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Protein Kinase C Activation Inhibits Stress-Induced Synthesis of Heat Shock Protein 27 in Osteoblast-Like Cells: Function of Arachidonic Acid

Atsushi Suzuki, Osamu Kozawa, Yutaka Oiso, and Kanefusa Kato

First Department of Internal Medicine, Nagoya University School of Medicine, Nagoya 466 (A.S., Y.O.), and Department of Biochemistry, Institute for Developmental Research, Aichi Human Service Center, Kasugai, Aichi 480-03 (O.K., K.K.), Japan

Abstract Exposure of osteoblast-like MC3T3-E1 cells to sodium arsenite (arsenite) increased the level of heat shock protein 27 (hsp27). The effect of arsenite was dose-dependent in the range of 50 to 200 μM. Arsenite also stimulated arachidonic acid release dose-dependently in the range between 50 and 200 μM in these cells. Both indomethacin, an inhibitor of cyclooxygenase, and nordihydroguaiaretic acid, a lipoxygenase inhibitor, significantly enhanced the arsenite-induced accumulation of hsp27. Melittin, an activator of phospholipase A_2, significantly enhanced the arsenite-induced accumulation of hsp27. 12-O-Tetradecanoylphorbol-13-acetate (TPA), a protein kinase C (PKC)-activating phorbol ester, inhibited the arsenite-induced accumulation of hsp27. In contrast, 4α-phorbol 12,13-didecanoate (4α-PDD), a PKC-nonactivating phorbol ester, had little effect. TPA suppressed the arsenite-induced arachidonic acid release, but 4α-PDD had little effect. Arsenite no longer affected cAMP accumulation, inositol phosphates formation nor the formation of choline and phosphocholine in these cells. These results suggest that the response to stress of hsp27 is coupled with the metabolic activity of the arachidonic acid cascade, and the activation of PKC inhibits the induction of hsp27 through the suppression of arachidonic acid release in osteoblast-like cells.

Key words: heat shock protein 27, arachidonic acid, protein kinase C, osteoblast

Cellular responses to biological stresses induce the expression of several multigene families of heat shock protein (hsp) that are grouped according to their apparent molecular weight, hsp27, hsp60, hsp70, hsp90, and hsp110 [Lindquist, 1986]. Among them, the mammalian small Mr hsp, hsp27, has been reported to be induced by heat-shock in various cells [Blake et al., 1990; Inaguma et al., 1993; Kato et al., 1993]. As for bone cells, it has been reported that hsp27 expression is induced by heat-shock in osteoblasts including osteoblast-like MC3T3-E1 cells and the down-regulation of their proliferation is accompanied by a transient increase of the expression of hsp27 mRNA [Shakoori et al., 1992; Cooper and Uoshima, 1994]. Although the function of small hsps is less well understood than that of larger hsps (60, 70, 90 kDa), it has recently been reported that small hsps can act as molecular chaperones as well as larger hsps [Benndorf et al., 1994; Groenen et al., 1994].

It is recognized that physiological factors modulate the responses to heat stress [Jurivich et al., 1994]. However, the exact mechanism of stress responses, especially the signaling system, has not yet been fully clarified. We have shown that the activation of PKC is involved in the process which suppresses the differentiation of osteoblast-like MC3T3-E1 cells and directs them toward proliferation [Kozawa et al., 1989]. In the present study, we investigated the mechanism of arsenite-induced hsp27 accumulation and the role of PKC activation in these cells. We herein show that the response to stress of hsp27 is coupled with the metabolic activity of the arachidonic acid cascade, and the activation of PKC inhibits the accumulation of hsp27 through the suppression of arachidonic acid release in osteoblast-like cells.

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Address reprint requests to Osamu Kozawa, M.D., Department of Pharmacology, Gifu University School of Medicine, Gifu 500, Japan.

MATERIALS AND METHODS

Materials

[5, 6, 8, 9, 11, 12, 14, 15-^{3}H]arachidonic acid (212.1 Ci/mmol), *myo*-[^{3}H]inositol (90 Ci/mmol) and [*methyl*-^{3}H]choline chloride (85 Ci/mmol) were purchased from Amersham Japan (Tokyo, Japan). The cAMP radioimmunoassay kit was kindly provided by Yamasa Shoyu Co. (Chiba, Japan). Indomethacin, nordihydroguaiaretic acid, melittin, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and 4α-phorbol 12,13-didecanoate (4α-PDD) were purchased from Sigma Chemical Co. (St. Louis, MO). Other materials and chemicals were obtained from commercial sources. Melittin, TPA, and 4α-PDD were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1% and this did not affect the assay for the accumulation of hsp27 nor the measurement of arachidonic acid release.

