Endothelin-1 stimulates heat shock protein 27 induction in osteoblasts: involvement of p38 MAP kinase

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Endothelin-1 stimulates heat shock protein 27 induction in osteoblasts: involvement of p38 MAP kinase. Am. J. Physiol. 277 (Endocrinol. Metab. 40): E1046–E1054, 1999.—We previously reported that endothelin-1 (ET-1) activates p42/p44 mitogen-activated protein (MAP) kinase in osteoblast-like MC3T3-E1 cells and consequently induces synthesis of interleukin-6. In the present study, we investigated the effect of ET-1 on the induction of heat shock protein 27 (HSP 27) in MC3T3-E1 cells. ET-1 time and dose dependently stimulated HSP 27 accumulation. ET-1 induced an increase in the levels of mRNA for HSP 27. Both staurosporine and calphostin C, inhibitors of protein kinase C (PKC), suppressed the ET-1-induced HSP 27 accumulation. 12-O-tetradecanoylphorbol 13-acetate (TPA), a PKC activator, induced the HSP 27 accumulation and the expression of mRNA for HSP 27. The ET-1-stimulated HSP 27 accumulation was reduced in PKC-downregulated MC3T3-E1 cells. The HSP 27 accumulation by ET-1 was not suppressed by PD-98059, an inhibitor of the activation of phosphatidylcholine-hydrolyzing phospholipase (PL) C and mobilizes intracellular Ca2+ (37, 38). In previous studies (35, 36), we have demonstrated that ET-1 induces the activation of phosphatidyglycerol-hydrolyzing PLD independently from the activation of phosphoinositide-hydrolyzing PLC in osteoblast-like MC3T3-E1 cells and that the ETA receptor mediates both of these activations. It is well known that diacylglycerol, a physiological activator of protein kinase C (PKC), is formed by the activation of PLs (26). In addition, we have recently reported that PKC activation is involved in ET-1-stimulated interleukin-6 (IL-6) synthesis in MC3T3-E1 cells (23).

When exposed to biological stress such as heat, cells produce heat shock proteins (HSPs; see Refs. 27 and 28). HSPs are classified into high-molecular-weight HSPs and low-molecular-weight HSPs according to their apparent molecular sizes. High-molecular-weight HSPs such as HSP 90, HSP 70, and HSP 60 are well recognized to function as molecular chaperones implicated in protein folding, oligomerization, and translocation (10). Low-molecular-weight HSPs such as HSP 27 and α-crystallin have significant similarities in terms of amino acid sequences (11, 14). Although the function of them is less understood than that of high-molecular-weight HSPs, it is recognized that low-molecular-weight HSPs may act as chaperones as well as high-molecular-weight HSPs (1). For bone cells, it has been reported that HSP 27 expression is induced by heat in osteoblasts, and pretreatment of estrogen facilitates the expression of HSP 27 (4, 32). Additionally, the downregulation of their proliferation has been shown to be accompanied by a transient increase of the expression of HSP 27 mRNA (4, 32). In a previous study (34), we have shown that a chemical stressor, sodium arsenite, stimulates the induction of HSP 27 in osteoblast-like MC3T3-E1 cells and that the HSP 27 induction is coupled with the increase of arachidonic acid release. However, the exact mechanism of HSP 27 induction in osteoblasts and its roles remain to be clarified.

Bone metabolism is strictly regulated by osteoblasts and osteoclasts, which are responsible for bone formation and bone resorption, respectively (24). The formation of bone structures and bone remodeling results from coupling bone resorption by activated osteoclasts with subsequent deposition of new matrix by osteoblasts. During bone remodeling, capillary endothelial cells provide the microvasculature. Thus it is currently recognized that the activity of osteoblasts, osteoclasts, and endothelial cells is closely coordinated (3, 7).
The mitogen-activated protein (MAP) kinase superfamily mediates intracellular signaling of various agonists and plays important roles in cellular functions, including proliferation, differentiation, and cell death in a variety of cells (25). In osteoblasts, p42/p44 MAP kinase among MAP kinases has been shown to be activated by several agonists such as estrogen, basic fibroblast growth factor, and parathyroid hormone (6, 12, 40). In addition, it has recently been demonstrated that SKF 86002, the cytokine-suppressant anti-inflammatory drug, suppresses IL-6 production by inhibiting p38 MAP kinase in cultured neonatal mouse calvaria and Saos-2 osteoblastic cells (2). We have recently reported that p42/p44 MAP kinase activation is involved in ET-1-induced IL-6 synthesis in osteoblast-like MC3T3-E1 cells (16). However, the roles of the MAP kinase superfamily in osteoblasts have not yet been fully elucidated.

