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Vasopressin Phosphorylates HSP27 in Aortic Smooth Muscle Cells

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Abstract Administration of arginine vasopressin (AVP) time-dependently induced the phosphorylation of heat shock protein 27 (HSP27) at Ser-15 and Ser-85 in smooth muscle of aorta in vivo. The AVP-induced phosphorylation of HSP27 at Ser-15 and Ser-85 was inhibited by a V1a receptor antagonist but not by a V2 receptor antagonist. In cultured aortic smooth muscle A10 cells, AVP markedly stimulated the phosphorylation of HSP27 at Ser-15 and Ser-85. The AVP-induced phosphorylation of HSP27 was attenuated by SB203580 and PD169316, inhibitors of p38 mitogen-activated protein (MAP) kinase, but not by PD98059, a MEK inhibitor. These results strongly suggest that AVP phosphorylates HSP27 via p38 MAP kinase in aortic smooth muscle cells. J. Cell. Biochem. 92: 1203–1211, 2004.

Key words: vasopressin; phosphorylation; protein kinase; vascular; smooth muscle

It is generally recognized that the physiological effects of arginine vasopressin (AVP) are exerted through binding V1 and V2 receptors, and AVP regulates free water reabsorption via V2 receptors [Scott-Burden et al., 1992; Thibonnier et al., 1994]. AVP acts as a vasoactive agent via stimulation of V1 receptors, which stimulates the proliferation of vascular smooth muscle cells and induces vasoconstriction [van Breemen, 1989; Scott-Burden et al., 1992]. In our previous studies [Kondo et al., 1989; Murase et al., 1992], we have reported that AVP induces phosphoinositide hydrolysis via a pertussis toxin-insensitive GTP-binding protein in primary cultured aortic smooth muscle cells and that AVP has an suppressive effect on proliferation of these cells by inhibiting progression from the late G_1 into the S phase of the cell cycle. In addition, we showed that γ12, a γ subunit of heterotrimeric GTP-binding proteins which play a crucial role in the signal transduction from cell surface receptors to cellular effectors, is phosphorylated by AVP in these cells [Morishita et al., 1995]. It has recently been recommended that AVP is an alternative to epinephrine clinically for the treatment during cardiopulmonary resuscitation. However, the exact mechanism of AVP in the cardiovascular system has not yet been precisely clarified.

It is well known that 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 and low-molecular-weight HSPs based on apparent molecular sizes. It is recognized that the high-molecular-weight HSPs such as HSP90

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and HSP70 act as molecular chaperones in protein folding, oligomerization, and translocation [Benjamin and McMillan, 1998]. Low-molecular-weight HSPs with molecular masses from 10 to 30 kDa, such as HSP27 and αB-crystallin have high homology in amino acid sequences [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]. In previous studies [Inaguma et al., 1993], we have shown that low-molecular-weight HSPs are present in various tissues and cells, especially in skeletal muscle and smooth muscle cells. The HSPs are present in significant amounts even in several unstressed cells including myocytes where they may have essential functions [Benjamin and McMillan, 1998]. In addition, it is known that HSP27 is phosphorylated at two sites (Ser-15 and Ser-85) of serine in rats [Benjamin and McMillan, 1998]. In previous studies [Kaida et al., 1999; Ito et al., 2000], we have shown that AVP stimulates the induction of HSP27 in aortic smooth muscle A10 cells. However, the exact role of HSP27 in arterial smooth muscle cells is not known.

In the present study, we investigated whether AVP stimulates the phosphorylation of HSP27 in rat arterial smooth muscle in vivo and rat arterial smooth muscle A10 cells, and the mechanism behind the phosphorylation. We here report that AVP stimulates the phosphorylation of HSP27 via a p38 mitogen-activated protein (MAP) kinase-dependent manner in these cells.

MATERIALS AND METHODS

Materials

AVP was purchased from Peptide Institute, Inc. (Minoh, Japan). Pentobarbital was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). PD98059, SB203580, and PD169316 were obtained from Calbiochem-Novabiochem (La Jolla, CA). [β-Mercapto-β, β-cyclopentamethylenepropionyl^{1}, *O*-me-Tyr^{2}, Arg^{8}]-Vasopressin (V1a receptor antagonist) and [Adamantaneacetyl^{1}, *O*-Et-D-Tyr^{2}, Val^{4}, Aminobutyryl^{6}, Arg^{8,9}]-Vasopressin (V2 receptor antagonist) were obtained from Sigma Chemical Co. (St. Louis, MO). 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 PD169316 were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect Western blot analysis.