Cell Culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [Kodama et al., 1981; Sudo et al., 1983] were generously provided by Dr. M. Kumegawa (Meikai University, Sakado, Japan) and maintained in α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells (5×10^4) were seeded into 35-mm diameter dishes in 2 ml of α-MEM containing 10% FCS. After 5 days, the medium was exchanged for 2 ml of α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h. In experiments for the formation of inositol phosphates, the medium was exchanged for 2 ml of inositol-free α-MEM containing 0.3% FCS.

Assay for the Accumulation of hsp27

The cultured cells were exposed to various doses of arsenite at 37°C for 1 h, and the medium was then exchanged for 1 ml of α-MEM containing 0.3% FCS. The cells were incubated at 37°C until harvest. After 16 h, the cells were rinsed twice with phosphate-buffered saline (PBS) and frozen at −20°C. The frozen cells on each dish were collected and suspended in 0.3 ml of PBS, and each suspension was sonicated and centrifuged at 125,000 × *g* for 20 min at 4°C. The supernatant was used for quantification of hsp27. When indicated, the cells were pretreated with indomethacin, nordihydroguaiaretic acid, melittin, TPA, or 4α-PDD for 20 min.

Immunoassay of hsp27

Concentration of hsp27 in extracts of cells was determined by specific immunoassay, as previously described [Inaguma et al., 1993].

Measurement of Arachidonic Acid Release

Measurement of arachidonic acid release was performed as previously described [Suzuki et al., 1993]. In brief, the cultured cells were labeled with [^{3}H]arachidonic acid (0.5 μCi/dish) for 24 h. The medium was then removed and the cells were washed four times with 1 ml of buffer A [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, and 1 mM $CaCl_2$]. The cells were preincubated subsequently with 1 ml of the buffer A containing 0.1% essentially fatty acid-free bovine serum albumin (BSA) at 37°C for 20 min, and then stimulated by various doses of arsenite. The reaction was terminated by collecting the medium and the radioactivity of the medium was determined. When indicated, the cells were pretreated with TPA or 4α-PDD for 20 min.

Assay for cAMP

The cultured cells were preincubated with 0.5 mM 3-isobutyl-1-methylxanthine for 10 min in 1 ml of buffer B [5 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 5.5 mM glucose, 0.8 mM $MgSO_4$, and 1 mM $CaCl_2$] containing 0.01% BSA. The cells were then stimulated by various doses of arsenite at 37°C for the indicated periods. The reaction was terminated by aspirating the medium, and the intracellular cAMP was then extracted with 1 ml of 90% *n*-propanol as previously described [Kozawa et al., 1992]. cAMP in the extracts was measured by a radioimmunoassay kit.

Measurement of the Formation of Inositol Phosphates

The cultured cells were labeled with *myo*-[^{3}H]inositol (3 μCi/dish) for 48 h. The labeled cells were preincubated with 10 mM LiCl for 10 min in 1 ml of buffer B containing 0.01% BSA, and then stimulated by various doses of arsenite for the indicated periods. The reaction was terminated by adding 1 ml of 30% trichloroacetic acid. The acid supernatant was treated with diethyl

ether to remove the acid and neutralized with 0.1 M NaOH. The radioactive inositol phosphates were measured as previously described [Suzuki et al., 1994].

Measurement of the Formations of Choline and Phosphocholine

The cultured cells were labeled with [*methyl*-^{3}H]choline chloride (2 μCi/dish) for 24 h. The labeled cells were preincubated in 1 ml of buffer B containing 0.01% BSA at 37°C for 20 min. The cells were then stimulated by various doses of arsenite for the indicated periods. The radioactive choline and phosphocholine were measured as previously described [Kozawa et al., 1994].

Other Methods

Concentrations of protein in soluble extracts were estimated with a protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard. Rat hsp 27, which was used as the standard for immunoassays, was purified from skeletal muscle [Inaguma et al., 1993].