In the present study, we investigated the effect of ET-1 on the induction of HSP 27 in osteoblast-like MC3T3-E1 cells and the potential involvement of the MAP kinase superfamily in the signaling of ET-1. We report here that p38 MAP kinase activation is required for ET-1-stimulated HSP 27 induction in MC3T3-E1 cells.

MATERIALS AND METHODS

Materials. ET-1 was obtained from the Peptide Institute (Minoh, Japan). Stauorosporine, calphostin C, 12-O-tetradecanoylphorbol 13-acetate (TPA), and 4k-phorbol 12,13-didecanoate (4k-PDD) were purchased from Sigma Chemical (St. Louis, MO). Cyclo-d-Trp-d-Asp-Pro-c-Val-Leu (BQ-123), N-cis-2,6-dimethyl-piperidinocarbonyl-L-γ-Meleu-d-Trp(COOMe)-d-Nle-Ona (BQ-788), genistein, and 1-6(17-3-methoxyestra-1,3,5(10)-trien-17-ylaminohexyl)-1H-pyrrrole-2,5-dione (U-73122) were purchased from Funakoshi Pharmaceutical (Tokyo, Japan). dl-Propranolol hydrochloride (propranolol) was purchased from Wako Pure Chemical (Osaka, Japan). PD-98059 and SB-203580 were obtained from Calbiochem-Novabiochem (La Jolla, CA). Phospho-specific p38 MAP kinase antibodies (rabbit polyclonal IgG, affinity purified), phospho-specific p42/p44 MAP kinase antibodies (rabbit polyclonal IgG, affinity purified), p38 MAP kinase antibodies (rabbit polyclonal IgG, affinity purified), and p42/p44 MAP kinase antibodies (rabbit polyclonal IgG, affinity purified) were purchased from New England Biolabs (Beverly, MA). An enhanced chemiluminescence (ECL) Western blotting detection system was obtained from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. Stauorosporine, calphostin C, TPA, U-73122, propranolol, PD-98059, SB-203580, 4k-PDD, and genistein were dissolved in DMSO. The maximum concentration of DMSO was 0.1%, which did not affect the immunoblotting of HSP 27, Northern blotting analysis of HSP 27, or the analysis of MAP kinases.

Cell culture. Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria (17) were maintained as previously described (16). In brief, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% FCS at 37°C in a humidified atmosphere of 5% CO2:95% air. The cells (5 × 104) were seeded in 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. When indicated, the cells were pretreated with BQ-123, BQ-788, PD-98059, or SB-203580 for 60 min before the stimulation of ET-1. The pretreatment with staurosporine, calphostin C, U-73122, propranolol, or genistein was performed for 20 min. For the experiment of the downregulation of PKC, the cultured cells were pretreated with 0.1 µM of TPA for 24 h and then were stimulated by ET-1.

Immunoassay of HSP 27. The concentration of HSP 27 in soluble extracts of cells was determined by sandwich-type enzyme immunoassays, as described previously (13). The cultured cells were stimulated by ET-1, TPA, or 4k-PDD in α-MEM for the indicated periods. The cells were then washed two times with 1 ml of 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 the specific immunoassay of HSP 27. In brief, we used an enzyme immunoassay system that employs polystyrene balls (3.2 mm in diameter; Immunno Chemicals, Okayama, Japan) carrying immobilized F(ab')2 fragments of antibody and the same F(ab')2 fragments labeled with β-galactosidase from Escherichia coli. A polystyrene ball carrying antibodies was incubated either with the purified standard for HSP 27 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% hydroyzed gelatin, 0.1% BSA, 1 mM MgCl2, and 0.1% NaN3. After being washed, each ball was incubated at 4°C overnight with 1.5 µg of galactosidase-labeled antibodies in a volume of 0.2 ml with 10 µM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl, 1 mM MgCl2, 0.1% BSA, and 0.1% NaN3. The galactosidase activity bound to the ball was assayed using a fluorogenic substrate, 4-methylumbelliferyl-β-galactosidase.

