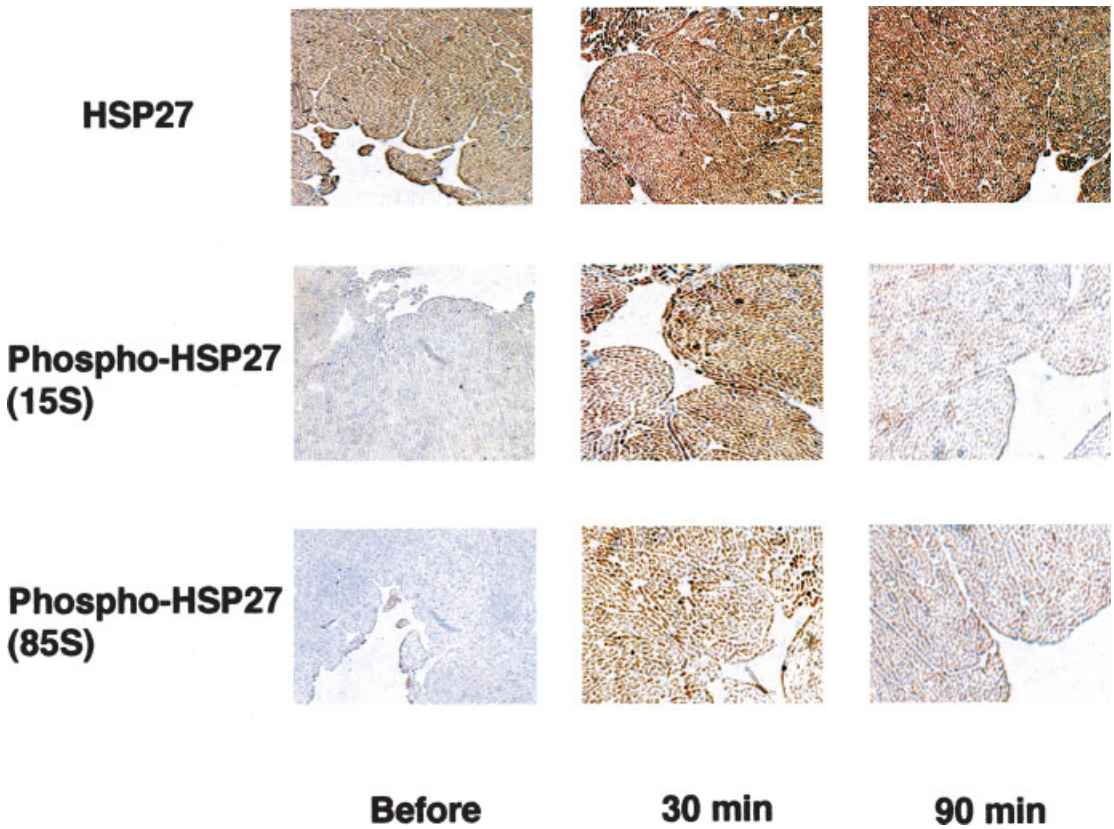

Fig. 1. Effects of platelet-derived growth factor (PDGF)-BB on the phosphorylation of heat shock protein 27 (HSP27) at serine residues (Ser-15 and -85) in mouse heart in vivo. PDGF-BB at a dose of 50 ng/ml was intravenously injected as a bolus via a right jugular vein. After the indicated periods, heart of each mouse was collected. The extracts of samples were analyzed using antibodies against HSP27 and phosphorylated Ser-15 (15S), Ser-85 (85S) in HSP27. Similar results were obtained with two additional and different preparations.

