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Contrasting Effects of Midazolam on Induction of Heat Shock Protein 27 by Vasopressin and Heat in Aortic Smooth Muscle Cells

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Abstract We previously showed that vasopressin stimulates the induction of heat shock protein (HSP) 27, a low molecular-weight HSP, through protein kinase C activation in aortic smooth muscle A10 cells. In the present study, we examined the effects of midazolam, an intravenous anesthetic, on the HSP27 induction stimulated by vasopressin, heat, or sodium arsenite (arsenite) in A10 cells. Midazolam inhibited the accumulation of HSP27 induced by vasopressin or 12-*O*-tetradecanoylphorbol 13-acetate (TPA), a direct activator of protein kinase C. Midazolam also reduced the vasopressin-induced level of the mRNA for HSP27. In contrast, midazolam enhanced the HSP27-accumulation induced by heat or arsenite. Midazolam also enhanced the heat-increased level of the mRNA for HSP27. However, midazolam had no effect on the dissociation of the aggregated form of HSP27 following stimulation by vasopressin, heat, or arsenite. These results suggest that midazolam suppresses vasopressin-stimulated HSP27 induction in vascular smooth muscle cells, and that this inhibitory effect is exerted at a point downstream from protein kinase C. In contrast, midazolam enhanced heat- or arsenite-stimulated HSP27 induction. Thus, midazolam has dual effects on the HSP27 induction stimulated by various stresses in vascular smooth muscle cells. J. Cell. Biochem. 84: 39–46, 2002.

Key words: aortic smooth muscle cells; cell culture; heat shock protein; midazolam; vasopressin

Midazolam is an intravenous anesthetic that is widely used for premedication, perioperative sedation, and the induction and maintenance of anesthesia [McNulty et al., 1994; Shafer, 1998]. It has been reported that midazolam affects hemodynamics and decreases systemic blood pressure [Windsor et al., 1988; Short and Chui, 1991; McNulty et al., 1994; Shafer, 1998], the latter effect being thought to be due to both direct and indirect effects on the cardiovascular system [Marty et al., 1986; Chang et al., 1994; Buljubasic et al., 1996; Gelissen et al., 1996; Yamaguchi et al., 1997]. As to the indirect effect, it has been reported that midazolam reduces sympathetic activity by an action on the central nervous system [Marty et al., 1986]. On the other hand, midazolam directly reduces myocardial contractility and systemic vascular resistance [Chang et al., 1994; Buljubasic et al., 1996; Gelissen et al., 1996; Yamaguchi et al., 1997]. It is well known that vascular smooth muscle cells play an important role in the regulation of vascular tone [Horowitz et al., 1996]. However, the exact mechanism underlying the effects of midazolam on these cells is not known.

Heat shock proteins (HSPs) are produced in cells after exposure to heat or chemical stress [Benjamin and Mcmillan, 1998]. HSPs are generally divided into high and low molecular-weight HSPs according to their apparent molecular sizes. Several HSPs are expressed constitutively in unstressed cells, in which they may perform essential functions [Benjamin and

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Mcmillan, 1998]. High molecular-weight HSPs (HSP70, HSP90, and HSP110) are recognized as playing the role of molecular chaperones, and are implicated in protein folding, oligomerization, and translocation [Benjamin and Mcmillan, 1998]. Low molecular-weight HSPs, with molecular masses of 15–30 kDa (such as HSP27 and αB-crystallin), may have chaperoning functions, like high molecular-weight HSPs [Jakob et al., 1993; Benjamin and Mcmillan, 1998]. Low molecular-weight HSPs are present in various tissues and cells, especially in skeletal muscle cells and smooth muscle cells [Kato et al., 1991; Inaguma et al., 1993]. Evidence is accumulating that HSP27 participates in several functions in a variety of cell types [Zuh et al., 1994; Guay et al., 1997; Benjamin and Mcmillan, 1998; Schneider et al., 1998; Hino et al., 2000]. In the vascular system, it has been reported that an expression of HSP27 is induced in aortic smooth muscle cells in restrained rats [Udelsman et al., 1993], and that HSP27 plays a major role in actin–filament dynamics in a number of cell types [Benjamin and Mcmillan, 1998]. It has been reported that thrombin-, serotonin-, and endothelin-induced contractions of vascular smooth muscle are all associated with an increased phosphorylation of HSP27 [Brophy et al., 1997, 1998; Yamboliev et al., 2000]. Thus, it is suggested that the HSP27 found in vascular smooth muscle cells serves to modulate systemic blood pressure.