Isolation of RNA and Northern blotting analysis of HSP 27. The cultured cells were stimulated by ET-1 or TPA in serum-free α-MEM for the indicated periods. Total RNA was isolated using a QuickPrep Total RNA Extraction kit (Pharmacia Biotech, Tokyo, Japan). Next, 20 µg of total RNA were subjected to electrophoresis on a 0.9% agarose:2.2 M formaldehyde gel and were blotted onto a nitrocellulose membrane. For Northern blotting, membrane was allowed to hybridize with a DNA probe that had been labeled using a Multiprime DNA labeling system (Amersham, Buckinghamshire, UK), as described previously (15). A BamH I-Hind III fragment of cDNA for mouse HSP 27 (4) was kindly provided by Dr. L. F. Cooper of the University of North Carolina. Analysis of p38 MAP kinase and p42/p44 MAP kinase by Western blotting. The cultured cells were stimulated by ET-1 in serum-free α-MEM for the indicated periods. The cells were washed two times with PBS and then lysed, homogenized, and sonicated in a lysis buffer containing 62.5 mM Tris·Cl, pH 6.8, 2% SDS, 50 mM dithiothreitol, and 10% glycerol. The cytosolic fraction was collected as the supernatant after centrifugation at 125,000 × g for 10 min at 4°C. SDS-PAGE was performed by the method of Laemml (15) in 10% polyacrylamide gel. Western blotting analysis was performed as described previously (15) by using phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, or p42/p44 MAP kinase antibodies and peroxidase-labeled antibodies raised in goat against rabbit IgG as secondary antibodies. Peroxidase activity on the nitrocellulose sheet was visualized on X-ray film by use of an ECL Western blotting detection system.

Statistical analysis. Each experiment was repeated three times with similar results. The data were analyzed by...
ANOVA followed by the Bonferroni method for multiple comparisons between pairs, and $P < 0.05$ was considered significant. All data are presented as means $\pm$ SD of triplicate determinations.

RESULTS

Effect of ET-1 on HSP 27 accumulation in MC3T3-E1 cells. ET-1 (3 nM) significantly increased the accumulation of HSP 27 in a time-dependent manner up to 24 h (Fig. 1A). The maximum effect of ET-1 on HSP 27 accumulation was observed at 12 h after the stimulation. The stimulatory effect of ET-1 on HSP 27 accumulation was dose dependent in the range between 10 pM and 0.1 µM (Fig. 1B). The maximum effect of ET-1 on HSP 27 accumulation was observed at 3 nM.

Northern blot analysis of the mRNA for HSP 27 in response to ET-1 in MC3T3-E1 cells. The expression levels of the mRNA for HSP 27 were markedly increased by 3 nM ET-1 (Fig. 2). The ET-1-induced increase in the mRNA levels for HSP 27 was observed at 2 h after the ET-1 stimulation. The levels of the mRNA at 4 or 6 h after the stimulation of ET-1 returned to the control levels.

Effects of BQ-123 or BQ-788 on the ET-1-induced HSP 27 accumulation in MC3T3-E1 cells. In a previous study (36), we have shown that the ET$_A$ receptor mediates the signaling of ET-1 in osteoblast-like MC3T3-E1 cells. We investigated whether the ET$_A$ receptor mediates the induction of HSP 27 in these cells. BQ-123, a selective ET$_A$ receptor antagonist (36) that alone did not affect the basal levels of HSP 27, inhibited the ET-1-induced HSP 27 accumulation (Table 1). On the contrary, BQ-788, a selective ET$_B$ receptor antagonist (36), had little effect on the ET-1-induced HSP 27 accumulation (Table 1).

