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Involvement of p42/p44 Mitogen-Activated Protein Kinase in Prostaglandin $F_{2\alpha}$-Stimulated Induction of Heat Shock Protein 27 in Osteoblasts

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Abstract We previously reported that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) activates both phosphoinositide-hydrolyzing phospholipase C and phosphatidylcholine-hydrolyzing phospholipase D in osteoblast-like MC3T3-E1 cells and then induces the activation of protein kinase C (PKC). In this study, we investigated the effect of $PGF_{2\alpha}$ on the induction of heat shock protein 27 (HSP27), a low-molecular-weight heat shock protein, in these cells. $PGF_{2\alpha}$ significantly induced the accumulation of HSP27 dose-dependently within the range of 10 nM to 10 μM. $PGF_{2\alpha}$ stimulated the increase in the levels of mRNA for HSP27. A total of 10 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA), an activator of PKC, induced the accumulation of HSP27. The stimulative effect of $PGF_{2\alpha}$ was reduced in the PKC down-regulated cells. Calphostin C, a specific inhibitor of PKC, suppressed the $PGF_{2\alpha}$-induced HSP27 accumulation as well as that induced by TPA. HSP27 induction by $PGF_{2\alpha}$ was reduced by U-73122, a phospholipase C inhibitor, or propranolol, a phosphatidic acid phosphohydrolase inhibitor. $PGF_{2\alpha}$ and TPA stimulated p42/p44 mitogen-activated protein (MAP) kinase. PD98059, an inhibitor of the upstream kinase that activates p42/p44 MAP kinase, suppressed the induction of HSP27 stimulated by $PGF_{2\alpha}$ or TPA. PD98059 and calphostin C reduced the levels of mRNA for HSP27 increased by $PGF_{2\alpha}$. These results indicate that $PGF_{2\alpha}$ stimulates the induction of HSP27 via p42/p44 MAP kinase activation, which depends on upstream PKC activation in osteoblasts. J. Cell. Biochem. 75:610–619, 1999.

Key words: heat shock protein 27; prostaglandin $F_{2\alpha}$; MAP kinase; protein kinase C; osteoblast

Cellular responses to biological stresses produce heat shock proteins (HSPs) [Nover, 1991; Nover and Sharf, 1991]. The HSPs are present in significant amounts even in several unstressed cells, where they may have essential functions [Schlessinger, 1990; Ang et al., 1991; Ciocca et al., 1993; Udelsman et al., 1993]. HSPs are divided into high-molecular-weight HSPs and low-molecular-weight HSPs according to their apparent molecular sizes. High-molecular-weight HSPs, including HSP90, HSP70, and HSP60, are recognized to act as molecular chaperones implicated in protein folding, oligomerization, and translocation [Ellis and van der Vies, 1991; Gething, 1992]. As for low-molecular-weight HSPs, including HSP27 and αB-crystallin, they have marked similarities in terms of amino acid sequences [Ingolia and Craig, 1982; Hickey et al., 1986]. It is recognized that low-molecular-weight HSPs may act as chaperones as well as high-molecular-weight HSPs [Benndorf et al., 1994; Groenen et al., 1994]. It has been reported that the expression of HSP27 participates in mediating physiological processes other than the stress response, including cellular differentiation in human keratinocytes and regulation of apoptosis in murine fibrosarcoma L929 cells [Kindas-Mügge and Trutinger, 1994]. In addition, it has recently been shown that HSP27 facilitates basic fibroblast growth factor release from endo-

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thelial cells [Piotiowicy et al., 1997]. As for bone cells, it has been reported that HSP27 expression is induced by heat stress in osteoblasts, including osteoblast-like MC3T3-E1 cells and pretreatment of estrogen, facilitates the expression [Shakoori et al., 1992; Cooper and Uoshima, 1994]. In addition, the down-regulation of their proliferation has been shown to be accompanied by a transient increase of the expression of HSP27 mRNA [Shakoori et al., 1992; Cooper and Uoshoma, 1994]. In a previous study [Suzuki et al., 1996], we have shown that a chemical stressor, sodium arsenite, stimulates the induction of HSP27 due to the increase of arachidonic acid release in osteoblast-like MC3T3-E1 cells. However, the regulatory mechanism in the induction of HSP27 in osteoblasts and the precise role it plays have not yet been clarified.

Prostaglandins are well known to act as autocrine/paracrine modulators in osteoblasts [Nijweide et al., 1986]. Among them, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is recognized to be a potent bone-resorptive agent [Raisz and Martin, 1984]. It has been reported that $PGF_{2\alpha}$ stimulates the proliferation of osteoblasts and inhibits the differentiation [Raisz and Martin, 1984; Koshihara and Kawamura, 1989]. We previously reported that $PGF_{2\alpha}$ activates protein kinase C (PKC) through both phosphoinositide hydrolysis by phospholipase C and phosphatidylcholine hydrolysis by phospholipase D in osteoblast-like MC3T3-E1 cells [Miwa et al., 1990; Kozawa et al., 1994]. It has recently been reported that $PGF_{2\alpha}$ stimulates mitogen-activated protein (MAP) kinase through PKC activation in these cells [Hakeda et al., 1997].