As to the intracellular signaling system for HSP induction, it is generally recognized that activation of one or more heat-shock transcriptional factors occurs in response to heat stress, and that these activated factors bind to specific DNA sequences (known as heat-shock elements) found in the promoter regions of the genes for HSP, with a resultant increase in the transcription of the respective genes [Rabindran et al., 1991; Sorger, 1991]. In an aortic smooth muscle cell line, A10 cells, we previously showed that vasopressin, a potent vasoactive agent, stimulates the induction of HSP27, a low molecular-weight-HSP, while having no effect on the induction of HSP70, a high molecular-weight-HSP [Kozawa et al., 2000]. We also showed that the induction occurs through the activation of protein kinase C [Kaida et al., 1999; Ito et al., 2000]. In the present study, we examined the effects of midazolam on the induction of HSP27 by vasopressin, heat, and sodium arsenite (arsenite) in A10 cells.

MATERIALS AND METHODS

Materials

Arginine vasopressin was purchased from Peptide Institute Inc. (Minoh, Japan). Midazolam and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) were purchased from Sigma Chemical Co. (St. Louis, MO). Other materials and chemicals were obtained from commercial sources. Midazolam, vasopressin, and arsenite were dissolved in an assay buffer (5 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 1 mM $CaCl_2$, and 5.5 mM glucose) containing 0.01% bovine serum albumin (BSA). We used the assay buffer containing 0.01% BSA as the vehicle control for midazolam. TPA was dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide in the culture medium was 0.1%, and this did not affect the immunoassay for HSP27.

Cell Culture

A10 cells derived from fetal rat aorta [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 in a humidified atmosphere of 5% CO_2 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.

Immunoassay of HSP27

Cultured cells were pretreated with midazolam (1 μM–0.1 mM) or vehicle, then stimulated by vasopressin (0.1 μM) or TPA (0.1 μM) for the indicated periods in serum-free Dulbecco's modified Eagle's medium. When cells were stimulated by arsenite (30 μM), the medium was replaced 1 h after the stimulation, and the cells were then incubated for a further 12 h. For heat treatment, dishes were floated in a water bath at the indicated temperature for 30 min, then incubated at 37°C for a further 12 h. The concentrations of HSP27 in soluble extracts of the cells were determined by means of a sandwich-type enzyme immunoassay, as described previously [Kaida et al., 1999; Ito et al., 2000]. 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 0.3 M NaCl, 0.5% hydrolyzed gelatin, 0.1% BSA, 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 0.1 M NaCl, 1 mM $MgCl_2$, 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.

Isolation of RNA and Northern Blot Analysis of the mRNA for HSP27

Cultured cells were pretreated with midazolam (0.1 mM) for 20 min and then stimulated by vasopressin (0.1 μM) for 12 h in serum-free Dulbecco's modified Eagle's medium. For heat treatment, dishes were floated in a water bath at 43°C for 30 min and then incubated at 37°C for a further 3 h. The cells were washed twice with phosphate-buffered saline, then frozen at −80°C for a few days before analysis. Total RNA was isolated using a QuickPrep Total RNA Extraction kit (Pharmacia Biotech, Tokyo, Japan). Twenty micrograms of total RNA was then subjected to electrophoresis on a 0.9% agarose/2.2 M formaldehyde gel before being blotted onto a nitrocellulose membrane. For Northern blot, the membrane was allowed to hybridize with a cDNA probe that had been labeled using a Multiprime DNA labeling system (Amersham, Buckinghamshire, UK), as described previously [Kaida et al., 1999; Ito et al., 2000]. A *Bam*HI–*Hind*III fragment of cDNA for mouse HSP27 was kindly provided by Dr. L. F. Cooper of the University of North Carolina [Cooper and Uoshima, 1994].