Effect of staurosporine on the ET-1-induced HSP 27 accumulation in MC3T3-E1 cells. In previous studies (35, 36), we have reported that ET-1 induces phosphoinositide hydrolysis by PLC and phosphatidylcholine hydrolysis by PLD via independent pathways in osteoblast-like MC3T3-E1 cells. It is well recognized that both phosphoinositide hydrolysis by PLC and phosphatidylcholine hydrolysis by PLD result in the formation of diacylglycerol, known as a physiological activator of PKC (26). Therefore, it is probable that ET-1 activates PKC through hydrolysis of both phosphoinositide and phosphatidylcholine in MC3T3-E1 cells. To clarify whether PKC activation is involved in ET-1-stimulated HSP 27 induction in MC3T3-E1 cells, we next examined the effect of staurosporine, an inhibitor of protein kinases including PKC (31), on the ET-1-stimulated HSP 27 accumulation. Staurosporine, which alone did not affect the basal levels of HSP 27, significantly suppressed the accumulation of HSP 27 induced by 3 nM ET-1 (Fig. 3). The inhibitory effect of staurosporine was dose dependent in the range between 1 nM and 0.1 µM. The maximum effect of the inhibitor was observed at 0.1 µM, a dose that caused $\sim$90% reduction in the effect of ET-1.

Effect of calphostin C on the ET-1-induced HSP 27 accumulation in MC3T3-E1 cells. In addition, we exam-
ined the effect of calphostin C, a highly potent and specific inhibitor of PKC (18), on the HSP 27 accumulation stimulated by ET-1. Calphostin C (0.5 µM), which by itself had little effect on the basal levels of HSP 27, significantly suppressed the ET-1-stimulated HSP 27 accumulation (Fig. 4).

Effects of phorbol esters on HSP 27 accumulation, the levels of mRNA for HSP 27, and p38 MAP kinase phosphorylation in MC3T3-E1 cells. To further clarify the role of PKC in the ET-1-stimulated HSP 27 induction in MC3T3-E1 cells, we examined the effects of phorbol esters on HSP 27 accumulation. TPA (10 nM), a PKC-activating phorbol ester (26), alone induced the HSP 27 accumulation. On the contrary, 4a-PDD (10 nM), a PKC-nonactivating phorbol ester (26), did not affect the HSP 27 accumulation (control, 4.4 ± 0.8 ng/mg protein; 10 nM TPA, 19.3 ± 1.6 ng/mg protein; 10 nM 4a-PDD, 4.8 ± 1.1 ng/mg protein; each value represents the mean ± SD of triplicate determinations of a representative experiment carried out 3 times, as measured during the stimulation for 12 h). The expression levels of mRNA for HSP 27 were increased by 10 nM TPA (Fig. 5A). The TPA-induced increase in the mRNA levels for HSP 27 was observed at 4 h after the TPA stimulation. In addition, TPA (10 nM) induced the phosphorylation of p38 MAP kinase in a time-dependent manner (Fig. 5B).

Table 1. Effects of BQ-123 or BQ-788 on ET-1-induced HSP 27 accumulation in MC3T3-E1 cells

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<tr>
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<th>HSP 27, ng/mg protein</th>
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<tr>
<td>Control</td>
<td>3.5 ± 0.7</td>
</tr>
<tr>
<td>ET-1</td>
<td>300.0 ± 12.3</td>
</tr>
<tr>
<td>BQ-123</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>BQ-123 + ET-1</td>
<td>8.9 ± 1.4*</td>
</tr>
<tr>
<td>BQ-788</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>BQ-788 + ET-1</td>
<td>287.1 ± 11.9</td>
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</table>

Each value represents the mean ± SD of triplicate determinations. ET, endothelin; HSP, heat shock protein. Cultured cells were pretreated with 2.2 µM (IC50 for ETa receptors × 100) BQ-123, 120 nM (IC50 for ETb receptor × 100) BQ-788, or vehicle for 60 min and then were stimulated by 3 nM ET-1 or vehicle for 12 h. Similar results were obtained with 2 additional and different cell preparations. *P < 0.05 vs. ET-1 alone.
Effect of PKC downregulation on the ET-1-induced HSP 27 accumulation in MC3T3-E1 cells. We previously showed that ET-1 induces phosphoinositide hydrolysis by PLC in MC3T3-E1 cells (35). To clarify the role of PKC activation in the HSP 27 induction by ET-1 in these cells, we examined the effect of U-73122, a PLC inhibitor (19), on the HSP 27 accumulation stimulated by ET-1. The ET-1-induced HSP 27 accumulation was significantly suppressed by U-73122 at 30 µM, which alone had little effect on the basal levels of HSP 27. U-73122 (30 µM) caused ~50% reduction in the effect of ET-1 (Table 2).