In the present study, we examined the effect of $PGF_{2\alpha}$ on the induction of HSP27 in osteoblast-like MC3T3-E1 cells. Our results suggest that $PGF_{2\alpha}$ stimulates the induction of HSP27 via p42/p44 MAP kinase activation, which depends on upstream PKC activation in these cells.

MATERIALS AND METHODS

Materials

p42/p44 MAP kinase enzyme assay system was purchased from Amersham Japan (Tokyo, Japan). $PGF_{2\alpha}$, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), 4α-phorbol 12, 13-didecanoate (4α-PDD) and calphostin C were purchased from Sigma Chemical Co. (St. Louis, MO). U-73122 was obtained from Funakoshi Pharmaceutical Co. (Tokyo, Japan). *dl*-Propranolol hydrochloride (propranolol) was purchased from Wako Pure Chemical Co. (Osaka, Japan). PD98059 was obtained from Calbiochem (La Jolla, CA). TPA, 4α-PDD, calphostin C, U-73122, propranolol, and PD98059 were dissolved in dimethylsulfoxide (DMSO). The maximum concentration of DMSO was 0.1%, and this did not affect the assay for the accumulation of HSP27.

Cell Culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [Kodama et al., 1981; Sudo et al., 1983] were maintained as previously described [Kozawa et al., 1992]. Briefly, the cells were cultured 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.

Assay for Accumulation of HSP27

The cultured cells were stimulated by $PGF_{2\alpha}$, TPA, or 4α-PDD in 1 ml of α-MEM containing 0.3% FCS for the indicated periods. The cells were washed twice with 1 ml of phosphate-buffered saline (PBS) 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 PBS, and each suspension was sonicated and centrifuged at 125,000*g* for 20 min at 4°C. The supernatant was used for immunoassay of HSP27.

Immunoassay of HSP27

The concentration of HSP27 in soluble extracts of cells was determined by a sandwich-type enzyme immunoassay as described previously [Kato et al., 1991; Inaguma et al., 1993]. 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 antibodies 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 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% bovine serum albumin (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.

Electrophoresis and Western Blotting Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli [1970] in 10% polyacrylamide gel. Western blotting analysis was performed as described previously [Kato et al., 1993], using affinity-purified antibodies (0.05 μg/ml) raised in rabbits against rat HSP27 [Inaguma et al., 1993], and peroxidase-labeled antibodies raised in goat against rabbit IgG as second antibodies. Peroxidase activity on the nitrocellulose sheet was visualized on X-ray film by use of a western blot chemiluminescence reagent (Renaissance, Du Pont-NEN, Boston, MA).

Isolation of RNA and Northern Blotting Analysis

Total RNA was isolated with a QuickPrep Total RNA Extraction kit (Pharmacia Bioteck, Tokyo, Japan). A total of 12.6 μg of total RNA was subjected to electrophoresis on a 0.9% agarose/2.2 M formaldehyde gel and blotted onto a nitrocellulose membrane. For Northern blotting analysis, membrane was allowed to hybridize with cDNA probes labeled with a Multiprime DNA labeling system (Amersham, Buckinghamshire, UK), as described previously [Kato et al., 1993]. A *Bam*HI-*Hin*dIII fragment of cDNA for mouse HSP27 [Cooper and Uoshima, 1994] was kindly provided by Dr. L.F. Cooper at the University of North Carolina.

Measurement of p42/p44 MAP Kinase Activity

The cultured cells were stimulated by $PGF_{2\alpha}$ or TPA in 1 ml of an assay buffer (5 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid, pH 7.4, 150 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 1 mM $CaCl_2$, 5.5 mM glucose) containing 0.01% BSA for the indicated periods. The cells were washed twice with 1 ml of PBS and then lysed, homogenized, and sonicated in a lysis buffer containing 10 mM Tris, 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10μg/ml leupeptin, 10μg/ml aprotinin and 1 mM sodium orthovanadate. The cytosolic fraction as a supernatant after centrifugation at 125,000*g* for 10 min at 4°C. MAP kinase activity of the cell extracts was then assayed with the use of the p42/p44 MAP kinase enzyme assay system.

Statistical Analysis

The data were analyzed by one-way analysis of variance (ANOVA), followed by the Bonferroni method for multiple comparison between pairs; a value of $P < 0.05$ was considered significant. All data are presented as the mean ± SD of triplicate determinations.