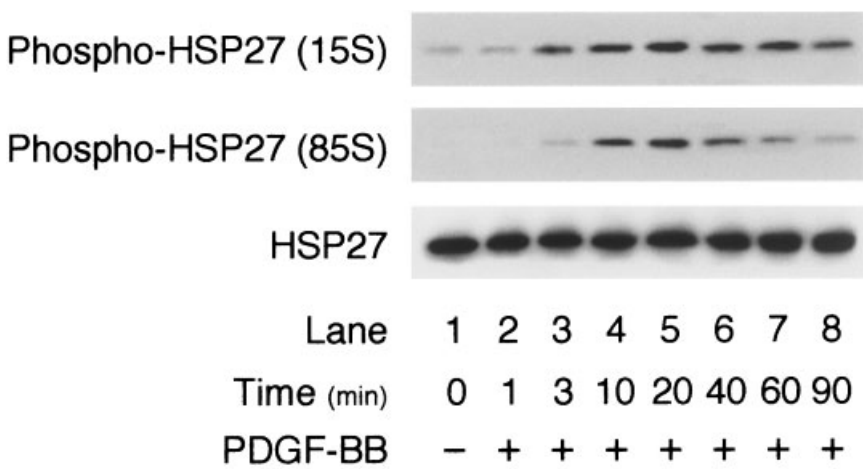

Fig. 2. Effects of PDGF-BB on the phosphorylation of HSP27 at serine residues (Ser-15 and -85) in cardiac myocytes. The cultured cells were stimulated by 50 ng/ml PDGF-BB for the indicated periods. The extracts of cells were subjected to SDS–PAGE using antibodies against phosphorylated Ser-15 (15S), Ser-85 (85S) in HSP27. Similar results were obtained with two additional and different cell preparations.

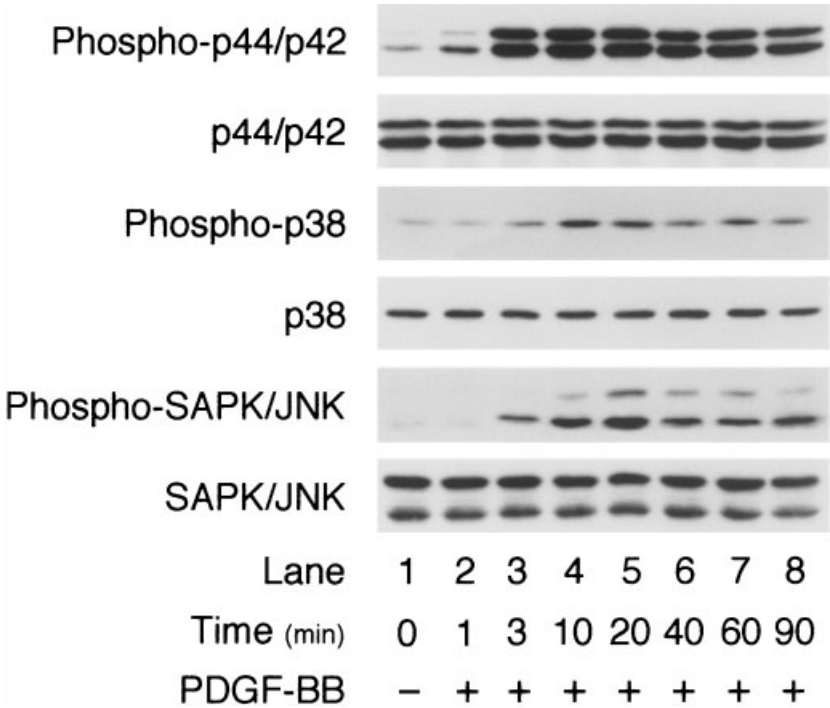

Fig. 3. Effects of PDGF-BB on the phosphorylation of p44/p42 mitogen-activated protein (MAP) kinase, p38 MAP kinase and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in cardiac myocytes. The cultured cells were stimulated by 50 ng/ml PDGF-BB for the indicated periods. The extracts of cells were subjected to SDS–PAGE against phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies, and SAPK/JNK antibodies. Similar results were obtained with two additional and different cell preparations.

suppressed the PDGF-BB-stimulated phosphorylations of HSP27 at both Ser-15 and -85 (Fig. 6). We found that SB203580 significantly reduced the PDGF-BB-induced phosphorylation of p38 MAP kinase in these cells (Fig. 7).

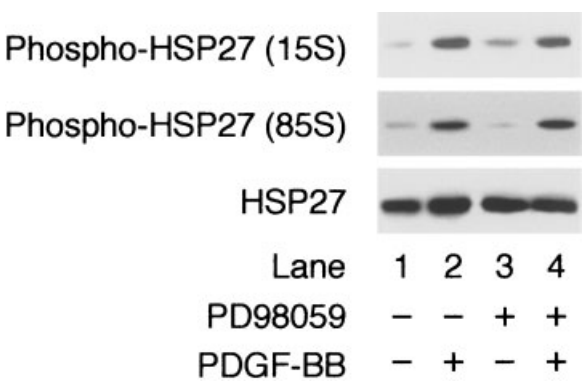

Fig. 4. Effect of PD98059 on the PDGF-BB-induced phosphorylation of HSP27 at serine residues (Ser-15 and -85) in cardiac myocytes. The cells were pretreated with 50 μM PD98059 or vehicle for 60 min, and then stimulated by 50 ng/ml PDGF-BB or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE using antibodies against phosphorylated Ser-15 (15S), Ser-85 (85S) in HSP27. Similar results were obtained with two additional and different cell preparations.