Experimental Protocol In Vivo and Immunohistochemical Observation

Wister rats (Wister Kyoto, SLC, Sizuoka, Japan) were divided into two groups, a control group ($n = 6$) and a group treated with AVP ($n = 6$, each time point) at a dose of 0.1 U/kg. Rats 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 AVP 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. AVP at a dose of 0.1 U/kg, chosen so that the generally AVP administered for in vivo experiments was 0.4 U/kg [Schwarz et al., 2002; Stadlbauer et al., 2003], was intravenously injected as a bolus via a right jugular vein. After the injection of AVP or saline, animals were sacrificed by an over dose of pentobarbital and then thoracic aorta of each rat were collected on each time point. Samples were excised in saline and frozen. These sections were first incubated with pre-immune 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 mmol/L Tris/HCL buffer, pH 7.6. The background was stained with hematoxylin. In separate experiments, AVP receptor antagonists (a V1a receptor antagonist at a dose of 10 μg/body or a V2 receptor antagonist at a dose of 100 μg/body) were treated in rats ($n = 6$, each). Antagonists were injected as a bolus via a right jugular vein and then AVP (0.1 U/kg) was injected. Ten minutes after an injection of AVP, mice were sacrificed by over dose of pentobarbital. Immunohistochemical stain was also performed as same as mention above. All surgical and anesthetic procedures were performed in accordance with institutional guidelines.

Cell Culture

A10 cells derived from fetal rat aortic smooth muscle [Kimes and Brandt, 1976] were obtained from the American Type Culture Collection (Rockville, MD). The cells (1×10^5 or 5×10^5) were seeded into 35- or 90-mm-diameter dishes and maintained at 37°C under a humidified atmosphere of 5% carbon dioxide and 95% air in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. After 5 days, the medium was exchanged for serum-free Dulbecco's modified Eagle's medium. The cells were used for experiments 48 h thereafter.

Western Blot Analysis

Cultured cells were stimulated by AVP 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 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 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–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; 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 second antibodies. Peroxidase activity on the nitrocellulose sheet was visualized on X-ray film by means of the ECL Western blotting detection system and was quantitated using NIH image software. When indicated, the cells were pretreated with PD98059, SB203580, or PD169316 for 60 min prior to stimulation by AVP.

RESULTS

Effect of AVP on the Phosphorylation of HSP27 in Rat In Vivo

AVP significantly induced the phosphorylation of HSP27 at Ser-15 and Ser-85 in rat aorta after its administration in a time-dependent manner (Fig. 1). The phosphorylation of HSP27 at both Ser-15 and Ser-85 was markedly observed at 3 min after the AVP administration. The phosphorylation reached its peak at 10 min after the stimulation, and was rapidly dephosphorylated within 90 min. Administration of vehicle alone did not affect the phosphorylation of HSP27 (Fig. 1). Blood pressure was elevated just after the administration of AVP while AVP had little effect on heart rate in rat (Fig. 1).

Effects of AVP Receptor Antagonists on the AVP-Induced Phosphorylation of HSP27 in Rat In Vivo