RESULTS

Effect of $PGF_{2\alpha}$ on the Accumulation of HSP27 in MC3T3-E1 Cells

$PGF_{2\alpha}$ (1 μM) significantly induced the accumulation of HSP27 in a time dependent manner (Fig. 1). The $PGF_{2\alpha}$-induced accumulation of HSP27 almost reached a maximum by 12 h. The stimulative effect of $PGF_{2\alpha}$ on the accumulation of HSP27 was dose dependent within the range of 10 nM to 10 μM (Fig. 2). The maximum effect of $PGF_{2\alpha}$ was observed at 1 μM.

Western Blotting Analysis of Response of HSP27 to $PGF_{2\alpha}$ in MC3T3-E1 Cells

Response of HSP27 to $PGF_{2\alpha}$ in MC3T3-E1 cells was also examined by Western blotting analysis of soluble extracts prepared from the cells that had been incubated for 12 h with various doses of $PGF_{2\alpha}$. Levels of HSP27 were induced by $PGF_{2\alpha}$ in a dose-dependent manner within the range of 10 nM to 10 μM (Fig. 3).

Northern Blotting Analysis of mRNA for HSP27 in MC3T3-E1 Cells

Expression of mRNA for HSP27 was markedly induced by 1 μM $PGF_{2\alpha}$ (Fig. 4). The stimulative effect of $PGF_{2\alpha}$ on the expression appeared at 2 h after the stimulation, and the effect was sustained until 6 h.

Effects of Phorbol Esters on the Accumulation of HSP27 in MC3T3-E1 Cells

We previously showed that 0.1 μM TPA, an activator of PKC [Nishizuka, 1986], had little effect on the accumulation of HSP27 in osteo-

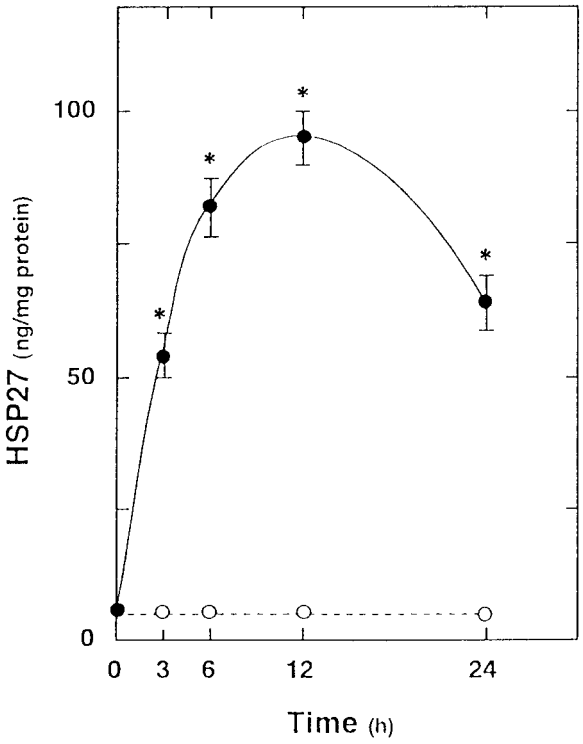

Fig. 1. Effect of $PGF_{2\alpha}$ on the accumulation of HSP27 in MC3T3-E1 cells. The cultured cells were stimulated by 1 μM $PGF_{2\alpha}$ (●) or vehicle (○) for the indicated periods. Each value represents the mean ±SD of triplicate determinations of a representative experiment carried out three times. $*P < 0.05$ vs. control value.

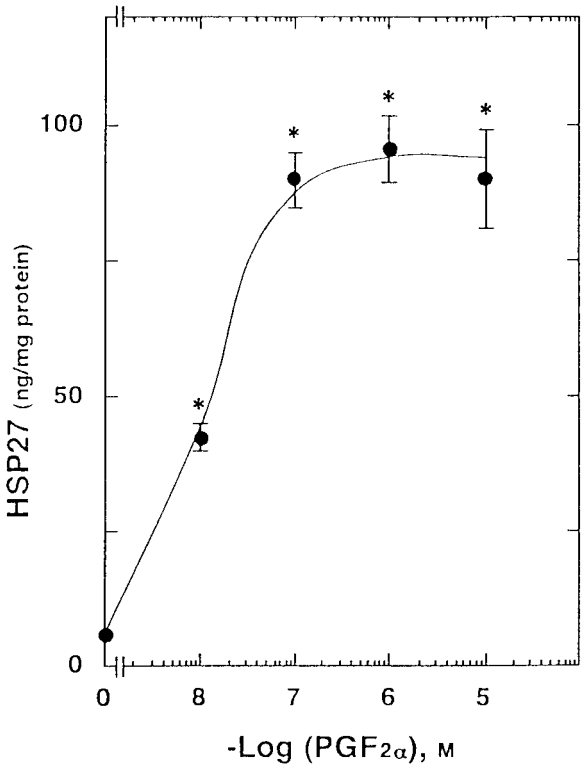

Fig. 2. Dose-dependent effect of $PGF_{2\alpha}$ on the accumulation of HSP27 in MC3T3-E1 cells. The cultured cells were stimulated by various doses of $PGF_{2\alpha}$ for 12 h. Each value represents the mean ±SD of triplicate determinations of a representative experiment carried out three times. $*P < 0.05$ vs. control value.

