Mechanism of prostaglandin E2-stimulated heat shock protein 27 induction in osteoblast-like MC3T3-E1 cells

H Tokuda, O Kozawa, M Niwa, H Matsuno, K Kato and T Uematsu

Department of Internal Medicine, Chubu National Hospital: National Institute for Longevity Sciences, Obu, Aichi 474–8511, Japan
1Department of Pharmacology, Gifu University School of Medicine, Gifu 500–8705, Japan
2Department of Biochemistry, Institute for Developmental Research, Aichi Human Service Center, Kasugai, Aichi 480–0392, Japan
(Requests for offprints should be addressed to O Kozawa; Email: okozawa@cc.gifu-u.ac.jp)

Abstract

We investigated the effect of prostaglandin E2 (PGE2) on the induction of heat shock protein 27 (HSP27) and HSP70, and the mechanism behind the induction in osteoblast-like MC3T3-E1 cells. PGE2 time-dependently increased the level of HSP27 without affecting the level of HSP70. PGE2 stimulated the accumulation of HSP27 dose-dependently in the range between 10 nM and 10 µM. PGE2 stimulated the increase in the level of the mRNA for HSP27. Staurosporine and calphostin C, inhibitors of protein kinase C (PKC), suppressed the PGE2-induced HSP27 accumulation. The effect of PGE2 on HSP27 accumulation was reduced in the PKC down-regulated cells. BAPTA/AM, a chelator of intracellular Ca2+, or TMB-8, an inhibitor of intracellular Ca2+ mobilization, reduced the accumulation of HSP27 induced by PGE2. Dibutyryl cAMP had little effect on the basal level of HSP27. PGE2 induced the phosphorylation of both p44/p42 mitogen-activated protein (MAP) kinase and p38 MAP kinase. PD98059 and U-0126, inhibitors of the upstream kinase of p44/p42 MAP kinase, reduced the accumulation of HSP27 induced by PGE2. SB203580, a specific inhibitor of p38 MAP kinase, suppressed the HSP27 accumulation induced by PGE2. U-73122, an inhibitor of phospholipase C, and calphostin C reduced the PGE2-induced phosphorylation of both p44/p42 MAP kinase and p38 MAP kinase. These results indicate that PGE2 stimulates the induction of HSP27 through PKC-dependent activations of both p44/p42 MAP kinase and p38 MAP kinase in osteoblasts.


Introduction

Prostaglandins are well known to act as autocrine/paracrine modulators in osteoblasts (Nijweide et al. 1986, Smith 1986). Among them, prostaglandin E2 (PGE2) is an important regulator of bone remodeling (Smith 1986, Pilbeam et al. 1996). As for the intracellular signaling mechanism of PGE2 in osteoblasts, cAMP formation by adenylate cyclase, Ca2+ influx, phosphoinositide hydrolysis by phospholipase C, and phosphatidylincholine hydrolysis by phospholipase D are reportedly involved in cultured osteoblast-like cells including MC3T3-E1 cells (Tokuda et al. 1991, 1992, Kozawa et al. 1992, Oiso et al. 1995, Pilbeam et al. 1996). It is well known that diacylglycerol produced through phosphoinositide hydrolysis and phosphatidylincholine hydrolysis is a physiological activator of protein kinase C (PKC) (Nishizuka 1992). PKC is recognized to mediate the mitogenic effect of PGE2 in osteoblasts (Pilbeam et al. 1996). On the other hand, it is known that the stimulatory effect of PGE2 on osteoblast differentiation is mainly mediated through cAMP-dependent protein kinase (PKA) (Pilbeam et al. 1996). We have recently reported that PGE2 stimulates interleukin-6 (IL-6) synthesis through cAMP formation and Ca2+ influx, and negatively regulates IL-6 synthesis via activating PKC in osteoblast-like MC3T3-E1 cells (Kozawa et al. 1998).