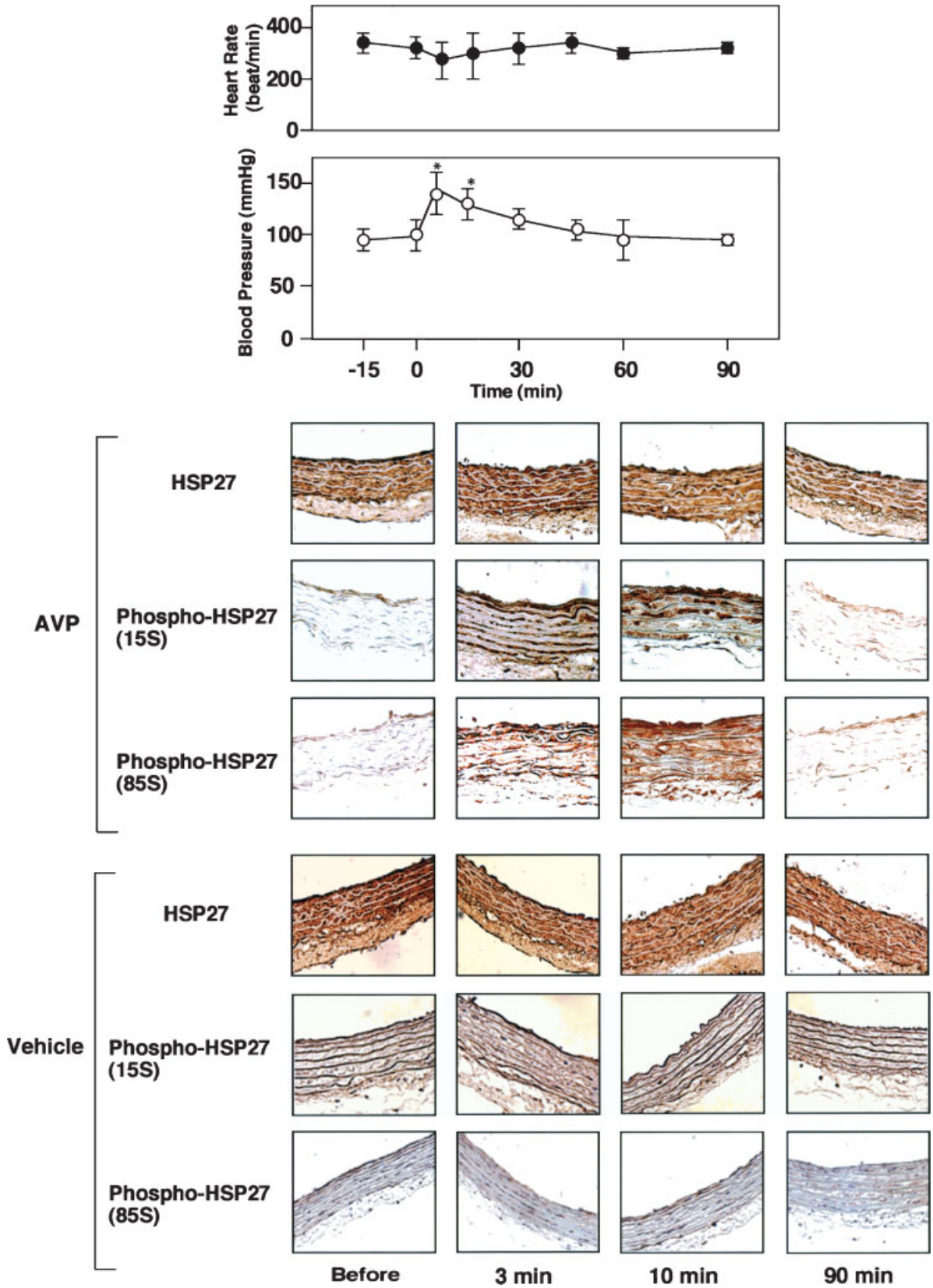
When [β-Mercapto-β, β-cyclopentamethylenepropionyl[1], *O*-me-Tyr[2], Arg[8]]-Vasopressin, a V1a receptor antagonist, was treated before an injection of AVP, the phosphorylation of HSP27 at both Ser-15 and Ser-85 were markedly reduced (Fig. 2). On the other hand, the treatment with [Adamantaneacetyl[1], *O*-Et-D-Tyr[2], Val[4], Aminobutyryl[6], Arg[8,9]]-Vasopressin, a V2 receptor antagonist, did not affect the phosphorylation of HSP27 induced by AVP (Fig. 2). V1a or V2 receptors antagonists alone did not induce the phosphorylation of HSP27 (Fig. 2).

Effect of AVP on the Phosphorylation of HSP27 in A10 Cells

AVP time dependently stimulated the phosphorylation of HSP27 at Ser-15 (15S) and Ser-85 (85S) in HSP27 in cultured aortic smooth muscle A10 cells (Fig. 3). The phosphorylation of HSP27 at both Ser-15 and Ser-85 reached a peak after 20 min AVP-stimulation. The phosphorylation at Ser-15 was remained for at least 90 min while the phosphorylation at Ser-85 was decrease after 60 min AVP-stimulation.

Effects of PD98059, SB203580, or PD169316 on the AVP-Induced Phosphorylation of HSP27 in A10 Cells

We have previously shown that AVP activates p44/p42 MAP kinase and p38 MAP kinase among the MAP kinase superfamily in aortic smooth muscle A10 cells [Ito et al., 2000]. In order to clarify whether these MAP kinases are involved in the AVP-induced phosphorylation of HSP27 in A10 cells, we next examined the effect of PD98059, a specific inhibitor of upstream kinase that activates p44/p42 MAP kinase [Alessi et al., 1995], on the HSP27 phosphorylation. However, PD98059 had little effect on the AVP-induced phosphorylation of HSP27 at Ser-15 and Ser-85 (Fig. 4).


Heart Rate (beat/min)
400
200
0
Blood Pressure (mmHg)
150
100
50
0
*
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-15
0
30
60
90
Time (min)
AVP
HSP27
Phospho-HSP27 (15S)
Phospho-HSP27 (85S)
Vehicle
HSP27
Phospho-HSP27 (15S)
Phospho-HSP27 (85S)
Before
3 min
10 min
90 min

Fig. 1.

In addition, we examined the effect of SB203580, a specific inhibitor of p38 MAP kinase [Cuenda et al., 1995], on the AVP-induced phosphorylation of HSP27. SB203580 significantly suppressed the phosphorylation of HSP27 stimulated by AVP (Fig. 5). Furthermore, PD169316, another inhibitor of p38 MAP kinase [Kummer et al., 1997], also significantly suppressed the phosphorylation of HSP27 at both Ser-15 and Ser-85 stimulated by AVP (Fig. 6). The inhibitory effects of PD169316 on the phosphorylation of HSP27 were dose-dependent over the range 10–30 μmol/L and the maximal effect of PD169316 was observed at 20 μmol/L, a dose that caused about 70% reduction in the AVP-induced HSP27 phosphorylation.

DISCUSSION

In the present study, we first investigated whether AVP phosphorylates HSP27 in rat aorta in vivo by histological analysis. The administration of AVP time dependently induced the HSP27 phosphorylation at two sites (Ser-15 and Ser-85) of serine in smooth muscle of rat aorta. Furthermore, the AVP-induced phosphorylation of HSP27 at Ser-15 and Ser-85 was inhibited by a V1a receptor antagonist but not by a V2 receptor antagonist. These results clearly showed that V1a receptors, but not V2 receptors, play a role of phosphorylation of HSP27 in vivo. We next examined the AVP-effect on the HSP27 phosphorylation and the mechanism behind the phosphorylation in cultured rat aortic smooth muscle A10 cells. We used pentobarbital as an anesthesia and administered it to rats with an intraperitoneal injection. However, we found that pentobarbital, which alone did not affect the phosphorylation of HSP27, had little effect on the AVP-induced phosphorylation of HSP27 in A10 cells (data not shown). AVP truly stimulated the phosphorylation of HSP27 at two sites also in these cells. The phosphorylation of HSP27 reached its peak at 10 min and HSP27 was rapidly dephosphorylated within 90 min of AVP-stimulation in rat in vivo. In contrast, the phosphorylation of HSP27 at Ser-15 and Ser-85 reached its peak after 20 min AVP-stimulation and remained for at least 90 min. These results implied that some mechanisms accounting for the rapid dephosphorylation of HSP27 after AVP-stimulation might exist in rat in vivo: some other cell types as endothelial cells or fibroblasts around the smooth muscle cells may affect the smooth muscle cells to activate some intracellular phosphatases against HSP27 at 10 min after the AVP-administration.