Effects of SP600125 on the PDGF-BB-Induced Phosphorylation of HSP27 and SAPK/JNK in Cardiac Myocytes

In addition, the effect of SP600125, a specific inhibitor of SAPK/JNK [Bennett et al., 2001], on the PDGF-BB-induced phosphorylation of HSP27 was investigated. SP600125 did not affect the PDGF-BB-stimulated phosphorylations of HSP27 at both Ser-15 and -85 (Fig. 8).

We examined the effect of SP600125 on the PDGF-BB-induced phosphorylation of SAPK/JNK. The PDGF-BB-induced phosphorylation of SAPK/JNK was truly reduced by SP600125 (Fig. 9).

DISCUSSION

In the present study, we demonstrated that PDGF-BB stimulated the phosphorylations of HSP27 at two sites (Ser-15 and -85) of serine in primary cultured mouse cardiac myocytes. In addition, we found that HSP27 in mouse heart muscle was truly phosphorylated by administration of PDGF-BB in vivo. It is currently known that the MAP kinase activation is

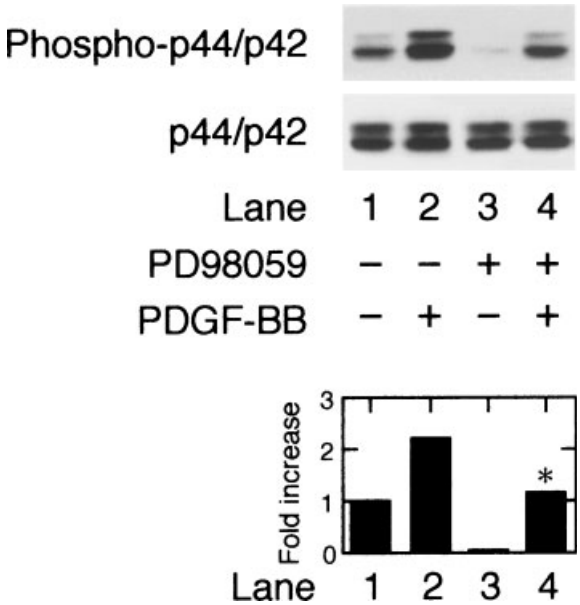

Fig. 5. Effect of PD98059 on the phosphorylation of p44/p42 MAP kinase induced by PDGF-BB in cardiac myocytes. The cultured cells were pretreated with 50 μM PD98059 or vehicle for 60 min, and then stimulated by 50 ng/ml PDGF-BB or vehicle for 10 min. The extracts of cells were subjected to SDS–PAGE against phospho-specific p44/p42 MAP kinase antibodies or p44/p42 MAP kinase antibodies. Similar results were obtained with two additional and different cell preparations. $^*P < 0.05$, compared with the value of PDGF-BB alone.

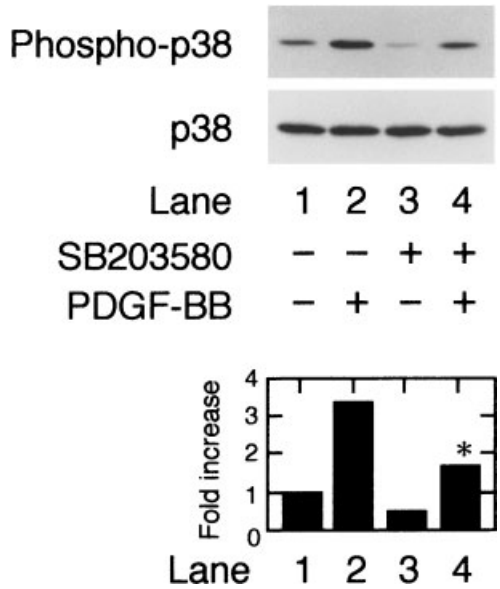

Fig. 7. Effect of SB203580 on the phosphorylation of p38 MAP kinase induced by PDGF-BB in cardiac myocytes. The cultured cells were pretreated with 30 μM SB203580 or vehicle for 60 min, and then stimulated by 50 ng/ml PDGF-BB or vehicle for 10 min. The extracts of cells were subjected to SDS–PAGE against phospho-specific p38 MAP kinase antibodies or p38 MAP kinase antibodies. Similar results were obtained with two additional and different cell preparations. $^*P < 0.05$, compared with the value of PDGF-BB alone.

involved in HSP27 phosphorylation in several types of cells [Welch, 1985; Benjamin and McMillan, 1998]. The MAP kinase superfamily plays a crucial role in intracellular signal transduction pathways initiated by a variety of extracellular stimuli [Widmann et al., 1999]. It is recognized that specificity of the cellular response is determined by the activation of a particular MAP kinase pathway in response to a given stimulus. Three MAP kinases, p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK, are generally recognized as being the central

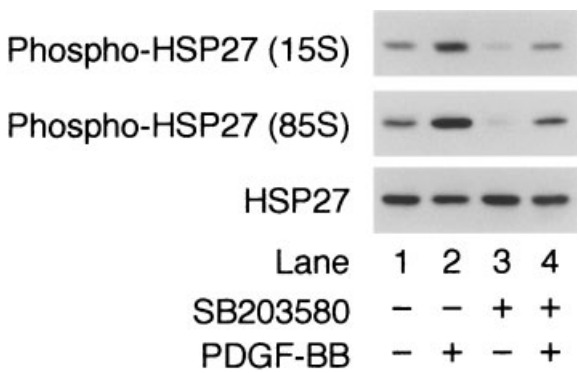

Fig. 6. Effect of SB203580 on the PDGF-BB-induced phosphorylation of HSP27 at serine residues (Ser-15 and -85) in cardiac myocytes. The cells were pretreated with 30 μM SB203580 or vehicle for 60 min, and then stimulated by 50 ng/ml PDGF-BB or vehicle for 10 min. The extracts of cells were subjected to SDS–PAGE using antibodies against phosphorylated Ser-15 (15S), Ser-85 (85S) in HSP27. Similar results were obtained with two additional and different cell preparations.

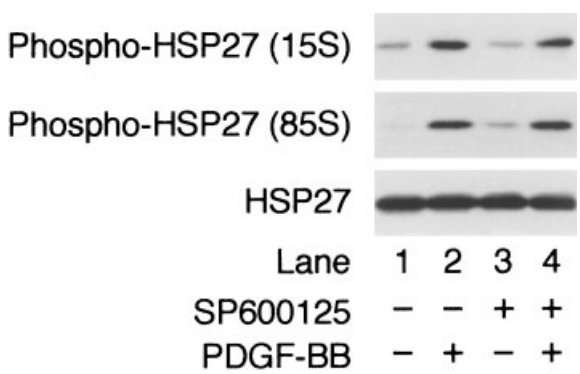

Fig. 8. Effect of SP600125 on the PDGF-BB-induced phosphorylation of HSP27 at serine residues (Ser-15 and -85) in cardiac myocytes. The cells were pretreated with 10 μM SP600125 or vehicle for 60 min, and then stimulated by 50 ng/ml PDGF-BB or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE using antibodies against phosphorylated Ser-15 (15S), Ser-85 (85S) in HSP27. Similar results were obtained with two additional and different cell preparations.

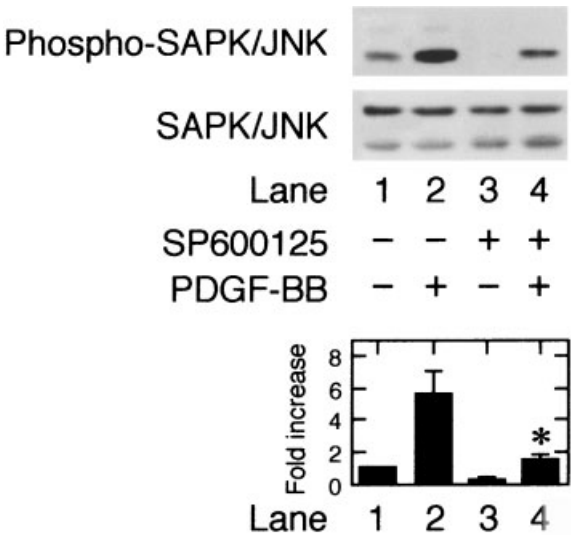

Fig. 9. Effect of SP600125 on the phosphorylation of SAPK/JNK induced by PDGF-BB in cardiac myocytes. The cultured cells were pretreated with 10 μM SP600125 or vehicle for 60 min, and then stimulated by 50 ng/ml PDGF-BB or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE against phospho-specific SAPK/JNK antibodies or SAPK/JNK antibodies. Similar results were obtained with two additional and different cell preparations. $^*P < 0.05$, compared with the value of PDGF-BB alone.