Determinations

The radioactivity of ^{3}H-samples was determined with a Beckman LS-6000IC liquid scintillation spectrometer. The radioactivity of ^{125}I-samples was determined with an Aloka ARC-600 auto well gamma system.

Statistical Analysis

The data were analyzed by Student's *t*-test and a $P < 0.05$ was considered significant. All data are presented as the mean ± S.D. of triplicate determinations.

RESULTS

Effect of Arsenite on hsp27 Accumulation in MC3T3-E1 Cells

The concentration of hsp27 in confluent cultures of osteoblast-like MC3T3-E1 cells was below 0.5 ng/mg protein. Arsenite significantly increased the level of hsp27 in MC3T3-E1 cells. The effect of arsenite was dose-dependent in the range between 50 and 200 μM (Fig. 1).

Effect of Arsenite on Arachidonic Acid Release in MC3T3-E1 Cells

To investigate the intracellular signaling system of arsenite in osteoblast-like MC3T3-E1 cells, we first examined the effect of arsenite on arachidonic acid release in these cells. Arsenite

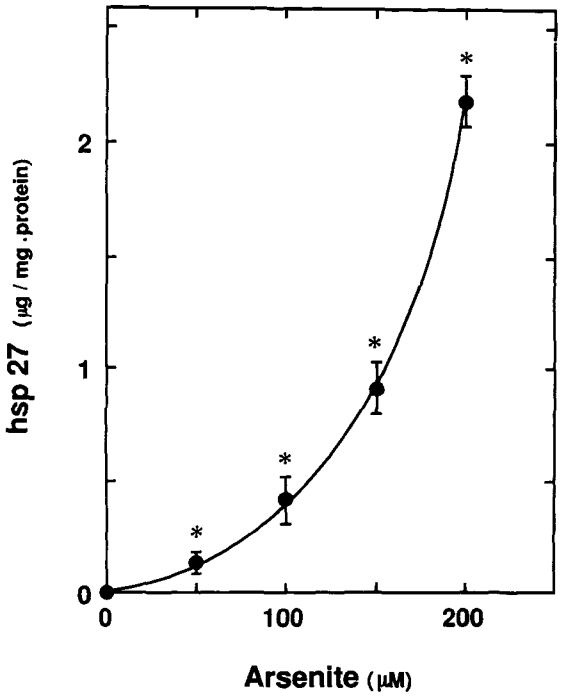

Fig. 1. Effect of arsenite on the accumulation of hsp27 in MC3T3-E1 cells. The cultured cells were stimulated by various doses of arsenite at 37°C for 1 h, and subsequently incubated at 37°C for 16 h in α-MEM containing 0.3% FCS. Each value represents the mean ± S.D. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$ compared to the value of control.

significantly stimulated arachidonic acid release dose-dependently in the range between 50 and 200 μM in MC3T3-E1 cells (Fig. 2). The effect of arsenite was time-dependent up to 60 min (data not shown).

Effects of Arsenite on cAMP Accumulation, and Formations of Inositol Phosphates, Choline, and Phosphocholine in MC3T3-E1 Cells

Arsenite did not affect cAMP accumulation in the range between 50 and 200 μM in MC3T3-E1 cells (30 ± 6 pmol/dish for control; 28 ± 5 pmol/dish for 200 μM arsenite, as measured during stimulation of 5 min). Arsenite did not stimulate the formation of inositol phosphates in these cells (2,254 ± 189 cpm for control; 2,111 ± 222 cpm for 200 μM arsenite, as measured during stimulation of 10 min). Arsenite had little effect on the formation of choline in these cells (3,654 ± 122 cpm for control; 3,933 ± 201 cpm for 200 μM arsenite, as measured during stimulation of 15 min). Arsenite did not affect the formation of phosphocholine in these cells (11,789 ± 1,021 cpm for control; 12,444 ± 987