Effect of U-73122 or propranolol on ET-1-induced HSP 27 accumulation in MC3T3-E1 cells. Effect of propranolol on the ET-1-induced HSP 27 accumulation in MC3T3-E1 cells. We previously showed that ET-1 induces phosphoinositide hydrolysis by PLC in MC3T3-E1 cells (35). To clarify the role of PKC in the ET-1-stimulated HSP 27 induction, we examined the effect of TPA (0.1 µM) long-term pretreatment on the HSP 27 accumulation stimulated by ET-1. The ET-1-induced HSP 27 accumulation was significantly suppressed by the long-term pretreatment of TPA, which caused ~60% reduction in the effect of ET-1 (Fig. 6).

Effect of propranolol on the ET-1-induced HSP 27 accumulation in MC3T3-E1 cells. We have demonstrated that ET-1 stimulates phosphatidylcholine hydrolysis by PLD independently from phosphoinositide hydrolysis by PLC in MC3T3-E1 cells (35). It is recognized that phosphatidic acid formed through phosphatidylcholine hydrolysis by PLD is degraded into diacylglycerol by phosphatidic acid phosphohydrolase (8). To clarify the role of PLD activation in the HSP 27 induction by ET-1 in these cells, we next examined the effect of propranolol, a phosphatidic acid phosphohydrolase inhibitor (19), on the HSP 27 accumulation stimulated by ET-1. The ET-1-stimulated HSP 27 accumulation was significantly suppressed by propranolol at 100 µM, which did not affect the basal levels of HSP 27. Propranolol (100 µM) caused ~40% reduction in the effect of ET-1 (Table 2).

Effect of PKC downregulation on the ET-1-induced HSP 27 accumulation in MC3T3-E1 cells. Effect of TPA (0.1 µM) on ET-1-induced HSP 27 accumulation in MC3T3-E1 cells. To further clarify the role of PKC in the ET-1-stimulated HSP 27 induction, we examined the effect of TPA (0.1 µM) long-term pretreatment on the HSP 27 accumulation stimulated by ET-1. The ET-1-induced HSP 27 accumulation was significantly suppressed by the long-term pretreatment of TPA, which caused ~60% reduction in the effect of ET-1 (Table 2).

Effect of propranolol on the ET-1-induced HSP 27 accumulation in MC3T3-E1 cells. We previously showed that ET-1 induces phosphatidylcholine hydrolysis by PLD in MC3T3-E1 cells (35). To clarify the role of PKC in the ET-1-stimulated HSP 27 induction, we examined the effect of U-73122, a PLC inhibitor (19), on the HSP 27 accumulation stimulated by ET-1. The ET-1-stimulated HSP 27 accumulation was significantly suppressed by U-73122 at 30 µM, which alone had little effect on the basal levels of HSP 27. U-73122 (30 µM) caused ~50% reduction in the effect of ET-1 (Table 2).

Effect of propranolol on the ET-1-induced HSP 27 accumulation in MC3T3-E1 cells. We previously showed that ET-1 induces phosphatidylcholine hydrolysis by PLD in MC3T3-E1 cells (35). To clarify the role of PKC in the ET-1-stimulated HSP 27 induction, we examined the effect of U-73122, a PLC inhibitor (19), on the HSP 27 accumulation stimulated by ET-1. The ET-1-stimulated HSP 27 accumulation was significantly suppressed by U-73122 at 30 µM, which alone had little effect on the basal levels of HSP 27. U-73122 (30 µM) caused ~50% reduction in the effect of ET-1 (Table 2).

Effect of PKC downregulation on the ET-1-induced HSP 27 accumulation in MC3T3-E1 cells. Effect of TPA (0.1 µM) on ET-1-induced HSP 27 accumulation in MC3T3-E1 cells. To further clarify the role of PKC in the ET-1-stimulated HSP 27 induction, we examined the effect of TPA (0.1 µM) long-term pretreatment on the HSP 27 accumulation stimulated by ET-1. The ET-1-induced HSP 27 accumulation was significantly suppressed by the long-term pretreatment of TPA, which caused ~60% reduction in the effect of ET-1 (Table 2).