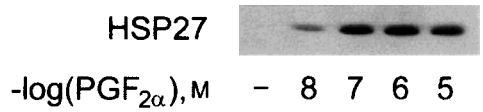

Fig. 3. Western blotting analysis of response of HSP27 in MC3T3-E1 cells. The cultured cells were exposed to various doses of $PGF_{2\alpha}$ for 12 h. The extracts of cells that contained 4 μg of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequent immunostaining with antibodies against HSP27.

blast-like MC3T3-E1 cells 16 h after the stimulation [Suzuki et al., 1996]. In the present study, we examined the effects of various doses of TPA or 4α-PDD, a PKC-nonactivating phorbol ester [Nishizuka, 1986], on the HSP27 accumulation. TPA (10 nM) alone significantly stimulated the accumulation of HSP27 (Table I). On the contrary, 4α-PDD did not affect the accumulation of HSP27. The TPA-effect was in a dose-dependent manner, attaining a maximum at 10 nM (data not shown). A dose of TPA of >10 nM caused less than maximal stimulation.

Effect of Down-Regulation of PKC on HSP27 Accumulation of $PGF_{2\alpha}$ in MC3T3-E1 Cells

It has been reported that 24 h pretreatment of TPA (0.1 μM) down-regulates PKC in osteoblast-like MC3T3-E1 cells [Sakai et al., 1992]. We also found that the binding capacity of phorbol-12,13-dibutyrate, a PKC-activating phorbol ester [Nishizuka, 1986], in PKC down-regulated MC3T3-E1 cells is reduced to about 30% of the capacity in intact cells [Kozawa et al., 1995]. To clarify the role of PKC in the $PGF_{2\alpha}$-stimulated HSP27 induction, we next examined the effect of TPA (0.1 μM) long-term pretreatment on the HSP27 accumulation by $PGF_{2\alpha}$. The effect of $PGF_{2\alpha}$ on HSP27 accumulation was significantly reduced in the PKC down-regulated cells compared with that in the cells without TPA pretreatment (Table II).

Effect of Calphostin C on $PGF_{2\alpha}$-Induced Accumulation of HSP27 in MC3T3-E1 Cells

We have shown that $PGF_{2\alpha}$ stimulates the activation of PKC through phosphoinositide hy-

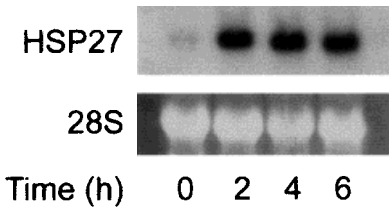

Fig. 4. Northern blotting analysis of mRNA for HSP27 in MC3T3-E1 cells. The cultured cells were exposed to 1 μM $PGF_{2\alpha}$ for 0, 2, 4, 6 h, respectively. The cells were harvested and total RNA was isolated. 12.6 μg of RNA from each sample was subjected to electrophoresis and blotted onto a nitrocellulose membrane. The membranes were then allowed to hybridize with the cDNA probe for HSP27. Bands of 28S RNA are shown for reference.

TABLE I. Effects of Calphostin C and PD98059 on the TPA-Induced Accumulation of HSP27 in MC3T3-E1 Cells†

	HSP27 (ng/mg protein)
Control	4.7 ± 0.8
4α-PDD	4.8 ± 0.9
TPA	15.5 ± 1.3*
Calphostin C	4.4 ± 1.1
Calphostin C + TPA	9.1 ± 0.9**
PD98059	4.3 ± 1.1
PD98059 + TPA	6.0 ± 0.8**

†Cultured cells were pretreated with 0.3 μM calphostin C, 50 μM PD98059, or vehicle, and then stimulated by 10 nM TPA, 10 nM 4α-PDD, or vehicle for 12 h. Each value represents the mean ± SD of triplicate determinations of a representative experiment carried out three times.
*$P < 0.05$ vs. control.
**$P < 0.05$ vs. the value of TPA alone.