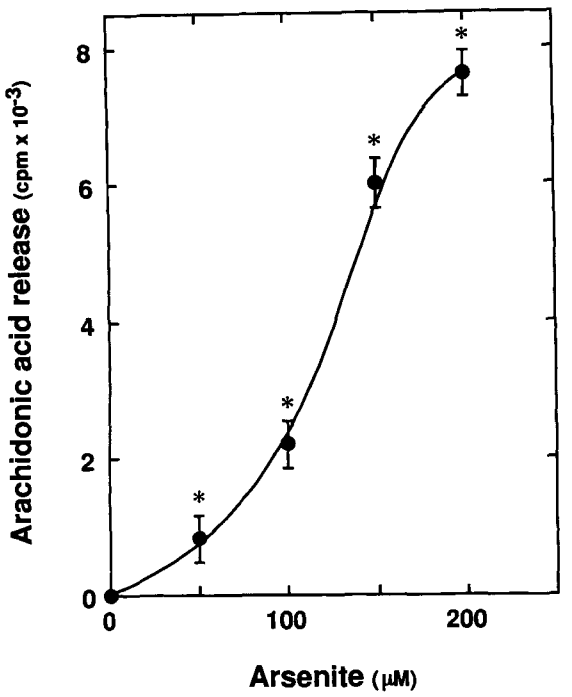

Fig. 2. Effect of arsenite on the arachidonic acid release in MC3T3-E1 cells. The labeled cells were stimulated by various doses of arsenite for 60 min. Values for unstimulated cells have been subtracted from each data point. Each value represents the mean ± S.D. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$ compared to the value of control.

cpm for 200 μM arsenite, as measured during stimulation of 15 min). Arsenite did not affect either the cAMP accumulation, or the formations of inositol phosphates, choline, and phosphocholine at any time point between 1 and 30 min after the stimulation.

Effects of Indomethacin, Nordihydroguaiaretic Acid, and Melittin on Arsenite-Induced Accumulation of hsp27 in MC3T3-E1 Cells

To clarify the role of arachidonic acid in the arsenite-induced hsp27 production, we first examined the effects of indomethacin, which is known to inhibit cyclooxygenase [Smith, 1989], and nordihydroguaiaretic acid, an inhibitor of lipoxygenase [Domin et al., 1994], on the arsenite-induced accumulation of hsp27 in MC3T3-E1 cells. The pretreatment with indomethacin, which by itself had little effect on the accumulation of hsp27, significantly enhanced the arsenite-induced accumulation of hsp27 in MC3T3-E1 cells (Table I). Nordihydroguaiaretic acid, which did not affect the basal level of hsp27, enhanced the arsenite-induced accumulation of hsp27 in these cells (Table I). In addition, melittin known to be an activator of phospholipase A_2 (Shier, 1979), which by itself had little effect on hsp27 accumulation, significantly enhanced the arsenite-induced accumulation of hsp27 in MC3T3-E1 cells (Table II).

TABLE I. Effects of Indomethacin and Nordihydroguaiaretic Acid on Arsenite-Induced Accumulation of hsp27 in MC3T3-E1 Cells*

	hsp27 (ng/mg protein)
Control	< 0.5
Arsenite	133 ± 12
Indomethacin	< 0.5
Indomethacin + arsenite	378 ± 28[a]
Nordihydroguaiaretic acid	< 0.5
Nordihydroguaiaretic acid + arsenite	279 ± 22[a]

*The cultured cells were pretreated by 50 μM of indomethacin, nordihydroguaiaretic acid, or vehicle for 20 min, and then stimulated by 50 μM of arsenite at 37°C for 1 h. The cells were subsequently incubated for 16 h at 37°C in α-MEM containing 0.3% FCS. Each value represents the mean ± S.D. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.
[a]$P < 0.05$ compared to the value of arsenite alone.

TABLE II. Effect of Melittin on Arsenite-Induced Accumulation of hsp27 in MC3T3-E1 Cells*

	hsp27 (ng/mg protein)
Control	< 0.5
Arsenite	141 ± 11
Melittin	< 0.5
Melittin + arsenite	211 ± 16[a]

*The cultured cells were pretreated with 1 μM melittin or vehicle for 20 min. The cells were then stimulated by 50 μM of arsenite at 37°C for 1 h. The cells were subsequently incubated for 16 h at 37°C in α-MEM containing 0.3% FCS. Each value represents the mean ± S.D. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.
[a]$P < 0.05$ compared to the value of arsenite alone.

Effects of Phorbol Esters on Arsenite-Induced Accumulation of hsp27 in MC3T3-E1 Cells

To clarify the role of PKC activation in the arsenite-induced hsp27 production, we next examined the effect of TPA, a PKC-activating phorbol ester [Nishizuka, 1986], on the arsenite-induced accumulation of hsp27 in MC3T3-E1 cells. TPA, which by itself had no effect on the accumulation of hsp27 (data not shown), significantly inhibited the arsenite-induced accumula-

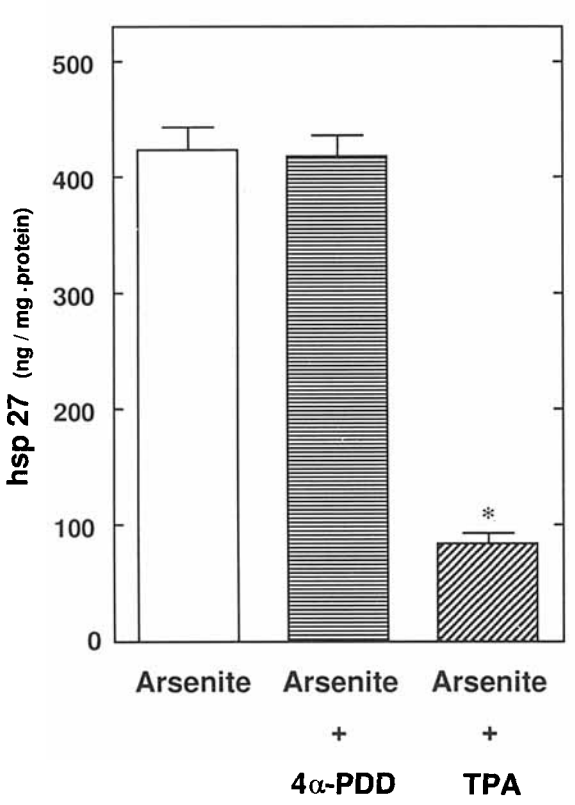

Fig. 3. Effect of phorbol esters on the arsenite-induced accumulation of hsp27 in MC3T3-E1 cells. The cultured cells were pretreated with 0.1 μM of TPA, 4α-PDD or vehicle for 20 min, and then stimulated by 100 μM of arsenite at 37°C for 1 h, and subsequently incubated at 37°C for 16 h in α-MEM containing 0.3% FCS. Each value represents the mean ± S.D. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$ compared to the value of arsenite alone.

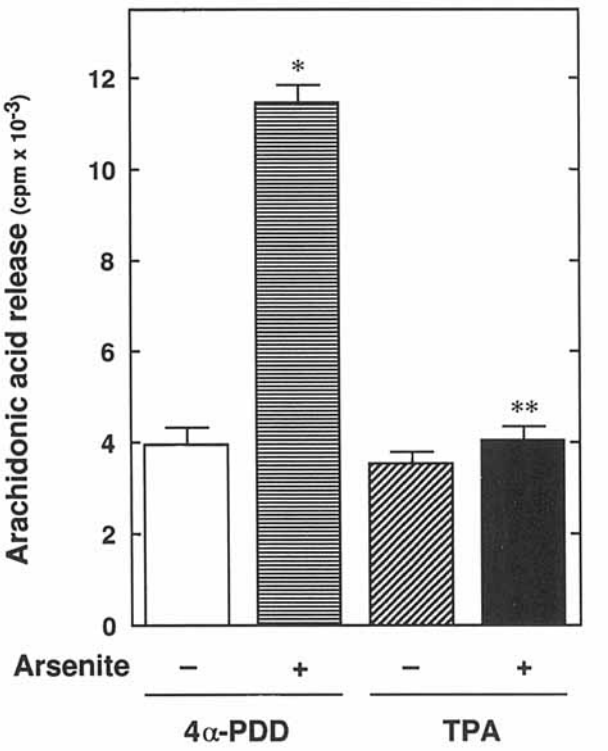

Fig. 4. Effect of phorbol esters on the arsenite-induced arachidonic acid release in MC3T3-E1 cells. The labeled cells were pretreated with 0.1 μM of TPA, 4α-PDD or vehicle for 20 min, and then stimulated by 200 μM of arsenite for 60 min. Each value represents the mean ± S.D. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$ compared to the value of 4α-PDD pretreatment alone. **$P < 0.05$ compared to the value of arsenite with 4α-PDD pretreatment.

tion of hsp27 in MC3T3-E1 cells (Fig. 3). However, 4α-PDD, which is known to be ineffective in PKC activation [Nishizuka, 1986], had little effect on the arsenite-induced accumulation of hsp27 (Fig. 3).

Effects of Phorbol Esters on Arsenite-Induced Arachidonic Acid Release in MC3T3-E1 Cells

The pretreatment with TPA, which by itself had little effect on arachidonic acid release, significantly suppressed the arsenite-induced arachidonic acid release in MC3T3-E1 cells (Fig. 4). On the other hand, 4α-PDD had little effect on the arsenite-induced arachidonic acid release in these cells.

DISCUSSION

It has been reported that hsp27 expression is induced by biological stress in osteoblast-like MC3T3-E1 cells [Cooper and Uoshima, 1994], and we showed here that arsenite-stress induced the accumulation of hsp27 in these cells. In the present study, we demonstrated that arsenite increased arachidonic acid release time- and dose-dependently in MC3T3-E1 cells. On the other hand, arsenite had little effect on the cAMP accumulation and formations of inositol phosphates, choline, and phosphocholine at any time point when we detect the effects of the other agents such as prostaglandins on cAMP accumulation and formations of inositol phosphates and choline in MC3T3-E1 cells [Kozawa et al., 1992, 1994, 1995]. It is well-known that phosphoinositides (PIs) are hydrolyzed by PI-phospholipase C, resulting in the formation of inositol phosphates and diacylglycerol [Berridge and Irvine, 1989], and that phosphatidylcholine (PC) is hydrolyzed by PC-phospholipase C and PC-phospholipase D [Exton, 1990]. PC hydrolysis by PC-phospholipase C results in the formation of phosphocholine and diacylglycerol, and

PC hydrolysis by PC-phospholipase D results in the formation of choline and phosphatidic acid, which can be dephosphorylated to give diacylglycerol [Exton, 1990]. Diacylglycerol is known to be a physiological activator of PKC [Nishizuka, 1986]. Our findings suggest that arsenite induced arachidonic acid release without affecting the activity of adenylate cyclase, PI-phospholipase C, PC-phospholipase C, nor PC-phospholipase D in MC3T3-E1 cells, and that arsenite by itself does not activate PKC in these cells. In addition, we showed that both indomethacin and nordihydroguaiaretic acid enhanced the arsenite-induced accumulation of hsp27 in MC3T3-E1 cells. It is well-known that indomethacin is an inhibitor of cyclooxygenase [Smith, 1989] and nordihydroguaiaretic acid is an inhibitor of lipoxygenase [Domin et al., 1994]. Furthermore, we demonstrated that melittin enhanced the arsenite-induced accumulation of hsp27 in these cells. Melittin is known to be a phospholipase A_2 activator [Shier, 1979], and we previously reported that melittin significantly increases arachidonic acid release in MC3T3-E1 cells [Tokuda et al., 1992]. Therefore, these results suggest that arsenite stimulates the induction of hsp27 due to the increase of arachidonic acid release in osteoblast-like MC3T3-E1 cells. However, the fact that melittin by itself did not induce the accumulation of hsp27 suggests that arachidonic acid release alone, without stress, is insufficient to induce hsp27.

Next, we showed that TPA suppressed the arsenite-induced accumulation of hsp27 in MC3T3-E1 cells. On the contrary, 4α-PDD had little effect. These results suggest that an activation of PKC inhibits the induction of hsp27 to the stress in these cells. In addition, we demonstrated that not 4α-PDD but TPA inhibited the arsenite-stimulated arachidonic acid release in MC3T3-E1 cells. Our findings suggest that an activation of PKC suppresses the response to stress of hsp27 through the inhibition of arachidonic acid release in MC3T3-E1 cells. We have previously reported that the activation of PKC increased arsenite-induced arachidonic acid release and accumulation of hsp27 in C6 rat glioma cells [Ito et al., 1995]. However, it has been reported that the inhibition of proliferation of osteoblasts corresponds temporally with an increase in hsp27 mRNA followed by a gradient decline during differentiation [Shakoori et al., 1992], and we have reported that the activation of PKC is involved in the process which suppresses the differentiation of osteoblast-like MC3T3-E1 cells and directs them toward proliferation [Kozawa et al., 1989]. These findings are compatible to the inhibitory effect of PKC activation on the arsenite-induced accumulation of hsp27. Further investigations would be required to clarify the role of hsp27 in osteoblasts.

In conclusion, our findings strongly suggest that the response to stress of hsp27 is coupled with the metabolic activity of the arachidonic acid cascade, and the activation of PKC inhibits the induction of hsp27 through the suppression of arachidonic acid release in osteoblast-like cells.

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