It is recognized that the activation of the MAP kinase superfamily is involved in HSP27 phosphorylation [Benjamin and McMillan, 1998]. We have previously shown that AVP activates p44/p42 MAP kinase and p38 MAP kinase but not stress-activated protein kinase/c-Jun N terminal kinase in aortic smooth muscle A10 cells [Ito et al., 2000]. Thus, we investigated whether two MAP kinases are involved in the AVP-induced HSP27 phosphorylation. However, PD98059 (10 μmol/L), a MEK inhibitor [Alessi et al., 1995], had little effect on the AVP-induced phosphorylation of HSP27. In a previous study [Ito et al., 2000], we demonstrated that AVP-induced p44/p42 MAP kinase is significantly suppressed by 10 μmol/L PD98059. Therefore, it seems unlikely that p44/p42 MAP kinase mediates the AVP-induced HSP27 phosphorylation in aortic smooth muscle cells. In the contrary, we showed that the AVP-induced HSP27 phosphorylations at two sites were markedly suppressed by SB203580, a p38 MAP kinase inhibitor [Cuenda et al., 1995]. Furthermore, we found that PD169316, another inhibitor of p38 MAP kinase [Kummer et al., 1997], reduced the HSP27 phosphorylation by AVP. Based on these findings, it is most likely that AVP stimulates HSP27 phosphorylation at two sites (Ser-15 and Ser-85) via p38 MAP kinase in aortic smooth muscle cells. To the best of our knowledge, this is probably the first report showing that as a physiological agonist, AVP, which is clinically available for

Fig. 1. Effect of arginine vasopressin (AVP) on the phosphorylation of heat shock protein 27 (HSP27) in rat aorta (n = 6, each time point). Blood pressure (**a**) and heart rates (**b**) were continuously monitored during the indicated periods. AVP at a dose of 0.1 U/kg or vehicle was intravenously injected as a bolus via a right jugular vein at time 0. The extracts of samples in each time point were analyzed using antibodies against phosphorylated Ser-15 (15S), Ser-85 (85S) in HSP27 and HSP27, respectively. Typical micro-photographs stained by DAB showed at before, 3, 10, or 90 min after an injection of AVP or vehicle. Data represent the mean ± SEM (n = 6). $^*P < 0.05$, compared to the value of blood pressure before AVP-injection.

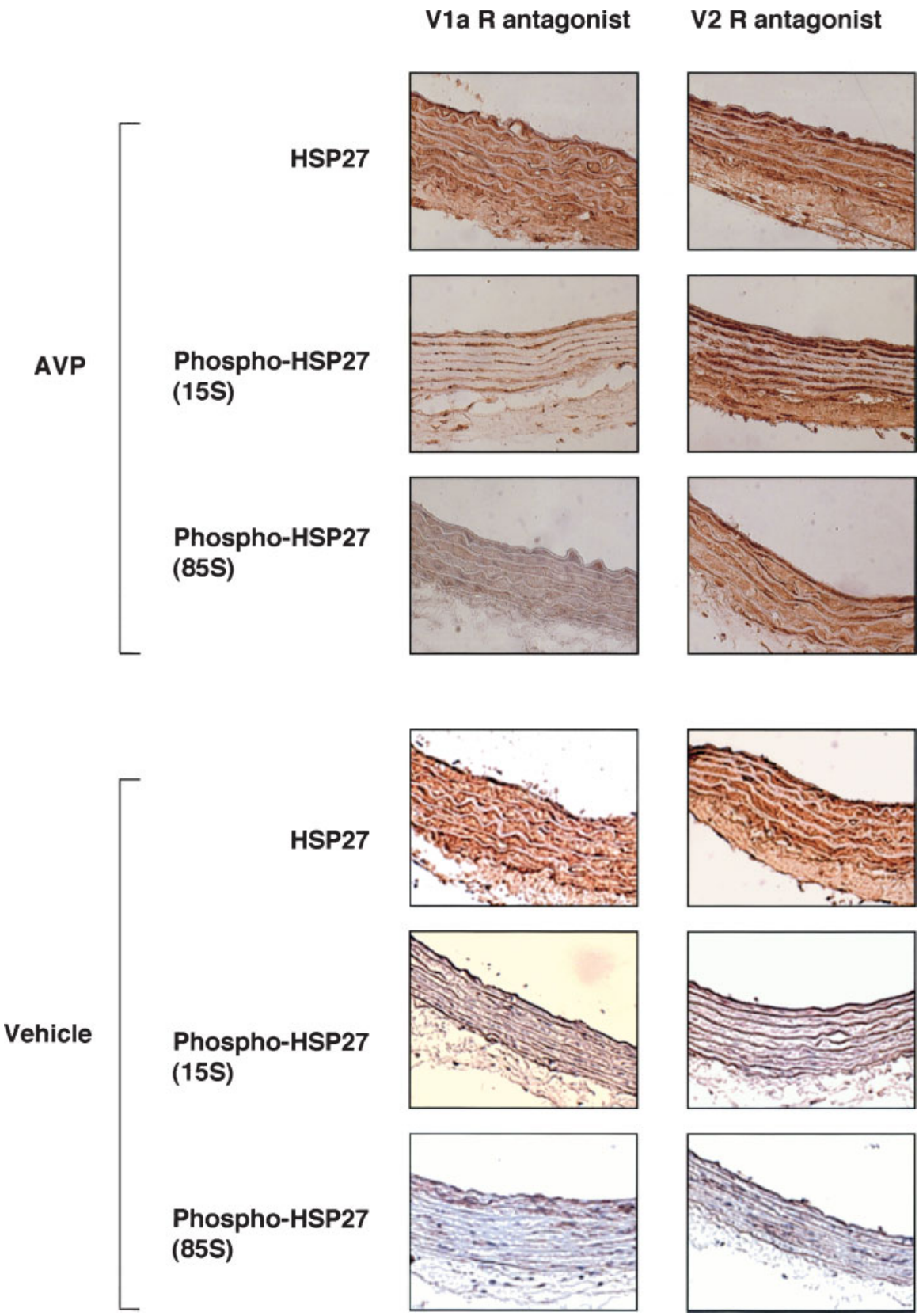

Fig. 2. Effect of AVP receptor antagonists on the AVP-induced phosphorylation of HSP27. Typical micrographs of rat aorta stained by DAB. The extracts of samples treated with a V1a receptor (V1a R) antagonist (10 μg) or a V2 receptor (V2 R) antagonist (100 μg) before an injection of AVP or vehicle were analyzed using antibodies against phosphorylated Ser-15 (15S), Ser-85 (85S) in HSP27 and HSP27, respectively.

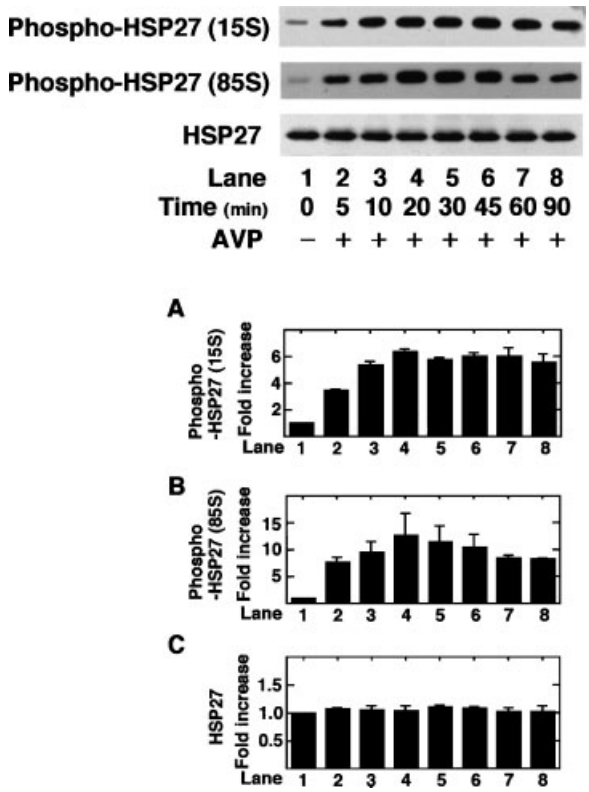

Fig. 3. Effect of AVP on the phosphorylation of HSP27 in A10 cells. The cultured cells were stimulated by 0.1 μmol/L AVP for the indicated periods. The extracts of cells were subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) using antibodies against phosphorylated Ser-15 (15S), Ser-85 (85S) in HSP27 and HSP27. **A**, **B**: The histograms showed quantitative representations of the levels of AVP-induced phosphorylation of HSP27 at Ser-15 (A) and Ser-85 (B) obtained from laser densitometric analysis after normalization to the levels of HSP27 of three independent experiments. The phosphorylation rates by AVP were shown the values of AVP-stimulated cells versus those of non-stimulated cells. Each value represents the mean ± SD of three independent experiments. **C**: The histogram showed quantitative representations of the levels of HSP27 obtained from laser densitometric analysis of three independent experiments. The levels of HSP27 were shown the values of AVP-stimulated cells versus those of non-stimulated cells.