Sucrose Density Gradient Centrifugation

Cultured cells were pretreated with midazolam (0.1 mM or 30 μM) for 20 min, then stimulated by vasopressin (0.1 μM) or arsenite (30 μM) in serum-free Dulbecco's modified Eagle's medium for 1 h. For heat treatment, dishes were floated in a water bath at 43°C for 20 min. The cells were washed twice with phosphate-buffered saline, then frozen at −80°C for a few days before analysis. An extract of the cells was layered over a 3.5 ml linear gradient of sucrose (10–40%) in 50 mM Tris/HCl, pH 7.0, that also contained 5 mM EDTA, and was centrifuged at 130,000*g* for 16 h at 4°C in a swinging-bucket rotor (RPS56T; Hitachi, Tokyo, Japan). After centrifugation, each sample was fractionated into 15 test tubes, each of which contained 0.25 ml of 0.1% BSA.

Other Methods

Protein concentrations in soluble extracts were determined using a protein assay kit (Bio-Rad, Hercules, CA), with BSA as the standard protein. Rat HSP27, which was used as the standard for the immunoassay, was purified from skeletal muscle [Kato et al., 1991].

Statistical Analysis

The data were analyzed by ANOVA followed by the Student–Newman–Keuls test. Probability values <0.05 were considered significant. Except where otherwise noted, data are presented as the mean ± SD of triplicate determinations.

RESULTS

Effects of Midazolam on the Accumulation of HSP27 Induced by Vasopressin or TPA

Midazolam (0.1 mM), which by itself had little effect on the basal levels of HSP27, time-dependently (up to 48 h) inhibited the HSP27 accumulation induced by vasopressin (0.1 μM) (Fig. 1A). This inhibitory effect was dose-dependent (1 μM to 0.1 mM) and it was significant at doses of 30 μM or more (Fig. 1B). In a previous study [Kaida et al., 1999], we reported that vasopressin stimulates the induction of HSP27 through activation of protein kinase C in A10 cells. In the present study, midazolam markedly inhibited the HSP27 accumulation induced by TPA (0.1 μM), a direct activator of protein kinase C [Nishizuka, 1986] (Fig. 2); this

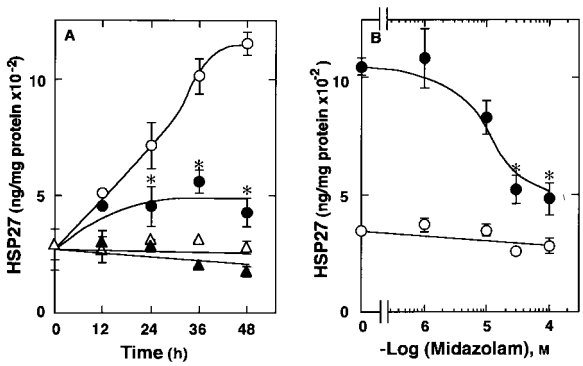

Fig. 1. Effect of midazolam on the vasopressin-induced accumulation of HSP27 in A10 cells. **A:** Time-course of the accumulation of HSP27 during the indicated period after vasopressin-stimulation. Cultured cells were pretreated with 0.1 mM midazolam (closed symbols) or vehicle (open symbols) for 20 min, then exposed to 0.1 μM vasopressin (circles) or vehicle (triangles) for the indicated period. **B:** Dose-dependent effect of midazolam on the accumulation of HSP27. Cultured cells were pretreated with various doses of midazolam for 20 min, and then exposed to 0.1 μM vasopressin (closed circles) or vehicle (open circles) for 48 h. The concentration of HSP27 in cell extracts was determined by a specific immunoassay. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with two other cell preparations. *$P < 0.05$ compared with vasopressin alone.

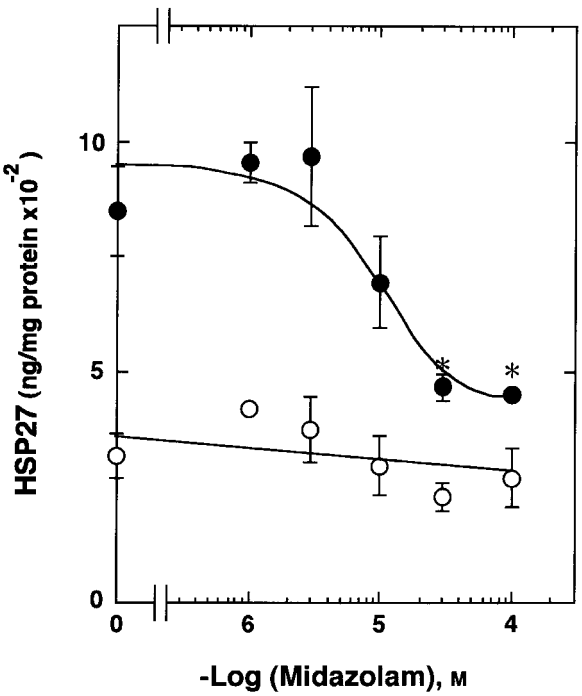

Fig. 2. Effect of midazolam on the TPA-induced accumulation of HSP27 in A10 cells. Cultured cells were pretreated with various doses of midazolam for 20 min, then exposed to 0.1 μM TPA (closed circles) or vehicle (open circles) for 48 h. The concentration of HSP27 in cell extracts was determined by a specific immunoassay. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with two other cell preparations. *$P < 0.05$ compared with TPA alone.

inhibitory effect was dose-dependent (1 μM to 0.1 mM) and it was significant at doses of 30 μM or more (Fig. 2).

Effects of Midazolam on the Accumulation of HSP27 Induced by Heat or Arsenite

We previously reported that heat significantly stimulates the induction of HSP27 in a time-dependent manner in A10 cells [Kaida et al., 1999]. Heat-stress stimulated the accumulation of HSP27 in a temperature-dependent manner (Fig. 3A). Midazolam (0.1 mM) significantly enhanced the HSP27 accumulation induced by heat (Fig. 3A); this effect of midazolam on heat (43°C)-induced HSP27 accumulation was dose-dependent (1 μM to 0.1 mM) and it was significant at doses of 30 μM or more (Fig. 3B). We found that arsenite, a chemical stress, stimulated the accumulation of HSP27 in A10 cells, and that midazolam strongly enhanced this effect of arsenite in a dose-dependent fashion (over the range 1–30 μM midazolam; Fig. 4).

Effects of Midazolam on the Vasopressin- or Heat-Increased Levels of the mRNA for HSP27

In order to clarify the effect of midazolam on the HSP27 induction stimulated by vasopressin or heat, we examined the effect of midazolam on the vasopressin- or heat-stimulated level of the mRNA for HSP27. Midazolam (0.1 mM), which itself hardly affected the basal mRNA level, significantly reduced the vasopressin (0.1 μM)-increased level of HSP27 mRNA as well as the vasopressin-induced HSP27 accumulation (Fig. 5A). In contrast, midazolam (0.1 mM) significantly enhanced the heat (43°C)-increased level of HSP27 mRNA, as it did the heat-induced HSP27 accumulation (Fig. 5B).

Effects of Midazolam on the Dissociation of the Aggregated Form of HSP27 by Vasopressin, Heat, or Arsenite

HSP27 exists in two forms, an aggregated form and a dissociated form [Kato et al., 1994]. It is known that dissociation of HSP27 occurs concomitantly with its phosphorylation [Kato et al., 1994]. Our specific immunoassay for HSP27 detects both the aggregated form and the dissociated form [Kato et al., 1994]. We previously showed that extracts of unstimulated A10 cells contains both forms, and that

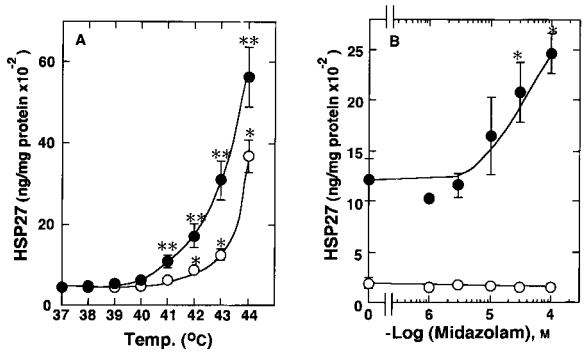

Fig. 3. Effect of midazolam on the heat-induced accumulation of HSP27 in A10 cells. **A:** Cultured cells were pretreated with 0.1 mM midazolam (closed circles) or vehicle (open circles) for 20 min, after which dishes were floated in a water bath at the indicated temperature for 30 min, and then incubated at 37°C for 12 h. **B:** Dose-dependent effect of midazolam on the accumulation of HSP27. Cultured cells were pretreated with various doses of midazolam for 20 min, after which dishes were floated in a water bath at 43°C (closed circles) or 37°C (open circles) for 30 min, and then incubated at 37°C for 12 h. The concentration of HSP27 in cell extracts was determined by a specific immunoassay. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with two other cell preparations. A, $*P < 0.05$ compared with control (37°C); $**P < 0.05$ compared with heat alone. B, $*P < 0.05$ compared with heat alone.

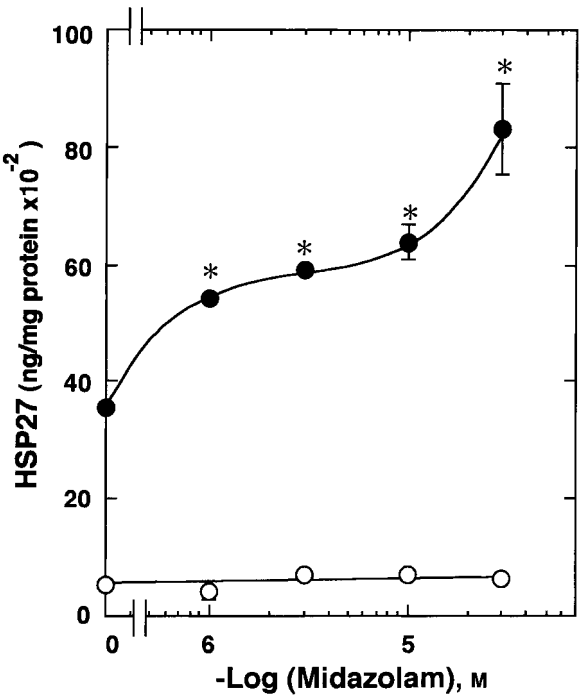

Fig. 4. Effect of midazolam on the arsenite-induced accumulation of HSP27 in A10 cells. Cultured cells were pretreated with various doses of midazolam for 20 min, then exposed to 30 μM arsenite (closed circles) or vehicle (open circles). After 1 h, the medium was exchanged, and then incubation continued for a further 12 h. Concentration of HSP27 in cell extracts was determined by a specific immunoassay. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with two other cell preparations. $*P < 0.05$ compared with arsenite alone.

vasopressin induces the dissociation of the aggregated form [Ito et al., 2000]. Midazolam (0.1 mM), which itself did not affect the dissociation of the aggregated form, had little effect on the vasopressin (0.1 μM)-induced dissociation (Fig. 6A). Arsenite (30 μM), too, induced dissociation of the aggregated form (Fig. 6B), and again midazolam (30 μM) had little effect on the dissociation (Fig. 6B). Likewise, midazolam (0.1 mM) did not affect heat (43°C)-stimulated dissociation (data not shown).

DISCUSSION

In the present study, we showed that midazolam inhibited the HSP27 accumulation induced by vasopressin, in an aortic smooth muscle cell line, A10 cells. In addition, midazolam reduced the vasopressin-stimulated level of the mRNA for HSP27 in these cells. These results strongly suggest that midazolam is an effective suppressor of vasopressin-stimulated HSP27 induction in vascular smooth muscle A10 cells. In addition, we demonstrated that midazolam also inhibited the accumulation of HSP27 induced by TPA, a direct activator of protein kinase C [Nishizuka, 1986], in these cells. In a previous study [Kaida et al., 1999], we showed that vasopressin induces HSP27 via an activation of protein kinase C through both phosphoinositide-hydrolyzing phospholipase C and phosphatidylcholine-hydrolyzing phospholipase D, and that TPA itself significantly stimulates HSP27 induction in A10 cells. Therefore, our findings suggest that the inhibitory effect of midazolam on vasopressin-stimulated HSP27 induction in these cells is exerted at a point downstream from protein kinase C. It is possible that midazolam directly affects protein kinase C activities or some unknown compound that is a target for protein kinase C. Further investigation is necessary to clarifying the exact mechanism of midazolam.

On the other hand, midazolam enhanced the HSP27 accumulation induced by heat or arsenite in A10 cells. Furthermore, we demonstrated that midazolam enhanced the level of HSP27 mRNA present following heat-stimulation. It is generally recognized that heat

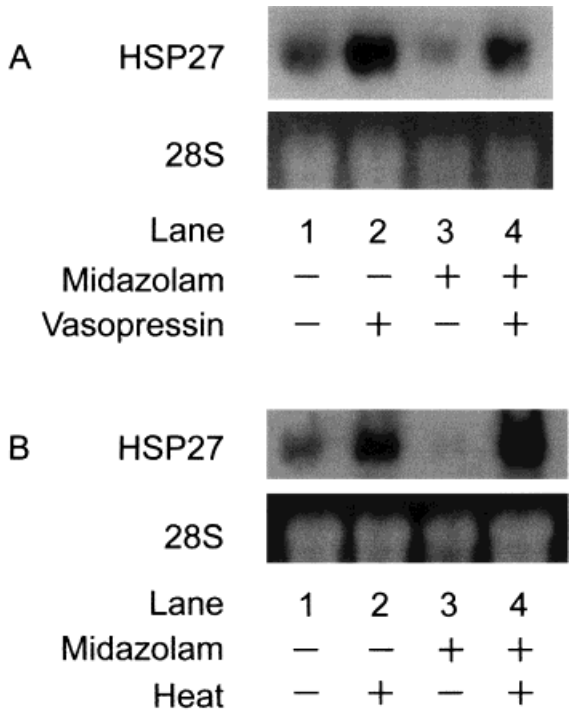

Fig. 5. Effect of midazolam on the vasopressin- or heat-stimulated levels of the mRNA for HSP27 in A10 cells. **A:** Cultured cells were pretreated with 0.1 mM midazolam or vehicle for 20 min, then exposed to 0.1 μM vasopressin or vehicle for 12 h. **B:** Cultured cells were pretreated with 0.1 mM midazolam or vehicle for 20 min, after which dishes were floated in a water bath at 43°C for 30 min, and then incubated at 37°C for 3 h. The cells were harvested and total RNA was isolated. Twenty micrograms of RNA from each sample was subjected to electrophoresis and blotted onto a nitrocellulose membrane. The membrane was then allowed to hybridize with the cDNA probe for HSP27. Bands representing 28S are shown for reference.

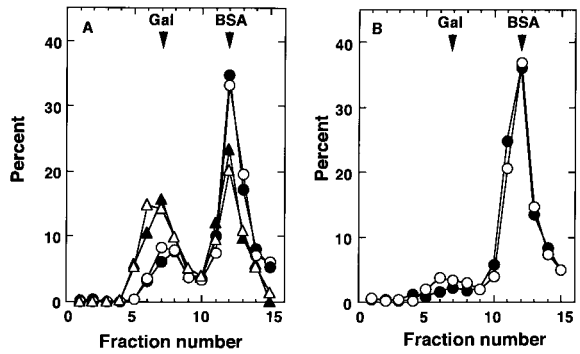

Fig. 6. Effect of midazolam on the dissociation of the aggregated form of HSP27 induced by vasopressin or arsenite in cells extracts. **A:** Cultured cells were pretreated with 0.1 mM midazolam (closed symbols) or vehicle (open symbols) for 20 min, then exposed to 0.1 μM vasopressin (circles) or vehicle (triangles) for 1 h. **B:** Cultured cells were pretreated with 30 μM midazolam (closed circles) or vehicle (open circles) for 20 min, then exposed to 30 μM arsenite for 1 h. In each case, a 200 μl aliquot of a soluble extract of the cells (containing 90 μg of protein) was fractionated by centrifugation on a sucrose-density gradient. Each point represents the average of duplicate assays. Arrows indicate the positions at which β-D-galactosidase from *E. Coli* (Gal, 540 kDa) and bovine serum albumin (BSA, 67 kDa) sedimented.

activates heat shock transcriptional factors, and that these activated factors bind to heat shock elements, with a resultant increase in the transcription of the respective HSP genes [Rabindran et al., 1991; Sorger, 1991]. An enhancement of heat shock element-binding activity by heat shock factor results in an enhanced expression of the heat-induced HSP27 genes [Rabindran et al., 1991; Sorger, 1991]. Our results suggest that midazolam enhances the induction of HSP27 by heat in vascular smooth muscle A10 cells. It is possible that midazolam interacts with heat shock transcription factor and this conjugate can bind more efficiently to the heat shock element of HSP27 gene. Further investigation would be required to clarifying the details.

We found that neither heat nor arsenite induced phosphoinositide hydrolysis by phospholipase C or phosphatidylcholine hydrolysis by phospholipase D in A10 cells (data not shown). In addition, we found that vasopressin had little effect on the binding of heat-shock elements (using a gel shift assay), but that heat did stimulate this binding (data not shown). Thus, it is probable that for HSP27 induction in A10 cells, there are at least two pathways. One involves activation of protein kinase C through phosphoinositide hydrolyzing-phospholipase C and phosphatidylcholine hydrolyzing-phospholipase D. The other involves activation of heat-shock transcriptional factors and does not require activation of protein kinase C. In the present study, we demonstrated that in vascular smooth muscle A10 cells, midazolam inhibited physiological agonist (vasopressin)-stimulated HSP27 induction, but increased both heat- and arsenite-stimulated HSP27 induction. On the basis of our findings, it is most likely that midazolam has dual effects: it inhibits the pathway involving activation of protein kinase C, but enhances the other pathway.

HSP27 exists in two forms, an aggregated form and a dissociated form [Kato et al., 1994]. It is known that the dissociation of HSP27 occurs as a result of phosphorylation of the aggregated form of HSP27 [Kato et al., 1994].

We have shown that extracts of unstressed A10 cells contained both forms. Dissociation of the aggregated form to the dissociated form can be induced by incubation of A10 cells with vasopressin [Ito et al., 2000]. In this study, we found that arsenite induces the dissociation of the aggregated form to the dissociated form in these cells. Thus, these findings suggest that vasopressin and arsenite phosphorylate HSP27. Taking our results into account, it is most likely that vasopressin and arsenite not only phosphorylate HSP27 but also stimulate HSP27 induction in aortic smooth muscle A10 cells. In the present study, we demonstrated that midazolam affects neither the vasopressin- nor the arsenite-stimulated dissociation of the aggregated form of HSP27. Therefore, these results suggest that midazolam does not affect the phosphorylation of HSP27. Based on our findings concerning the effects of midazolam, it seems that the phosphorylation of HSP27 is independent of the HSP27 induction in vascular smooth muscle A10 cells.

It is recognized that low molecular-weight HSPs act as chaperones, like high molecular-weight HSPs, in various cells [Jakob et al., 1993; Benjamin and Mcmillan, 1998]. Recently, it has been reported that HSP27 is involved in the regulation of actin assembly [Benjamin and Mcmillan, 1998], and it has been speculated that HSP27 plays an important role in vascular smooth muscle contraction [Brophy et al., 1997, 1998; Yamboliev et al., 2000]. Vasopressin, the HSP27-inducing action of which is inhibited by midazolam, is a potent vasoactive agent [Scott-Burden et al., 1992]. The systemic blood pressure in patients treated with a continuous intravenous infusion of midazolam is reportedly decreased [Shafer, 1998], and midazolam has been shown to have direct effects on the peripheral vasculature [Chang et al., 1994; McNulty et al., 1994; Yamaguchi et al., 1997]. These findings lead us to speculate that the decrease in systemic blood pressure seen under midazolam may be associated with its inhibitory effect on vasopressin-stimulated HSP27 induction.

In conclusion, these results suggest that midazolam has dual effects on the induction of HSP27 by various stresses in vascular smooth muscle cells. It suppresses vasopressin-stimulated HSP27 induction, an inhibitory effect is exerted at a point downstream from protein kinase C. On the other hand, it enhances both the heat- and arsenite-stimulated induction of HSP27 in the same cells.

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