elements used by mammalian cells to transduce such diverse messages [Widmann et al., 1999]. We next investigated the mechanism behind the PDGF-BB-induced phosphorylation of HSP27 in cardiac myocytes. In the present study, we showed that PDGF-BB induced the phosphorylations of three MAP kinases 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; Widmann et al., 1999]. Taking our findings into account, it is probable that PDGF activates p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK in cardiac myocytes.

We next investigated the involvement of three MAP kinases in the PDGF-BB-induced HSP27 phosphorylation in cardiac myocytes. PD98059 [Alesso et al., 1995] and SP600125 [Bennett et al., 2001] had no effects on the HSP27 phosphorylation stimulated by PDGF-BB. We found that the PDGF-induced phosphorylations of p44/p42 MAP kinase and SAPK/JNK were attenuated by PD98059 and SP600125, respectively. Therefore, it seems unlikely that p44/p42 MAP kinase and SAPK/JNK are involved in the PDGF-induced HSP27 phosphorylation at two sites in cardiac myocytes. On the other hand, we showed that the PDGF-BB-induced HSP27 phosphorylations at two sites were markedly suppressed by a specific p38 MAP kinase inhibitor, SB203580 [Cuenda et al., 1995], which reduced the phosphorylation of p38 MAP kinase stimulated by PDGF-BB. Based on these results, it is most likely that PDGF-BB stimulates HSP27 phosphorylation at two sites (Ser-15 and -85) via p38 MAP kinase in ventricular myocytes. It has previously been reported that hypoxia induces HSP27 phosphorylation in cardiac myocytes [Kacimi et al., 2000]. To the best of our knowledge, this is probably the first study showing that as a physiological agonist, PDGF phosphorylates HSP27 at two sites of serine in cardiac myocytes.

HSP27 is present at two forms, an aggregated form and a dissociated small form in unstressed cells [Benjamin and McMillan, 1998]. It has been shown that HSP27 is constitutively expressed at high levels in various tissues and cells, especially in skeletal muscle cells and smooth muscle cells including cardiomyocytes [van de Klundert et al., 1998]. It is generally recognized that post-translational modifications such as phosphorylation are crucial regulators of its functions [Benjamin and McMillan, 1998]. We have previously demonstrated that HSP27 is dissociated concomitantly with the phosphorylation of the aggregated form of HSP27 and that dephosphorylation of the dissociated HSP27 causes aggregation [Kato et al., 1994]. Additionally, we have reported that conversion of the non-phosphorylated, aggregated form of HSP27 to the phosphorylated, dissociated form results in decreased tolerance to heat stress [Kato et al., 1994]. Accumulating evidence suggests 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]. In human neutrophils, it has recently been reported that Akt in addition to p38 MAP kinase-activated protein kinase-2 phosphorylates HSP27, and HSP27 regulates apoptosis through Akt activity [Rane et al., 2003]. Interestingly, Akt has been shown to increase cell size or protein synthesis in cultured cardiac myocytes [Haq et al., 2000; Morisco et al., 2000]. On the other hand, Akt signaling pathway is activated by PDGF in several cell types [Cartel et al., 2002; Jhonson

et al., 2002; Zhang et al., 2002; Reif et al., 2003]. Here, we showed that PDGF-BB phosphorylates HSP27 at Ser-15 and -85 via p38 MAP kinase in cardiac myocytes. Based on our data and previous reports, it is possible that PDGF-BB regulates cardiomyocyte function through HSP27 phosphorylation induced by p38 MAP kinase or Akt. However, the physiological role of HSP27 in heart is not precisely known. Further investigation is required to clarify the exact role of phosphorylated HSP27 in cardiac myocytes.

In conclusion, these results strongly suggest that PDGF-BB phosphorylates HSP27 at Ser-15 and -85 via p38 MAP kinase in cardiac myocytes.

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