In response to various stresses such as heat stress and chemical stress, cells produce heat shock proteins (HSPs) (Nover 1991, Nover & Scharf 1991). HSPs are mainly divided into high molecular weight HSPs and low molecular weight HSPs according to their apparent molecular sizes. High molecular weight HSPs, including HSP110, HSP90 and HSP70, are well recognized to act as molecular chaperones implicated in protein folding, oligomerization and translocation (Ellis & van der Vies 1991, Gething & Sambrook 1992). Low molecular weight HSPs, including HSP27 and αB-crystallin, have marked similarities in terms of amino acid sequences (Ingolia & Craig 1982, Hickey et al. 1986). It is speculated that small molecular weight HSPs may act as chaperones like high molecular weight HSPs (Benndorf et al. 1994, Groenen...
et al. 1994). 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). In 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 & Uoshima 1994). In addition, the down-regulation of proliferation of osteoblasts has been shown to be accompanied by a transient increase in the expression of HSP27 mRNA (Shakoori et al. 1992, Cooper & Uoshima 1994). We have reported that prostaglandin (PG) F2a, endothelin-1, sphingosine 1-phosphate, basic fibroblast growth factor (bFGF) and PGD2 induce HSP27 accumulation in MC3T3-E1 cells (Kawamura et al. 1999, Kozawa et al. 1999a,b, 2001a,b). Among these, PGE2, endothelin-1, bFGF and PGD2 reportedly stimulate proliferation of these cells (Hakeda et al. 1987, Takuda et al. 1989, Tsushita et al. 1992, Suzuki et al. 1996), and sphingosine 1-phosphate is well known as a mitogen of a variety of cells (Hannun 1994). These results could lead us to speculate that the induction of HSP27 couples with the regulation of osteoblast-like cell proliferation. As for HSP27 induction in osteoblasts, we have demonstrated that endothelin-1 and sphingosine 1-phosphate induce the induction of HSP27 not through p44/p42 mitogen-activated protein (MAP) kinase but through p38 MAP kinase (Hannun 1994). These results could lead us to speculate that the induction of HSP27 couples with the regulation of osteoblast-like cell proliferation. As for HSP27 induction in osteoblasts, we have demonstrated that endothelin-1 and sphingosine 1-phosphate induce the induction of HSP27 not through p44/p42 mitogen-activated protein (MAP) kinase but through p38 MAP kinase activation in these cells (Kawamura et al. 1999, Kozawa et al. 1999b). However, the exact mechanism behind the induction of HSP27 in osteoblasts, which may play an important role in osteoblast cell function, remains to be elucidated.

PGE2 reportedly shows a mitogenic effect on osteoblast-like MC3T3-E1 cells (Hakeda et al. 1986, Miwa et al. 1991). In the present study, we examined the effect of PGE2 on the induction of HSP27 and HSP70 in osteoblast-like MC3T3-E1 cells and the exact mechanism of the induction. We herein show that PGE2 stimulates the induction of HSP27 through Ca2+ mobilization and PKC-dependent activation of both p44/p42 MAP kinase and p38 MAP kinase in these cells.

Materials and Methods

Materials

PGE2, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), staurosporine, calphostin C and dibutyryl cAMP (Bt2cAMP) were purchased from Sigma Chemical Co. (St Louis, MO, USA). 1,2-bis-(O-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester (BAPTA/AM) and 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate (TMB-8) were obtained from BIOMOL Research Laboratories, Inc. (Plymouth, PA, USA). 1-[6-(amino)hexyl]-1H-pyrrole-2,5-dione (U-73122) was obtained from Funakoshi Pharmaceutical Co. (Tokyo, Japan). 2'-Amino-3'-methoxyflavone (PD98059), 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB203580), 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U-0126) and 1-[3-(aminomethyl)propyl]-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide; bisindolylmaleimide IX, metnanesulfonate (Ro-31–8220) were obtained from Calbiochem (La Jolla, CA, USA). HSP70 antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA). Phospho-specific p44/p42 MAP kinase antibodies (rabbit polyclonal IgG, affinity purified), p44/p42 MAP kinase antibodies (rabbit polyclonal IgG, affinity purified), phospho-specific p38 MAP kinase antibodies (rabbit polyclonal IgG, affinity purified), and p38 MAP kinase antibodies (rabbit polyclonal IgG, affinity purified) were purchased from New England Biolabs, Inc. (Beverly, MA, USA). An enhanced chemiluminescence (ECL) Western blotting detection system was obtained from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. PGE2 and TMB-8 were dissolved in ethanol. BAPTA/AM, TPA, staurosporine, calphostin C, Ro-31–8220, U-73122, SB203580 and PD98059 were dissolved in dimethyl sulfoxide. The maximum concentration of ethanol or dimethyl sulfoxide was 0.1%, which did not affect Western blot analysis or immunoassay of HSP27.

Cell culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria by Kodama and colleagues (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 bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO2/95% air. The cells (5 × 104) were seeded into 35-mm diameter dishes in 2 ml α-MEM containing 10% FBS. After 5 days, the medium was exchanged for 2 ml α-MEM containing 0.3% FCS. The cells were then used for experiments after 48 h. In each series of experiments, we confirmed that the cells possessed high alkaline phosphatase activity, a marker of the osteoblast phenotype (Robinson et al. 1973), as previously described (Miwa et al. 1991). We have reported that MC3T3-E1 cells have the capacity to form calcified bone tissue in vitro (Sudo et al. 1983). We have also confirmed that these cells possess the capacity to accumulate Ca into extracellular matrix (Kozawa et al. 1992). These findings indicate that MC3T3-E1 cells have osteoblastic features.

Western blotting analysis of HSP27, HSP70, p44/p42 MAP kinase and p38 MAP kinase

The cultured cells were stimulated by PGE2 in α-MEM containing 0.3% FBS for the indicated periods of time.

The cells were washed twice with 1 ml phosphate-buffered saline (PBS) and then lysed, homogenized and sonicated in a lysis buffer containing 62.5 mM Tris/HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at 125,000 g for 10 min at 4 °C. SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (1970) in 10% polyacrylamide gels. Western blotting analysis was performed as previously described (Kato et al. 1993) by using HSP27 antibodies. HSP70 antibodies, phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies or p38 MAP kinase antibodies, with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second antibodies. Peroxidase activity on the nitrocellulose sheet was visualized on X-ray film by means of the ECL Western blotting detection system. The HSP27 antibodies were raised in rabbits against purified rat HSP27, and the cross reactivity with HSP90, HSP70 or αB-crystallin was under 1% (Inaguma et al. 1993).

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. 1993). The cultured cells were stimulated by PGE2 in 1 ml α-MEM containing 0.3% FBS for the indicated periods of time. The cells were then washed twice with 1 ml 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 PBS, and each suspension was sonicated and centrifuged at 125,000 g for 20 min at 4 °C. The supernatant was used for the specific 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-n-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. The incubation was carried out at 30 °C for 5 h in a final volume of 0.5 ml 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 MgCl2 and 0.1% NaNO3. After washing, each ball was incubated at 4 °C overnight with 1.5 mM 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 MgCl2, 0.1% BSA and 0.1% NaNO3. The galactosidase activity bound to the ball was assayed using a fluorogenic substrate, 4-methylumbelliferyl-β-n-galactoside.

Isolation of RNA and Northern blotting analysis of HSP27

The cultured cells were stimulated by PGE2 in α-MEM containing 0.3% FBS for the indicated periods of time. Total RNA was isolated with a QuickPrep Total RNA Extraction kit (Pharmacia Biotech, Tokyo, Japan). Twenty micrograms total RNA were 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 that had been labeled with a Multiprime DNA labeling system (Amersham International, Amersham, Bucks, UK), as described previously (Kato et al. 1993). A BamHI–HindIII fragment of cDNA for mouse HSP27 (Cooper & Uoshima 1994) was kindly provided by Dr L F Cooper of the University of North Carolina, USA.

Other methods

Protein concentrations in soluble extracts were determined using a protein assay kit (Bio–Rad, Hercules CA, USA), with BSA as the standard protein. Rat HSP27, which was used as the standard for the immunoassay, was purified from skeletal muscle as described previously (Kato et al. 1993). The densitometric analysis was performed using Molecular Analyst/Macintosh (Bio–Rad Laboratories).

Statistical analysis

The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between pairs, and a P<0.05 was considered significant. All data are presented as the mean ± s.e.m. of triplicate determinations.

Results

Effect of PGE2 on the induction of HSP70 and HSP27 in MC3T3-E1 cells

We first examined whether PGE2 affects the levels of HSP70, a high molecular weight HSP, and HSP27, a low molecular weight HSP, in osteoblast-like MC3T3-E1 cells. HSP70 clearly exists before the stimulation by PGE2, whereas the level of HSP27 seems to be quite low in unstimulated cells (Fig. 1). PGE2 had little effect on the level of HSP70 in MC3T3-E1 cells (Fig. 1). On the other hand, PGE2 significantly increased the level of HSP27 in the same sample (Fig. 1). The stimulatory effect of PGE2 on HSP27 induction was time-dependent up to 9 h.

Effect of PGE2 on HSP27 accumulation in MC3T3-E1 cells

To clarify more precisely the effect of PGE2 on HSP27 induction in MC3T3-E1 cells, we determined the level of
HSP27 by a specific enzyme immunoassay. PGE$_2$ (10 µM) significantly stimulated the accumulation of HSP27 in a time-dependent manner up to 12 h (Fig. 2A). The stimulatory effect of PGE$_2$ on the accumulation of HSP27 was dose-dependent in the range between 10 nM and 10 µM (Fig. 2B). The maximum effect of PGE$_2$ was observed at 10 µM.

Expression of the mRNA for HSP27 was markedly induced by 10 µM PGE$_2$ (Fig. 3). The stimulatory effect of PGE$_2$ on the expression appeared at 2 h after the stimulation, and the expression level was much more induced at 6 h.

Effects of staurosporine and calphostin C on the PGE$_2$-induced accumulation of HSP27 in MC3T3-E1 cells

We have previously shown that PGE$_2$ stimulates phosphoinositol hydrolysis by phospholipase C, and phosphatidylinositol hydrolysis by phospholipase D, resulting in the activation of PKC in osteoblast-like MC3T3-E1 cells (Tokuda et al. 1992, Oiso et al. 1995). To elucidate whether or not the activation of PKC is involved in the PGE$_2$-stimulated HSP27 induction, we examined the effect of staurosporine, a potent inhibitor of PKC (Tamaoki et al. 1986), on the PGE$_2$-induced accumulation of HSP27. Staurosporine, which alone had little effect on the level of HSP27, significantly reduced the PGE$_2$-induced accumulation of HSP27 (Table 1). Next, we
tested the effect of calphostin C, a highly potent and specific inhibitor of PKC (Kobayashi et al. 1989), on the PGE₂-induced HSP27 accumulation. Calphostin C, which by itself did not affect the accumulation of HSP27, significantly inhibited the accumulation of HSP27 induced by PGE₂ (Table 1). Furthermore, Ro-31-8220, another potent and specific inhibitor of PKC (Davis et al. 1992, McKenna & Hanson 1993), also reduced the accumulation of HSP27 induced by PGE₂ (data not shown).

**Effect of down-regulation of PKC on HSP27 accumulation of PGE₂ in MC3T3-E1 cells**

It has been reported that a 24-h pretreatment with 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 1992), in PKC +/− TPA pretreatment (Table 2). To further clarify the role of PKC in the PGE₂-stimulated HSP27 induction, we next examined the effect of long-term pretreatment with 0·1 μM TPA on the HSP27 accumulation by PGE₂. The effect of PGE₂ on HSP27 accumulation was significantly reduced in the PKC down-regulated cells compared with that in the cells without TPA pretreatment (Table 2).

**Effects of BAPTA/AM and TMB-8 on the PGE₂-induced accumulation of HSP27 in MC3T3-E1 cells**

It is well known that inositol trisphosphate resulting from phosphoinositide hydrolysis by phospholipase C induces Ca²⁺ mobilization from the intracellular Ca²⁺ store (Berridge & Irvine 1989). In addition, we previously showed that PGE₂ induces Ca²⁺ influx independently from phosphoinositide hydrolysis in MC3T3-E1 cells (Tokuda et al. 1992). To investigate whether or not the intracellular Ca²⁺ mobilization is involved in the PGE₂-stimulated HSP27 induction in these cells, we examined the effect of BAPTA/AM, an intracellular Ca²⁺ chelator (Preston et al. 1991), on the PGE₂-induced HSP27 accumulation. BAPTA/AM, which by itself had little effect on the level of HSP27, markedly inhibited the HSP27 accumulation induced by PGE₂ (Table 3). We next examined the effect of TMB-8, an inhibitor of intracellular Ca²⁺ mobilization (Chiou & Malagodi 1975), on the PGE₂-induced accumulation of HSP27. TMB-8, which alone had little effect on the level of HSP27, dose-dependently suppressed the accumulation of HSP27 stimulated by PGE₂ in the range between 1 and 30 μM (Fig. 4).

PGE₂ is known to stimulate adenylate cyclase, resulting in the formation of cAMP, in cultured osteoblasts including MC3T3-E1 cells (Kozawa et al. 1992, Pilbeam et al. 1996). We examined the effect of Bt₂cAMP on the level of HSP27 in these cells. However, Bt₂cAMP alone had little effect on the HSP27 accumulation (data not shown).

**Table 2 Effect of protein kinase C down-regulation on the PGE₂-induced accumulation of HSP27 in MC3T3-E1 cells. Each value represents the mean ± S.E.M. of triplicate determinations of a representative experiment carried out three times**

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>PGE₂</th>
<th>HSP27 (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>−</td>
<td>17·5 ± 1·6</td>
</tr>
<tr>
<td>Vehicle</td>
<td>+</td>
<td>13·5 ± 12·0</td>
</tr>
<tr>
<td>TPA</td>
<td>−</td>
<td>15·6 ± 1·5</td>
</tr>
<tr>
<td>TPA</td>
<td>+</td>
<td>20·2 ± 2·5*</td>
</tr>
</tbody>
</table>

The cultured cells were pretreated with 0·1 μM TPA or vehicle for 24 h, and then stimulated by 10 μM PGE₂ or vehicle for 12 h. *P<0·05 compared with the value of PGE₂ alone.
Effects of PGE2 on the phosphorylation of p44/p42 MAP kinase and p38 MAP kinase in MC3T3-E1 cells

In order to investigate whether PGE2 activates p44/p42 MAP kinase and/or p38 MAP kinase in MC3T3-E1 cells, we examined the effects of PGE2 on the phosphorylation of these MAP kinases. PGE2 time-dependently induced the phosphorylation of both p44/p42 MAP kinase and p38 MAP kinase (Fig. 5). The maximum stimulatory effects of PGE2 on the phosphorylation of p44/p42 MAP kinase and p38 MAP kinase were observed at 10 min and 3 min, respectively, after the stimulation.

Effects of PD98059, U-0126 and SB203580 on the PGE2-induced accumulation of HSP27 in MC3T3-E1 cells

To investigate whether p44/p42 MAP kinase is involved in the PGE2-stimulated HSP27 induction in MC3T3-E1 cells, we next examined the effects of PD98059 and U-0126, inhibitors of upstream kinase that activates p44/p42 MAP kinase (Alessi et al. 1995, Favata et al. 1998), on the PGE2-induced accumulation of HSP27. PD98059 inhibited the accumulation of HSP27 induced by PGE2 in a dose-dependent manner in the range between 1 and 30 µM (Fig. 6A). The maximum effect of PD98059 was observed at 30 µM, a dose that caused about 60% reduction in the effect of PGE2. The HSP27 accumulation induced by PGE2 was markedly inhibited by U-0126 (Fig. 6B). The effect of U-0126 on the PGE2-induced HSP27 accumulation was dose-dependent in the range between 1 and 30 µM. The maximum effect of U-0126 was observed at 30 µM, a dose that caused about 70% reduction in the effect of PGE2. In addition, we examined the effect of SB203580, an inhibitor of p38 MAP kinase (Cuenda et al. 1995), on the PGE2-induced HSP27 accumulation in these cells. SB203580 dose-dependently suppressed the PGE2-stimulated HSP27 accumulation in the range between 1 and 30 µM (Fig. 6C). The maximum effect of

Table 3 Effects of BAPTA/AM on the PGE2-induced accumulation of HSP27 in MC3T3-E1 cells. Each value represents the mean ± S.E.M. of triplicate determinations of a representative experiment carried out three times

<table>
<thead>
<tr>
<th>BAPTA/AM (µM)</th>
<th>PGE2</th>
<th>HSP27 (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>−</td>
<td>18.5 ± 1.7</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>134.2 ± 13.0</td>
</tr>
<tr>
<td>5</td>
<td>−</td>
<td>156.6 ± 2.7</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>92.5 ± 9.0*</td>
</tr>
<tr>
<td>10</td>
<td>−</td>
<td>162.2 ± 2.1</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>251.1 ± 3.2*</td>
</tr>
</tbody>
</table>

The cultured cells were pretreated with BAPTA/AM or vehicle for 60 min, and then stimulated by 10 µM PGE2 or vehicle for 12 h. *P<0.05 compared with the value of PGE2 alone.
SB203580 was observed at 30 µM, a dose that caused about 70% reduction in the effect of PGE2.

**Effect of PD98059 on the PGE2-induced phosphorylation of p44/p42 MAP kinase and effect of SB203580 on the phosphorylation of p38 MAP kinase induced by PGE2 in MC3T3-E1 cells**

We examined the effect of PD98059 on the PGE2-induced phosphorylation of p44/p42 MAP kinase in these cells. PD98059 markedly reduced the phosphorylation of p44/p42 MAP kinase induced by PGE2 (Fig. 7A). In addition, we found that SB203580 significantly inhibited the PGE2-induced phosphorylation of p38 MAP kinase (Fig. 7B).

**Effects of calphostin C or U-73122 on the PGE2-induced phosphorylation of p44/p42 MAP kinase and p38 MAP kinase in MC3T3-E1 cells**

To clarify whether or not PGE2 activates these MAP kinases in a PKC-dependent fashion in MC3T3-E1 cells, we examined the effects of calphostin C on the phosphorylation of these MAP kinases induced by PGE2. Calphostin C, which by itself had little effect on the phosphorylation of p44/p42 MAP kinase or p38 MAP kinase, significantly reduced the PGE2-induced phosphorylation of these two MAP kinases (Fig. 8). We also examined the effects of U-73122, an inhibitor of phospholipase C (Blessedale et al. 1990) on the phosphorylation of p44/p42 MAP kinase and p38 MAP kinase in these cells. U-73122, which alone did not affect the phosphorylation of these MAP kinases, markedly inhibited the phosphorylation of these two MAP kinases induced by PGE2 (Fig. 8).
Discussion

In the present study, we demonstrated that PGE₂ stimulated the level of HSP27 by means of Western blotting analysis in osteoblast-like MC3T3-E1 cells. In addition, PGE₂ induced the accumulation of HSP27 time- and dose-dependently as detected by a specific immunoassay, and stimulated the level of the mRNA for HSP27. These results strongly suggest that PGE₂ stimulates the induction of HSP27 in MC3T3-E1 cells. As far as we know, this is probably the first report showing the induction of HSP27 by PGE₂ in osteoblasts. On the other hand, we showed that PGE₂ failed to affect the level of HSP70 in MC3T3-E1 cells. It is recognized that high molecular weight HSPs such as HSP90 and HSP70 act as molecular chaperones and protect cells under potentially hazardous conditions (Ellis & van der Vies 1991, Gething & Sambrook 1992). Parathyroid hormone reportedly up-regulates HSP70 transcription in osteosarcoma SaOS-2 and ROS17/2·8 cells (Fukuyama et al. 1996), suggesting that HSP70 not only plays a role in the stress responses but also has physiological functions in osteoblasts. Compared with the high molecular weight HSPs, the roles of low molecular weight HSPs are still poorly understood, although it is speculated that they, too, act as molecular chaperones (Benndorf et al. 1994, Groenen et al. 1994). Our present results suggest that HSP27 but not HSP70 is involved in the effect of PGE₂ on osteoblast cell functions.

In patients with osteosarcomas, it has been shown that overexpression of HSP27 in the biopsy specimens is associated with poor prognosis (Uozaki et al. 1997). As for HSP27 in osteoblasts, it has been reported that the down-regulation of their proliferation is accompanied by a transient increase in the expression of HSP27 mRNA (Shakoori et al. 1992, Cooper & Uoshima 1994). Taking account of our previous reports showing that several mitogenic agonists induce HSP27 accumulation in MC3T3-E1 cells (Kawamura et al. 1999, Kozawa et al. 1999, 2001a, b), it is most likely that HSP27 is involved in the regulation of osteoblast proliferation. Further investigations would be required to clarify the detailed roles of HSP27 in osteoblasts.

We next investigated the exact mechanism underlying the PGE₂-stimulated HSP27 induction in osteoblast-like MC3T3-E1 cells. As for the intracellular signaling of PGE₂ in osteoblast-like MC3T3-E1 cells, we have shown that PGE₂ induces the activation of adenylate cyclase, Ca²⁺ influx, phosphoinositide hydrolysis by phospholipase C and phosphatidylcholine hydrolysis by phospholipase D (Tokuda et al. 1991, 1992, Kozawa et al. 1992, Oiso et al. 1995). Diacylglycerol, resulting from the hydrolysis of phosphoinositides and phosphatidylcholine, is well known to be a physiological activator of PKC (Nishizuka 1992). Herein, we showed that the stimulatory effect of PGE₂ on HSP27 accumulation was markedly suppressed in the PKC down-regulated cells compared with that in intact cells. In addition, staurosporine inhibited the PGE₂-induced accumulation of HSP27. Moreover, calphostin C and Ro-31-8220 reduced the accumulation of HSP27. Thus, these findings suggest that PKC activation is involved in the PGE₂-stimulated HSP27 induction in osteoblast-like MC3T3-E1 cells. In this study, the suppression of the PGE₂-induced HSP27 accumulation in PKC down-regulated cells was almost complete, whereas the inhibition by staurosporine or calphostin C of the HSP27 accumulation induced by PGE₂ was incomplete. Doses of these inhibitors higher than those presented here were toxic in our experimental conditions. We next showed that BAPTA/AM and TMB-8 suppressed the...
HSP27 accumulation induced by PGE₂. It is well known that inositol trisphosphate, a product of phosphoinositide hydrolysis by phospholipase C, acts as an intracellular messenger of the mobilization of Ca²⁺ from the intracellular Ca²⁺ store (Berridge & Irvine 1989). We have previously reported that PGE₂ induces Ca²⁺ influx independently from phosphoinositide hydrolysis by phospholipase C in MC3T3-E1 cells (Tokuda et al. 1992). The suppression by TMB-8 of the HSP27 accumulation induced by PGE₂ was partial, suggesting that not only the intracellular Ca²⁺ mobilization but also Ca²⁺ influx plays a role in the induction of HSP27 accumulation. Thus, it is probable that Ca²⁺ mobilization from both the intracellular Ca²⁺ store and the extracellular space takes part in the PGE₂-stimulated HSP27 induction in osteoblast-like MC3T3-E1 cells. On the other hand, we found that Br₂cAMP had little effect on HSP27 accumulation. Thus, it seems unlikely that adenylyl cyclase activation plays a role in the PGE₂-stimulated induction of HSP27 in MC3T3-E1 cells.

MAP kinases play important roles in intracellular signaling of a variety of agonists (Nishida & Gotoh 1993, Widmann et al. 1999). We have recently reported that endothelin-1- and sphingosine 1-phosphate-stimulated HSP27 induction is mediated by p38 MAP kinase in MC3T3-E1 cells (Kawamura et al. 1999, Kozawa et al. 1999b). In the present study, we showed that PGE₂ elicited the phosphorylation of both p44/p42 MAP kinase and p38 MAP kinase in MC3T3-E1 cells. It is well recognized that MAP kinases are activated by phosphorylation of threonine and tyrosine residues by dual specificity MAP kinase kinase (Nishida & Gotoh 1993, Widmann et al. 1999). Therefore, our present results suggest that PGE₂ activates both p44/p42 MAP kinase and p38 MAP kinase in osteoblast-like MC3T3-E1 cells. Thus, we tested the effects of PD98059, U-0126 and SB203580 on the PGE₂-stimulated induction of HSP27 in these cells, and showed the reduction of HSP27 accumulation by them. We found that PD98059 actually suppressed the PGE₂-induced phosphorylation of p44/p42 MAP kinase, and that SB203580 inhibited the phosphorylation of p38 MAP kinase induced by PGE₂. These results suggest that PGE₂-stimulated HSP27 induction is dependent upon both p44/p42 MAP kinase and p38 MAP kinase in MC3T3-E1 cells. In the present study, PD98059, U-0126 or SB203580 partially suppressed the PGE₂-induced HSP27 accumulation, suggesting that p44/p42 MAP kinase and p38 MAP kinase are parallel pathways in HSP27 induction. On the other hand, the partial inhibition of the phosphorylation by PD98059 or SB203580 are possibly due to the experimental conditions using whole cells in these series. Furthermore, we demonstrated that calphostin C and U-73122 inhibited the phosphorylation of both p44/p42 MAP kinase and p38 MAP kinase induced by PGE₂. Based on our findings as a whole, it is most likely that PGE₂ stimulates PKC activation, and that the activation of both p44/p42 MAP kinase and p38 MAP kinase acts as a positive regulator at a point downstream from PKC activation in the induction of HSP27 in osteoblast-like MC3T3-E1 cells. We previously reported that PGE₂-induced IL-6 synthesis is negatively regulated by PKC activation in these cells. Therefore, in MC3T3-E1 cells it is probable that PGE₂-activated PKC acts as a dual effector, which positively regulates HSP27 induction and negatively regulates IL-6 synthesis. PGE₂ is well known to act as an autocrine/paracrine regulator of osteoblasts (Nijweide et al. 1986). Thus, the concentration of PGE₂ affecting osteoblasts seems to become rather high when the cells secrete PGE₂. It is possible that the PGE₂-stimulated HSP27 induction shown here takes part in the regulation of osteoblast function in the physiological state of osteoblasts. Further investigation would be required to clarify the detail.

In conclusion, our present results strongly suggest that PGE₂ stimulates the induction of HSP27 through Ca²⁺ mobilization and PKC-dependent activation of both p44/p42 MAP kinase and p38 MAP kinase in osteoblasts.

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