Effect of PKC downregulation on the ET-1-induced HSP 27 accumulation in MC3T3-E1 cells. Effect of TPA (0.1 µM) on ET-1-induced HSP 27 accumulation in MC3T3-E1 cells. To further clarify the role of PKC in the ET-1-stimulated HSP 27 induction, we examined the effect of TPA (0.1 µM) long-term pretreatment on the HSP 27 accumulation stimulated by ET-1. The ET-1-induced HSP 27 accumulation was significantly suppressed by the long-term pretreatment of TPA, which caused ~60% reduction in the effect of ET-1 (Table 2).

Table 2. Effects of U-73122 or propranolol on ET-1-induced HSP 27 accumulation in MC3T3-E1 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HSP 27, ng/mg protein</th>
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<tbody>
<tr>
<td>Control</td>
<td>7.4 ± 1.6</td>
</tr>
<tr>
<td>ET-1</td>
<td>294.4 ± 12.3</td>
</tr>
<tr>
<td>U-73122</td>
<td>143.3 ± 3.8</td>
</tr>
<tr>
<td>U-73122 + ET-1</td>
<td>167.8 ± 14.3*</td>
</tr>
<tr>
<td>Propranolol</td>
<td>9.6 ± 1.3</td>
</tr>
<tr>
<td>Propranolol + ET-1</td>
<td>190.4 ± 19.5*</td>
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Each value represents the mean ± SD of triplicate determinations. *P < 0.05 vs. ET-1 alone.

Fig. 6. Effect of protein kinase C (PKC) downregulation on ET-1-induced HSP 27 accumulation in MC3T3-E1 cells. Cultured cells were pretreated with 0.1 µM TPA for 24 h and then were stimulated by 3 nM ET-1 or vehicle for 12 h. Each value represents mean ± SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *P < 0.05 vs. ET-1 alone.
Effects of calphostin C or U-73122 on the ET-1-induced p38 MAP kinase phosphorylation in MC3T3-E1 cells. We next investigated the effect of calphostin C or U-73122 on the ET-1-stimulated p38 MAP kinase phosphorylation in MC3T3-E1 cells. Both inhibitors markedly suppressed the ET-1-stimulated phosphorylation of p38 MAP kinase (Fig. 8B).

Effect of SB-203580 on the mRNA levels for HSP 27 in response to ET-1 in MC3T3-E1 cells. We further investigated the effect of SB-203580 on the ET-1-increased levels of the mRNA for HSP 27 in MC3T3-E1 cells. SB-203580 significantly suppressed the expression levels in the mRNA for HSP 27 by ET-1 (Fig. 9).

Effects of PD-98059 on the ET-1-induced phosphorylation of MAP kinases in MC3T3-E1 cells. We examined the effects of PD-98059 on ET-1-induced phosphorylation of p38 MAP kinase and p42/p44 MAP kinase in the same samples. PD-98059 suppressed the phosphorylation of p42/p44 MAP kinase induced by ET-1 (Fig. 10A). On the other hand, PD-98059 had little effect on the phosphorylation of p38 MAP kinase (Fig. 10B).

Effects of genistein on the ET-1-induced HSP 27 accumulation and phosphorylation of p38 MAP kinase in MC3T3-E1 cells. Staurosporine is a mixed inhibitor of PKC and protein tyrosine kinases (31). To clarify the role of tyrosine kinases in the ET-1-stimulated HSP 27 induction, we examined the effect of genistein, an inhibitor of protein tyrosine kinases (19), on the ET-1-induced HSP 27 accumulation in MC3T3-E1 cells. Genistein (10 mg/ml) caused ~60% reduction in the effect of ET-1 on the HSP 27 accumulation (data not shown).

Fig. 7. Effect of PD-98059 or SB-203580 on ET-1-induced HSP 27 accumulation in MC3T3-E1 cells. A: cultured cells were pretreated with various doses of PD-98059 for 60 min and then were stimulated by 3 nM ET-1 (●) or vehicle (○) for 12 h. B: cultured cells were pretreated with various doses of SB-203580 for 60 min and then were stimulated by 3 nM ET-1 (●) or vehicle (○) for 12 h. Each value represents mean ± SD of triplicate determinations. *P < 0.05 vs. ET-1 alone.