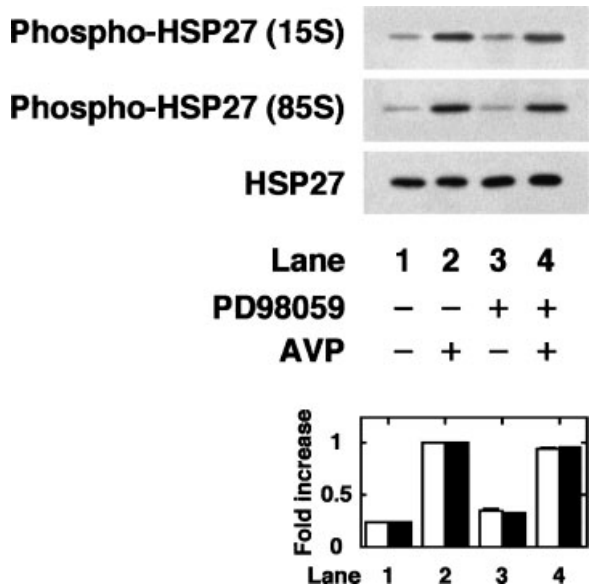

Fig. 4. Effect of PD98059 on the AVP-induced phosphorylation of HSP27 in A10 cells. The cultured cells were pretreated with 10 μmol/L PD98059 or vehicle for 60 min, and then stimulated by 0.1 μmol/L AVP or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE using antibodies against phosphorylated Ser-15 (15S), Ser-85 (85S) in HSP27 and HSP27. Western blot analysis was performed from three independent cell preparations and the signal intensities visualized on X-ray film were quantitated by using NIH image software. The levels of phospho-HSP27 for each cells were normalized by levels of total HSP27. The histograms showed the fold increase of levels of phospho-HSP27 at Ser-15 (open bars) and Ser-85 (filled bars) in each cells versus those of AVP-stimulated cells. Values on the vertical axis represent mean ± SD from three independent experiments. **Lanes 1**, **2**, without PD98059-pretreatment; **lanes 3**, **4**, with PD98059-pretreatment; lanes 1, 3, without AVP-stimulation; lanes 2, 4, with AVP-stimulation.

patients undergoing cardiopulmonary resuscitation, phosphorylates HSP27 at two sites of serine in aortic smooth muscle.

It is well recognized that 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 [Benjamin and McMillan, 1998]. Post-translational modifications such as phosphorylation and oligomerization are crucial regulators of its functions [Benjamin and McMillan, 1998]. In a previous study [Kato et al., 1994], we have reported that HSP27 is dissociated concomitantly with the phosphorylation of the aggregated form of HSP27 and that dephosphorylation of the dissociated HSP27 causes aggregation. In addition, we have shown 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]. We previously reported that AVP induces the protein level of HSP27 via p38 MAP kinase in aortic smooth muscle A10 cell [Kaida et al., 1999; Ito et al., 2000]. The effect of AVP was significantly observed at 36 h after the AVP-stimulation [Kaida et al., 1999]. In contrast, in this report we showed that AVP induces the phosphorylation of HSP27 at 20 min after the

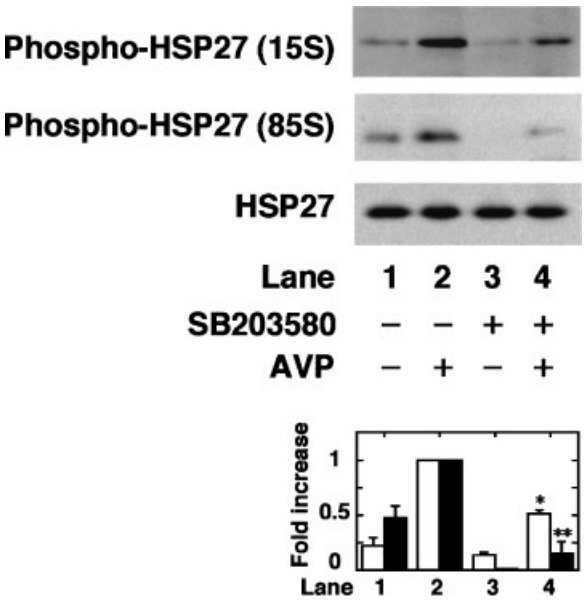

Fig. 5. Effect of SB203580 on the AVP-induced phosphorylation of HSP27 in A10 cells. The cultured cells were pretreated with 20 μmol/L SB203580 or vehicle for 60 min, and then stimulated by 0.1 μmol/L AVP or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE using antibodies against phosphorylated Ser-15 (15S), Ser-85 (85S) in HSP27 and HSP27. Western blot analysis was performed from three independent cell preparations and the signal intensities visualized on X-ray film were quantitated by using NIH image software. The levels of phospho-HSP27 for each cells were normalized by levels of total HSP27. The histograms showed the fold increase of levels of phospho-HSP27 at Ser-15 (open bars) and Ser-85 (filled bars) in each cells versus those of AVP-stimulated cells. Values on the vertical axis represent mean ± SD from three independent experiments. **Lanes 1**, **2**, without SB203580-pretreatment; **lanes 3**, **4**, with SB203580-pretreatment; lanes 1, 3, without AVP-stimulation; lanes 2, 4, with AVP-stimulation. $*P < 0.05$, compared to the value of AVP alone (15S). $**P < 0.05$, compared to the value of AVP alone (85S).

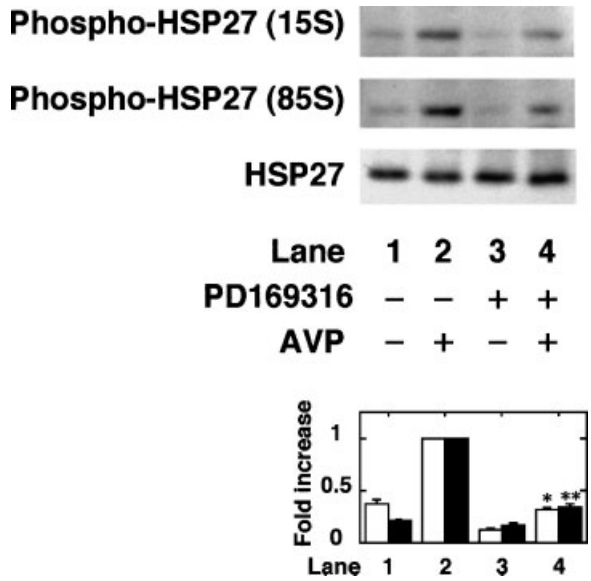

Fig. 6. Effect of PD169316 on the AVP-induced phosphorylation of HSP27 in A10 cells. The cultured cells were pretreated with 20 μmol/L PD169316 or vehicle for 60 min, and then stimulated by 0.1 μmol/L AVP or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE using antibodies against phosphorylated Ser-15 (15S), Ser-85 (85S) in HSP27 and HSP27. Western blot analysis was performed from three independent cell preparations and the signal intensities visualized on X-ray film were quantitated by using NIH image software. The levels of phospho-HSP27 for each cells were normalized by levels of total HSP27. The histograms showed the fold increase of levels of phospho-HSP27 at Ser-15 (open bars) and Ser-85 (filled bars) in each cells versus those of AVP-stimulated cells. Values on the vertical axis represent mean ± SD from three independent experiments. **Lanes 1**, **2**, without PD169316-pretreatment; **lanes 3**, **4**, with PD169316-pretreatment; lanes 1, 3, without AVP-stimulation; lanes 2, 4, with AVP-stimulation. $*P < 0.05$, compared to the value of AVP alone (15S). $**P < 0.05$, compared to the value of AVP alone (85S).

AVP-stimulation in the same cell line. Therefore, it is implied that the AVP-induced phosphorylation of HSP27 is short term effect of AVP on A10 cells, and that the AVP-induced expression of HSP27 is long term effect of AVP on those cells. Although the physiological significance of these phenomena is not known, we speculate that the rapid increase in the phosphorylation of HSP27 might be an early defense mechanism of the cells for protection against stress, and subsequent expression of HSP27 might be related to the acquisition of tolerance to such stress. Taking our findings into account, it is probable that AVP directly affects aortic smooth muscle through the stimulation of HSP27 phosphorylation. However, the physiological significance of HSP27 in aortic smooth muscle has not yet been precisely clarified. Further investigations are necessary to clarify the exact roles of non-phosphorylated- and phosphorylated-HSP27 in aortic smooth muscle cells.

In conclusion, these results strongly suggest that AVP phosphorylates HSP27 via p38 MAP kinase in aortic smooth muscle cells.

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