TABLE II. Effect of PKC Down-Regulation on the $PGF_{2\alpha}$-Induced Accumulation of HSP27 in MC3T3-E1 Cells†

TPA pretreatment (0.1 μM)	$PGF_{2\alpha}$ (1 μM)	HSP27 (ng/ml protein)
−	−	5.5 ± 1.0
−	+	94.5 ± 7.0
+	−	3.6 ± 1.5
+	+	24.7 ± 3.5*

†Cultured cells were pretreated with 0.1 μM TPA or vehicle for 24 h, and then stimulated by 1 μM $PGF_{2\alpha}$ or vehicle for 12 h. Each value represents the mean ± SD of triplicate determinations of a representative experiment carried out three times.
*$P < 0.05$ vs. the value of $PGF_{2\alpha}$ alone.

drolysis by phospholipase C and phosphatidylcholine hydrolysis by phospholipase D in osteoblast-like MC3T3-E1 cells [Miwa et al., 1990; Kozawa et al., 1994]. Thus, we next examined the effect of calphostin C, a highly potent and specific inhibitor of PKC [Kobayashi et al., 1989], on the $PGF_{2\alpha}$-induced accumulation of HSP27. Calphostin C, which by itself did not affect the accumulation of HSP27, significantly inhibited the accumulation of HSP27 induced by $PGF_{2\alpha}$ (Fig. 5). Calphostin C (0.5 μM) caused about 80% reduction in the effect of $PGF_{2\alpha}$. Calphostin C also suppressed the TPA (10 nM)-induced HSP27 accumulation (Table I).

Effects of U-73122 and Propranolol on $PGF_{2\alpha}$-Induced Accumulation of HSP27 in MC3T3-E1 Cells

To clarify the roles of these two phospholipases activation on the induction of HSP27, we next examined the effects of U-73122, an inhibitor of phospholipase C [Bleasedale et al., 1990], and propranolol, an inhibitor of phosphatidic acid phosphohydrolase on the $PGF_{2\alpha}$-induced accumulation of HSP27 [Pappu and Hausser, 1983]. U-73122 (30 μM), which by itself had little effect on the accumulation of HSP27, significantly suppressed the $PGF_{2\alpha}$-induced accumulation of HSP27, reducing it by 40% (Table III). In addition, propranolol, which alone did not affect HSP27 accumulation, dose-dependently inhibited the accumulation of HSP27 stimulated by $PGF_{2\alpha}$ (Fig. 6). Propranolol (100 μM) caused about 70% reduction in the effect of $PGF_{2\alpha}$.

Effect of PD98059 on the $PGF_{2\alpha}$-Induced Accumulation of HSP27 in MC3T3-E1 Cells

It has recently been reported that PKC activation is involved in $PGF_{2\alpha}$-stimulated p42/p44 MAP kinase activation in MC3T3-E1 cells [Hakeda et al., 1997]. We also found that $PGF_{2\alpha}$ activates p42/p44 MAP kinase and that PD98059, a selective inhibitor of the upstream kinase that activates p42/p44 MAP kinase [Dudley et al., 1995; Alessi et al., 1995], which alone had little effect on the basal level, significantly suppressed the $PGF_{2\alpha}$-induced p42/p44 MAP kinase activation in these cells (data not shown). The inhibitory effect of PD98059 was dose dependent within the range of 0.1 to 50 μM.

We next examined the effect of PD98059 on the $PGF_{2\alpha}$-induced accumulation of HSP27 in MC3T3-E1 cells. The HSP27 accumulation induced by $PGF_{2\alpha}$ was markedly inhibited by PD98059 (Fig. 7). The effect of PD98059 on the $PGF_{2\alpha}$-induced HSP27 accumulation was dose dependent within the range of 0.1 to 50 μM. The maximum effect of PD98059 was observed

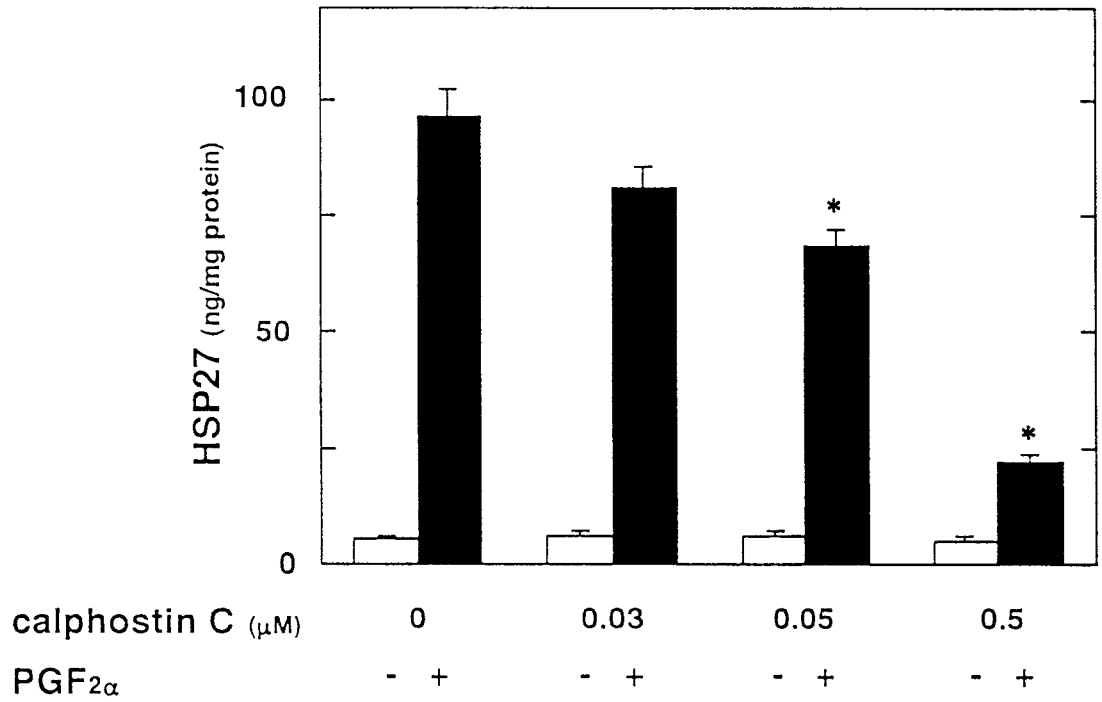

Fig. 5. Effects of calphostin C on the $PGF_{2\alpha}$-induced accumulation of HSP27 in MC3T3-E1 cells. The cultured cells were pretreated with various doses of calphostin C or vehicle for 20 min and then stimulated by 1 μM $PGF_{2\alpha}$ or vehicle for 12 h. Each value represents the mean ±SD of triplicate determinations of a representative experiment carried out three times. *$P < 0.05$ vs. control value.

TABLE III. Effect of U-73122 on the $PGF_{2\alpha}$-Induced Accumulation of HSP27 in MC3T3-E1 Cells†

	HSP27 (ng/mg protein)
Control	5.9 ± 0.7
U-73122	4.6 ± 0.5
$PGF_{2\alpha}$	94.0 ± 4.8
U-73122 + $PGF_{2\alpha}$	57.5 ± 3.6*

†Cultured cells were pretreated with 30 μM U-73122 or vehicle, and then stimulated by 1 μM $PGF_{2\alpha}$ or vehicle for 12 h. Each value represents the mean ± SD of triplicate determinations of a representative experiment carried out three times.
*$P < 0.05$ vs. the value of $PGF_{2\alpha}$ alone.

at 50 μM, a dose that caused about 50% reduction in the effect of $PGF_{2\alpha}$. In addition, the HSP27 accumulation induced by TPA (10 nM) was markedly reduced by PD98059 (Table I).

Effect of PD98059 and Calphostin C on the Levels of mRNA for HSP27 Induced by $PGF_{2\alpha}$ in MC3T3-E1 Cells

In addition, we examined the effects of PD98059 and calphostin C on the increase in the levels of mRNA for HSP27 induced by $PGF_{2\alpha}$. PD98059 significantly reduced the levels of mRNA or HSP27, and the level of mRNA was almost completely suppressed by calphostin C (Fig. 8).

DISCUSSION

In the present study, we showed that $PGF_{2\alpha}$ stimulated the induction of HSP27 as detected by specific immunoassay and Western blotting analysis in osteoblast-like MC3T3-E1 cells. In addition, we demonstrated that $PGF_{2\alpha}$ significantly induced the expression of mRNA for HSP27 in these cells, suggesting that $PGF_{2\alpha}$ stimulates de novo synthesis of HSP27 in MC3T3-E1 cells. This is probably the first report showing the stimulatory effect of $PGF_{2\alpha}$, as a physiological agonist for osteoblasts, on the induction of HSP27, a low-molecular-weight HSP, as far as we know.

We next investigated the precise mechanism underlying the $PGF_{2\alpha}$-stimulated HSP27 induction in osteoblast-like MC3T3-E1 cells. As for the intracellular signaling of $PGF_{2\alpha}$ in osteoblasts, we previously reported that $PGF_{2\alpha}$ induces PKC activation through both phosphoinositide hydrolysis by phospholipase C and phosphatidylcholine hydrolysis by phospholi-

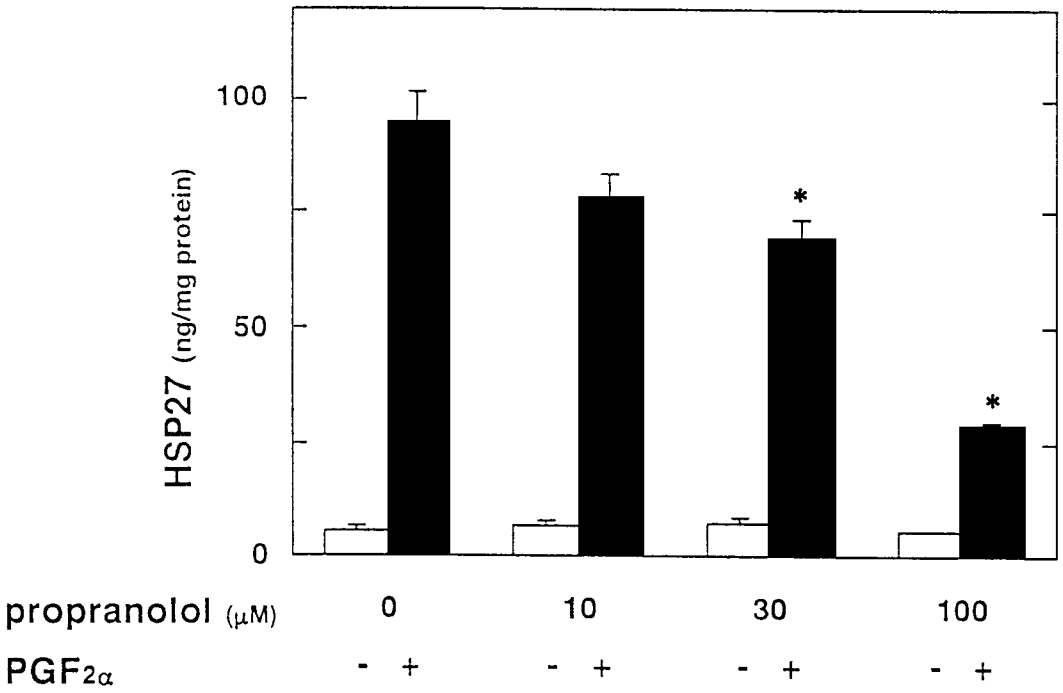

Fig. 6. Effect of propranolol on the $PGF_{2\alpha}$-induced accumulation of HSP27 in MC3T3-E1 cells. The cultured cells were pretreated with various doses of propranolol or vehicle for 12 h. The cells were then stimulated by 1 μM $PGF_{2\alpha}$ or vehicle for 12 h. Each value represents the mean ± SD of triplicate determinations of a representative experiment carried out three times. $^*P < 0.05$ vs. control value.

pase D in MC3T3-E1 cells [Miwa et al., 1990; Kozawa et al., 1994]. In this study, we found that not 4α-PDD but TPA significantly stimulated the accumulation of HSP27, suggesting the possible induction of HSP27 through PKC activation. In addition, a PKC inhibitor, calphostin C [Kobayashi et al., 1989] inhibited the $PGF_{2\alpha}$-induced accumulation of HSP27 as well as that induced by TPA. Furthermore, we showed that the stimulative effect of $PGF_{2\alpha}$ on HSP27 accumulation was markedly reduced in the PKC down-regulated cells compared with that in intact cells, and that the $PGF_{2\alpha}$-stimulated increase in the levels of mRNA for HSP27 was reduced by calphostin C. Thus, these findings suggest that PKC activation is involved in the $PGF_{2\alpha}$-stimulated HSP27 induction in osteoblast-like MC3T3-E1 cells. We also demonstrated that both inhibitors for phosphoinositide-hydrolyzing phospholipase C and phosphatidic acid phosphohydrolase suppressed the $PGF_{2\alpha}$-induced HSP27 accumulation. It is well known that phosphatidylinositol is hydrolyzed by phospholipase C, resulting in the formation of diacylglycerol, a physiological activator of PKC [Nishizuka, 1986]. In addition, phospholipase D hydrolyzes phosphatidylcholine to yield phosphatidic acid. Phophatidic acid is subsequently degraded into diacylglycerol by phosphatidic acid phosphohydrolase. On the basis of our findings, it is most likely that $PGF_{2\alpha}$ stimulates HSP27 induction via PKC activation though phosphoinositide hydrolysis by phospholipase C and phosphatidylcholine hydrolysis by phospholipase D in osteoblast-like MC3T3-E1 cells.

It has recently been shown that $PGF_{2\alpha}$ stimulates p42/p44 MAP kinase activation at a point downstream from PKC activation in osteoblast-like MC3T3-E1 cells [Hakeda et al., 1997]. Thus, we next investigated whether or not p42/p44 MAP kinase activation is involved in $PGF_{2\alpha}$-induced HSP27 induction in these cells. We showed that PD98059 significantly suppressed the HSP27 accumulation induced by $PGF_{2\alpha}$ as well as that induced by TPA. It is generally accepted that MAP kinase cascade consists of several kinase modules and that MAP kinase is activated by MAP kinase kinase [Nishida and Gotoh, 1993]. These results suggest that p42/p44 MAP kinase activation has a stimulatory effect on the HSP27 induction by $PGF_{2\alpha}$ in MC3T3-E1 cells. Furthermore, we demon-

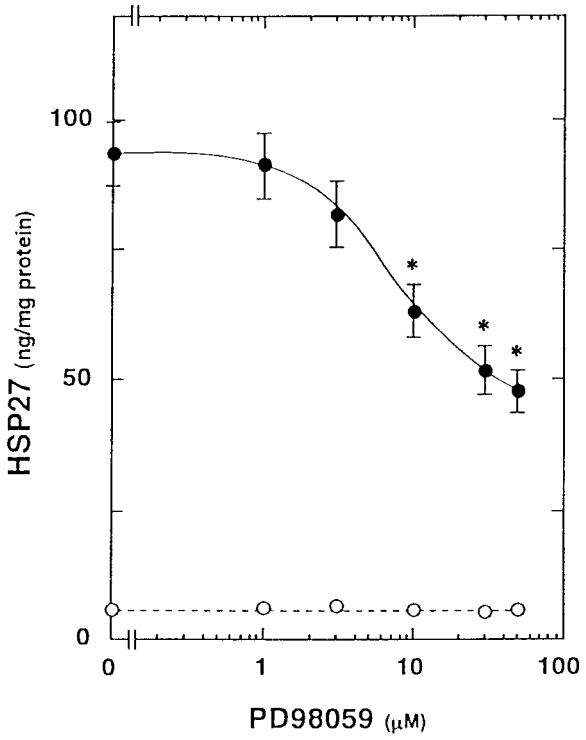

Fig. 7. Effect of PD98059 on the $PGF_{2\alpha}$-induced accumulation of HSP27 in MC3T3-E1 cells. The cultured cells were pretreated with various doses of PD98059, and then stimulated by 1 μM $PGF_{2\alpha}$ (●) or vehicle (○) for 12 h. Each value represents the mean ±SD of triplicate determinations of a representative experiment carried out three times. $*P < 0.05$ vs. control value.

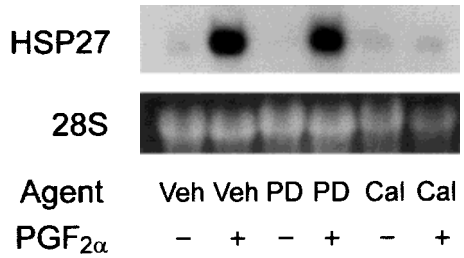

Fig. 8. Effects of PD98059 and calphostin C on the levels of mRNA for HSP27 induced by $PGF_{2\alpha}$ in MC3T3-E1 cells. The cultured cells were pretreated with 50 μM PD98059 (PD), 0.3 μM calphostin C (Cal), or vehicle for 20 min, and then exposed to 1 μM $PGF_{2\alpha}$ or vehicle for 2 h. The cells were harvested and total RNA was isolated. A total of 12.6 μg of mRNA from each sample was subjected to electrophoresis and blotted onto a nitrocellulose membrane. The membranes were then allowed to hybridize with cDNA probe for HSP27. Bands of 28S RNA are shown for reference.

strated that PD98059 markedly suppressed the $PGF_{2\alpha}$-stimulated increase in the levels of mRNA for HSP27. Taking our findings into account, therefore, it is most likely that $PGF_{2\alpha}$ stimulates HSP27 induction through p42/p44 MAP kinase activation, which acts at a point downstream from PKC in osteoblast-like MC3T3-E1 cells.

Concerning the relationship between p42/p44 MAP kinase and HSP27, it has been reported that the phosphorylation of HSP27 stimulated by interleukin-1 is mediated by p42/p44 MAP kinase activation in human epidermoid carcinoma cells, KB cells [Bird et al., 1994]. In addition, Yamada et al. [1995] demonstrated that p42/p44 MAP kinase translocates accompanied by HSP27 during bombesin-induced contraction of rabbit rectosigmoid smooth muscle. However, there are no reports showing the involvement of p42/p44 MAP kinase in the induction of HSP27, as far as we know. Therefore, this is probably the first report showing that p42/p44 MAP kinase activation has a stimulatory effect on the HSP27 induction stimulated by $PGF_{2\alpha}$ in osteoblasts. It is generally recognized that low-molecular-weight HSPs, including HSP27, may act as molecular chaperones, a role also played by high-molecular-weight HSPs such as HSP70 and HSP90 [Ursula et al., 1993]. As for HSP27 in osteoblasts, it has been reported that the down-regulation of their proliferation is accompanied by a transient increase of the expression of HSP27 mRNA [Shakoori et al., 1992]. Because $PGF_{2\alpha}$ is known to be a potent stimulator of proliferation of osteoblasts including MC3T3-E1 cells, it is possible that the self-induction of HSP27 regulates the proliferative effect of $PGF_{2\alpha}$ in osteoblasts. Further investigations would be required to clarify the physiological roles of the HSP27 induction stimulated by $PGF_{2\alpha}$ in osteoblasts.

In conclusion, these results strongly suggest that $PGF_{2\alpha}$ stimulates the induction of HSP27 in osteoblasts, and that p42/p44 MAP kinase activation, which depends on upstream PKC activation, plays an key role in the HSP27 induction.

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