Fig. 8. Effect of ET-1 on phosphorylation of p38 MAP kinase and effects of calphostin C or U-73122 on ET-1-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. A: cultured cells were stimulated by 3 nM ET-1 for the indicated periods. Extracts of cells were subjected to SDS-PAGE against phospho-specific p38 MAP kinase antibodies or p38 MAP kinase antibodies. Lane 1, control cells; lane 2, stimulation for 1 min; lane 3, stimulation for 3 min; lane 4, stimulation for 5 min; lane 5, stimulation for 10 min; lane 6, stimulation for 20 min; lane 7, stimulation for 30 min. B: cultured cells were pretreated with vehicle (lanes 1 and 2), 0.5 µM calphostin C (lanes 3 and 4), or 30 µM U-73122 (lanes 5 and 6) for 20 min and then were stimulated by vehicle (lanes 1, 3, and 5) or 3 nM ET-1 (lanes 2, 4, and 6) for 20 min.

Fig. 9. Effect of SB-203580 on mRNA levels for HSP 27 in response to ET-1 in MC3T3-E1 cells. Cultured cells were pretreated with 30 µM SB-203580 for 60 min and then were stimulated by 3 nM ET-1 or vehicle for 2 h. Cells were then harvested, and total RNA was isolated. RNA from each sample (20 µg) was subjected to electrophoresis and blotted on a nitrocellulose membrane. Membrane was then allowed to hybridize with cDNA probe for HSP 27. Bands of 28S RNA are shown for reference.
In the present study, we showed that ET-1 mediated IL-6 synthesis through p42/p44 MAP kinase activation in osteoblast-like MC3T3-E1 cells. In a previous study (36), we have shown that ET-1 activates phosphoinositide-hydrolyzing PLC and phosphatidylcholine-hydrolyzing PLD through the ETA receptor in osteoblast-like MC3T3-E1 cells. In the present study, we found that ET-1 induces phosphoinositide hydrolysis by PLC and phosphatidylcholine hydrolysis by PLD, which plays an important role as a positive regulator of ET-1-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells.

On the other hand, we previously reported that ET-1 activates phosphoinositide-hydrolyzing PLC and phosphatidylcholine-hydrolyzing PLD through the ETA receptor in osteoblast-like MC3T3-E1 cells (16). Thus, to clarify whether p42/p44 MAP kinase activation is involved in the HSP 27 induction by ET-1. Based on our findings, it is most likely that both phosphoinositide-hydrolyzing PLC activation and phosphatidylcholine-hydrolyzing PLD activation are involved in the ET-1-stimulated HSP 27 induction in osteoblast-like MC3T3-E1 cells. It is well recognized that PKC is activated through both hydrolysis of phosphoinositide by PLC and phosphatidylcholine by PLD (26). We next investigated whether PKC activation mediates the effect of ET-1 on HSP 27 induction in osteoblast-like MC3T3-E1 cells. Staurosporine and calphostin C significantly reduced the accumulation of HSP 27 induced by ET-1. These results suggest the involvement of PKC in ET-1-stimulated HSP 27 induction in osteoblast-like MC3T3-E1 cells. In addition, TPA but not 4α-PDD stimulated the HSP 27 accumulation and the expression of mRNA for HSP 27. Furthermore, we demonstrated that the effect of ET-1 on HSP 27 accumulation in PKC-downregulated MC3T3-E1 cells was much weaker than that in the intact cells. Therefore, it is most likely that PKC activation through phosphoinositide-hydrolyzing PLC and phosphatidylcholine-hydrolyzing PLD plays an important role as a positive regulator of ET-1-stimulated HSP 27 induction in osteoblast-like MC3T3-E1 cells.

DISCUSSION

In the present study, we showed that ET-1 stimulated the accumulation of HSP 27, as detected by specific enzyme immunoassay, in a time- and dose-dependent manner in osteoblast-like MC3T3-E1 cells. Additionally, we found that ET-1 increased the expression levels of mRNA for HSP 27 in these cells. