Pentobarbital, but not Propofol, Suppresses Vasopressin-stimulated Heat Shock Protein 27 Induction in Aortic Smooth Muscle Cells

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Background: Although it is known that systemic blood pressure decreases after the administration of pentobarbital or propofol, the mechanisms underlying the cardiovascular effects of these anesthetics are still poorly understood. The authors previously showed that vasopressin stimulates the induction of heat shock protein (HSP) 27, a low-molecular-weight HSP, by a protein kinase C–dependent manner in aortic smooth muscle A10 cells. It is recognized that HSP27 may act as a chaperone like high-molecular-weight HSPs such as HSP70. HSP27 is reportedly associated with agonist-induced contraction of vascular smooth muscle cells. The authors examined the effects of pentobarbital and propofol on the vasopressin-stimulated HSP27 induction in A10 cells.

Methods: Cultured A10 cells were pretreated with pentobarbital or propofol and then stimulated by vasopressin or 12-o-tetradecanoylphorbol 13-acetate (TPA). The effect of vasopressin on HSP70 was evaluated by Western blot analysis and compared with its effect on HSP27. The concentrations of HSP27 were determined by a specific immunoassay. The effect of pentobarbital on the expression levels of mRNA for HSP27 by vasopressin was evaluated by Northern blot analysis.

Results: Vasopressin induced HSP27 but had little effect on HSP70. At concentrations used clinically, pentobarbital inhibited the accumulation of HSP27 by vasopressin or TPA. Pentobarbital reduced the levels of mRNA for HSP27 induced by vasopressin. In contrast, propofol affected neither the vasopressin- nor TPA-induced HSP27 accumulation.

Conclusions: These results suggest that pentobarbital suppresses the vasopressin-stimulated HSP27 induction in vascular smooth muscle cells. This inhibitory effect is probably exerted at a point downstream from protein kinase C. (Key words: A10 cells; anesthetic; stress; vascular.)

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PENTOBARBITAL and propofol reduce systemic blood pressure after intravenous administration.[1-3] It has been shown that these anesthetics have direct and indirect effects on the cardiovascular system.[2,3] These anesthetics have been reported to depress systemic blood pressure *via* a redistribution of blood volume through the decrease of systemic vascular resistance.[2,3] In addition, it has been shown that the suppression of myocardial contractility is correlated with the reduction of systemic blood pressure.[2,4,5] However, the exact mechanisms behind the effects of these anesthetics on the cardiovascular system have not yet been fully clarified.

Vascular smooth muscle cells play important roles in the regulation of vascular tone and in atherosclerosis.[6] Vasopressin is a potent vasoactive agent that has been reported to bind to V_1 receptors in vascular smooth muscle cells.[7] Vasopressin induces phosphoinositide hydrolysis by phospholipase C and phosphatidylcholine hydrolysis by phospholipase D in vascular smooth muscle cells.[7-9] It has been shown that phosphatidylcholine breakdown occurs downstream from phosphoinositide hydrolysis by phospholipase C in a vascular smooth

muscle cell line, A10 cells.[8] Both of these types of hydrolysis form diacylglycerol, which is a physiologic activator of protein kinase C.[10] It is well recognized that the activation of protein kinase C plays important roles in signal transduction after vasopressin binding in vascular smooth muscle cells.[11]

Diverse stresses (such as exposure to heat or chemicals) produce heat shock proteins (HSPs) in cells.[12,13] In addition, several HSPs are expressed constitutively in unstressed cells, where they may perform essential functions.[13,14] There are high- and low-molecular-weight HSPs.[14] High-molecular-weight HSPs such as HSP70 and HSP90 are well known to act as molecular chaperones implicated in protein folding and oligomelization.[15,16] Low-molecular-weight HSPs with molecular masses of 15–30 kd, such as HSP27 and αB-crystallin, have significant similarities in terms of amino acid sequences.[17,18] In previous studies,[19,20] we have shown that HSP27 and αB-crystallin are present in various tissues and cells, especially in skeletal and smooth muscle cells. It is recognized that low-molecular-weight HSPs may also act as chaperone proteins.[14,21] Among low-molecular-weight HSPs, evidence is accumulating that HSP27 participates in several functions in a variety of cells types, such as modification of growth in epidermal cells,[22] regulation of apoptosis in murine fibrosarcoma cells,[23] secretion of granules in human platelets,[24] and contraction of vascular smooth muscle cells.[25,26] These observations suggest that HSP27 may play important roles in a variety of physiologic cellular functions. We previously reported that vasopressin stimulates the induction of HSP27 through protein kinase C activation in A10 cells.[27] Because HSP27 is involved in contraction of vascular smooth muscle cells[25,26] and intravenous anesthetics such as pentobarbital and propofol have blood pressure–lowering effects,[1–3] we thought it possible that the anesthesia by such agents might be associated with a reduction in HSP27 in vascular smooth muscle cells. In the present study, we investigated the effects of pentobarbital and propofol on the vasopressin-stimulated induction of HSP27 in A10 cells.

Materials and Methods

Materials

Arginine vasopressin was purchased from Peptide Institute Inc. (Minoh, Japan). Pentobarbital was purchased from Nacalai Tesque, Inc. (Tokyo, Japan). Propofol was purchased from Aldrich (Tokyo, Japan). 12-*o*-tetradeca-noylphorbol 13-acetate (TPA) was purchased from Sigma Chemical Co. (St. Louis, MO). HSP70 antibodies were purchased from Santa Cruz (Santa Cruz, CA). An ECL Western blotting detection system was obtained from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. Vasopressin and pentobarbital were dissolved in an assay buffer (consisting of 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 pentobarbital. Propofol was dissolved in ethanol. The maximum concentration of ethanol in the culture medium was 0.1%, and this did not affect the immunoassay of HSP27. We used the assay buffer containing 0.01% BSA and 0.1% ethanol as the vehicle control for propofol.

Cell Culture

A10 cells derived from rat aortic smooth muscle cells[28] were obtained from the American Type Culture Collection (Rockville, MD). The cells (1 × 10^5) were seeded into 35- or 90-mm-diameter dishes and maintained at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air in 2 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum. After 5 days, the medium was exchanged for 2 ml of serum-free Dulbecco's modified Eagle's medium. The cells were used for experiments 48 h later. The cells were treated with pentobarbital or propofol in serum-free Dulbecco's modified Eagle's medium for the indicated periods.

Western Blot Analysis of HSP70 and HSP27

Cultured cells were stimulated by vasopressin for 48 h. The stimulated cells were rinsed twice with the assay buffer, lysed, homogenized and sonicated in a lysis buffer containing 62.5 mM Tris/Cl, pH 6.8, 2% sodium dodecyl sulfate, 50 mM dithiothreitol, and 10% glycerol. The cytosolic fraction was collected as the supernatant after centrifugation at 125,000g for 10 min at 4°C. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed by the method of Laemmli[29] in 10% polyacrylamide gel. Western blotting analysis was performed as described previously[19,20] using HSP70 antibodies or HSP27 antibodies, with peroxidase-labeled antibodies raised in goat against rabbit immunoglobulin G 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.

Immunoassay of HSP27

The concentrations of HSP27 in soluble extracts of the cells were determined by means of a sandwich-type enzyme immunoassay, as described previously.[27] Cultured cells were pretreated with pentobarbital (0.1–0.8 mM), propofol (1 μM to 0.1 mM), or vehicle and then stimulated by vasopressin (0.1 μM) or TPA (0.1 μM) for the indicated periods in 1 ml of serum-free Dulbecco's modified Eagle's medium. The stimulated cells were washed twice with 1 ml of phosphate-buffered saline and 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 125,000g 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 the samples. This incubation was conducted 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 mU 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 mRNA for HSP27

Cultured cells were pretreated with pentobarbital (0.8 mM) for 20 min and then stimulated by vasopressin (0.1 μM) in 1 ml of serum-free Dulbecco's modified Eagle's medium. After 12 h, the stimulated cells were washed twice with 1 ml of phosphate-buffered saline and 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 and 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 sys-
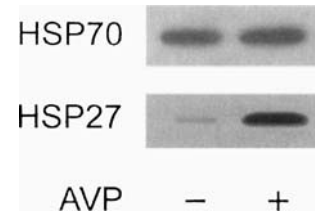

Fig. 1. Effect of vasopressin (AVP) on levels of HSP70 and HSP27 in A10 cells. Cultured cells were exposed to 0.1 μM of AVP or vehicle for 48 h. Cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by Western blot analysis using antibodies against HSP70 or HSP27.

tem (Amersham, Buckinghamshire, United Kingdom), as described previously.[30] A *Bam*HI-*Hind*III fragment of cDNA for mouse HSP27 was provided by Dr. L. F. Cooper of the University of North Carolina.[31]

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.[19,20]

Statistical Analysis

The data were analyzed by analysis of variance followed by the Student-Newman-Keuls test. P values < 0.05 were considered significant. All data are presented as the mean $\pm$ SD of triplicate determinations.

Results

Effects of Vasopressin on the Induction of HSP70 and HSP27

Vasopressin had little effect on the levels of HSP70 in vascular smooth muscle A10 cells while increasing the levels of HSP27 in the same sample (fig. 1).

Effects of Pentobarbital and Propofol on the Vasopressin-induced Accumulation of HSP27

Pentobarbital (0.4 mM), which by itself had little effect on the basal levels of HSP27, significantly (time-dependently up to 48 h) inhibited the HSP27 accumulation induced by vasopressin (0.1 μM; fig. 2A). On the contrary, propofol (0.1 mM) did not affect the vasopressin-stimulated accumulation of HSP27 up to 48 h (data not shown). The inhibitory effect of pentobarbital on the HSP27 accumulation was dose-dependent (0.1–0.8 mM), and it was significant at doses of 0.2 mM or more (fig.

Fig. 2. Effect of pentobarbital on vaso-
pressin-induced accumulation of HSP27
in A10 cells. (*A*) Time course of the accu-
mulation of HSP27 during the incubation
period after vasopressin stimulation. Cul-
tured cells were pretreated with 0.4 mм
pentobarbital (closed symbols) or vehicle
(open symbols) for 20 min, then exposed
to 0.1 µм of vasopressin (circles) or vehi-
cle (triangles) for the indicated periods.
(*B*) Dose-dependent effect of pentobarbi-
tal on the accumulation of HSP27. Cul-
tured cells were pretreated with various
doses of pentobarbital for 20 min, then
exposed to 0.1 µм vasopressin (closed
circles) or vehicle (open circles) for 48 h.
The concentrations of HSP27 in cell ex-
tracts were determined by a specific im-
munoassay. Each value represents the
mean ± SD of triplicate determinations.
Similar results were obtained with two
other cell preparations. *P* < 0.05 com-
pared with vasopressin alone.

Time (h)
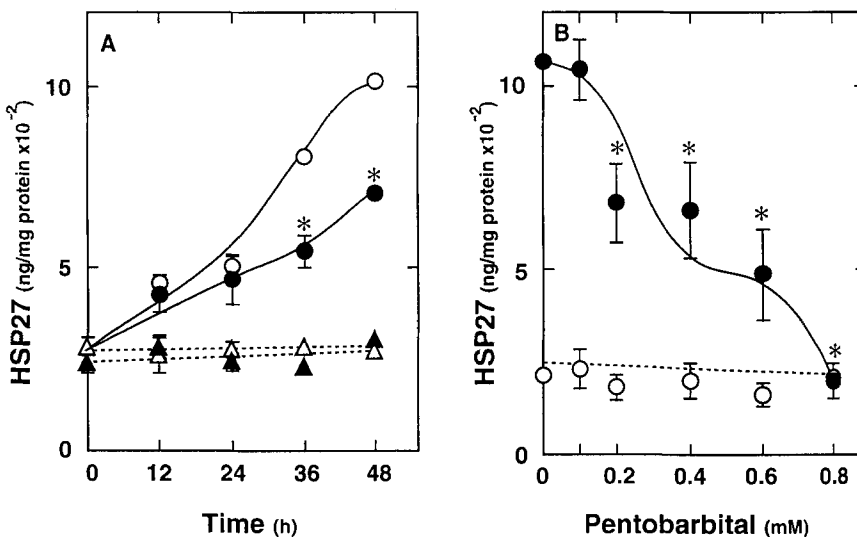
Pentobarbital (mM)

2B). In contrast, propofol had little effect on the HSP27
accumulation induced by vasopressin over the range 1
µм to 0.1 mм (fig. 3). Long-term treatment (48 or 96 h)
with pentobarbital did not affect the basal levels of
HSP27 (table 1). In addition, pentobarbital reduced the
vasopressin-stimulated HSP27 accumulation regardless
of whether the cells had or had not been pretreated with
pentobarbital (table 1).

Effect of Pentobarbital on the Vasopressin-stimulated Levels of mRNA for HSP27

We previously showed that vasopressin stimulates the
expression levels of mRNA for HSP27.[27] We next exam-
ined the effect of pentobarbital on the levels of mRNA
for HSP27 induced by vasopressin. Pentobarbital (0.8
mм), which alone did not affect the basal levels, signifi-
cantly reduced the vasopressin (0.1 µм)-stimulated levels
of mRNA for HSP27 (fig. 4).

Effects of Pentobarbital and Propofol on the TPA-induced Accumulation of HSP27

We previously reported that TPA alone, an activator of
protein kinase C,[32] significantly stimulates HSP27 induc-
tion in A10 cells and that vasopressin stimulates the
induction of HSP27 through protein kinase C activa-
tion.[27] Pentobarbital markedly inhibited the HSP27 ac-
cumulation induced by TPA (0.1 µм; fig. 5). This inhib-
itory effect of pentobarbital on the HSP27 accumulation
was dose-dependent (0.1–0.8 mм), and it was significant
at doses of 0.1 mм or more (fig. 5). In contrast, propofol
(0.1 mм) had no such effect (284 ± 18 ng/mg protein for

vehicle; 900 ± 144 ng/mg protein for 0.1 µм TPA; 312 ±
46 ng/mg protein for 0.1 mм propofol; 961 ± 228 ng/mg
protein for 0.1 µм TPA with 0.1 mм propofol, as mea-
sured during a stimulation of 48 h).

Discussion

We previously showed that in an aortic smooth muscle
cell line, A10 cells, vasopressin stimulates the induction
of HSP27.[27] In the present study, we examined the
effects of pentobarbital and propofol on the HSP27 in-

Table 1. Effect of Pretreatment with Pentobarbital on the Accumulation of HSP27 by Vasopressin in A10 Cells

Pretreatment with Pentobarbital	Agent	HSP27 (ng/mg protein)
−	Control	305 ± 32
−	Vasopressin	805 ± 57
−	Pentobarbital	277 ± 24
−	Vasopressin + pentobarbital	347 ± 33*
+	Control	342 ± 33
+	Vasopressin	967 ± 44
+	Pentobarbital	328 ± 35
+	Vasopressin + pentobarbital	309 ± 29*

Cultured cells were pretreated with pentobarbital (0.8 mм) or vehicle. After
48 h, the medium was replaced and the cells were exposed to 0.1 µм
vasopressin or vehicle in the presence or absence of pentobarbital (0.8 mм)
for 48 h. The concentrations of HSP27 in cell extracts were 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.
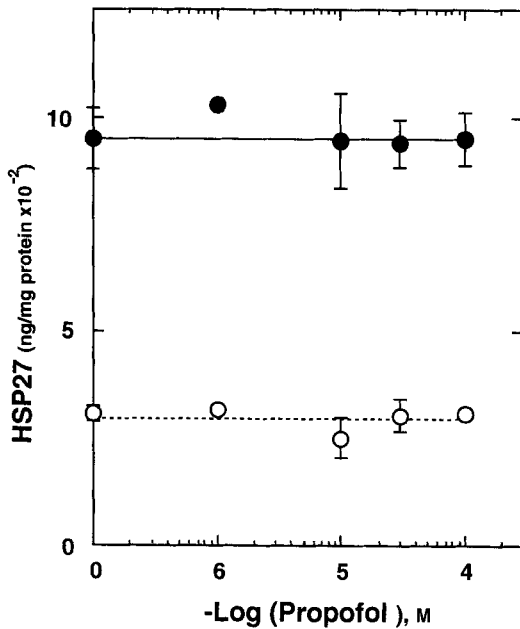HSP = heat shock protein.

Fig. 3. Effect of propofol on vasopressin-induced accumulation of HSP27 in A10 cells. Cultured cells were pretreated with various doses of propofol for 20 min, then exposed to 0.1 μM vasopressin (closed circles) or vehicle (open circles) for 48 h. The concentrations of HSP27 in cell extracts were determined by a specific immunoassay. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained with two other cell preparations.

duction stimulated by vasopressin in these cells. Pentobarbital significantly reduced the vasopressin-induced accumulation of HSP27 in a time- and dose-dependent fashion. In contrast, propofol had no such effect. In addition, we demonstrated that pentobarbital suppressed the vasopressin-stimulated expression levels of mRNA for HSP27. Thus, our findings suggest that pento-
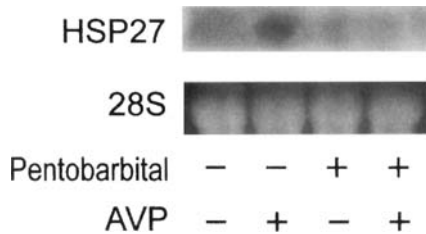

Fig. 4. Effect of pentobarbital on vasopressin (AVP)-stimulated levels of mRNA for HSP27 in A10 cells. Cultured cells were pretreated with 0.8 mM of pentobarbital or vehicle for 20 min, then exposed to 0.1 μM of AVP or vehicle for 12 h. The cells were collected, and total RNA was isolated. Twenty micrograms 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 of 28S are shown for reference.

barbital, but not propofol, inhibits the vasopressin-stimulated HSP27 induction in aortic smooth muscle cells.

High-molecular-weight HSPs such as HSP70 are well known to act as molecular chaperones.[14] It is recognized that low-molecular-weight HSPs such as HSP27 may also act as chaperones.[14,21] It has been shown that heat stress induces both HSP70 and HSP27 in vascular smooth muscle cells.[14] In addition, an expression of HSP27 is reportedly induced in aortic smooth muscle cells in restrained rats.[14] In our study, vasopressin did not affect the levels of HSP70, but it did stimulate the levels of HSP27 in A10 cells. As for the roles played by HSP27 in the vascular system, it has recently been reported that thrombin and serotonin induce contraction of vascular smooth muscle, and that the contraction is associated with an increased phosphorylation of HSP27.[25,26] These findings lead us to speculate that HSP27 may have important effects on aortic smooth muscle cell functions after stimulation by vasoactive agents. It is well recognized that the administration of pentobarbital or propofol reduces systemic blood pressure.[2,3] These anesthetics have direct and indirect effects on the cardiovascular system.[2,3] As for the direct effect on the cardiovascular system, it has been shown that they reduce the systemic vascular resistance through affecting vascular smooth muscle cells.[2,3,33,34] Investigations of HSPs, especially HSP27, in vascular smooth muscle cells suggest that HSP27 may contribute to physiologic and pathologic processes such as contraction, atherosclerosis, and aging of the vasculature.[14] In addition, intravenous anesthetics are administered to patients suffering from various stresses and complications. Consequently, further investigations of the relationship between intravenous anesthetics and HSPs in vascular smooth muscle cells could make an important contribution to clarify the exact mechanism underlying the effects of these agents on the vascular system.

In the present study, we showed that pentobarbital also reduced the TPA-induced accumulation of HSP27. We previously reported that TPA alone significantly stimulates HSP27 induction in A10 cells and that protein kinase C activation is involved in the vasopressin-stimulated induction of HSP27.[27] Therefore, it is probable that the inhibitory effect of pentobarbital is exerted at a point downstream from protein kinase C in these cells. On the other hand, in the present study, propofol did not affect the accumulation of HSP27 induced by vasopressin or TPA. We have reported that in A10 cells, propofol inhibits protein kinase C activation through a suppression of the activation of both phosphatidylinositol-hydrolyzing
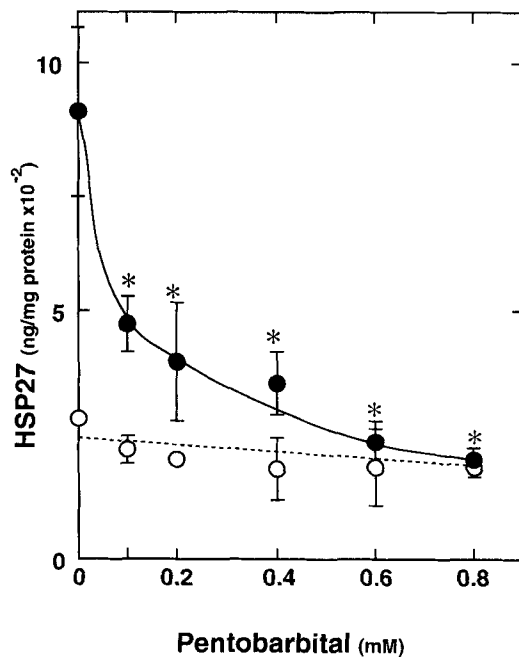
Pentobarbital (mM)

Fig. 5. Effect of pentobarbital on TPA-induced accumulation of HSP27 in A10 cells. Cultured cells were pretreated with various doses of pentobarbital for 20 min, then exposed to 0.1 μM TPA (closed circles) or vehicle (open circles) for 48 h. The concentrations of HSP27 in cell extracts were 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.

phospholipase C and phosphatidylcholine-hydrolyzing phospholipase D by endothelin-1 and vasopressin.[35,36] Clinically, propofol decreases systemic blood pressure after a bolus injection.[3] However, systemic blood pressure remains stable at the lower level during continuous infusion.[37,38] Therefore, it is conceivable that propofol inhibits short-acting signaling by vasoactive agents, resulting in a more-or-less immediate suppression of systemic blood pressure, but does not affect long-acting signaling, such as the induction of HSP27 seen in aortic smooth muscle cells.

Pentobarbital is used to control intracranial pressure, with serum concentrations reported to reach as high as 0.45 mM during a 4-mg · kg^{-1} · h^{-1} continuous infusion.[1] It has been shown that the protein binding of pentobarbital is 60%.[39] Thus, the free concentration of pentobarbital could reach 0.18 mM. If this is so, it is probable that a suppression of the induction of HSP27 by pentobarbital would occur in vascular smooth muscle cells at the doses used clinically. On the other hand, the plasma concentration of propofol has been reported to be 56–190 μM in patients in whom general anesthesia is main-

tained with continuous intravenous infusion of propofol alone.[40] It has been shown that approximately 97% of propofol is bound to plasma proteins.[41] Thus, free concentration of propofol is estimated to be 2–6 μM. In the current study, propofol had no effect on the vasopressin-stimulated HSP27 induction over the range 1 μM to 0.1 mM. Therefore, it is possible that propofol dose not affect on the induction of HSP27 stimulated by vasopressin, not only at doses use clinically, but also at the doses over clinical concentration.

In conclusion, these results suggest that pentobarbital suppresses the vasopressin-stimulated HSP27 induction in vascular smooth muscle cells. This inhibitory effect is probably exerted at a point downstream from protein kinase C.

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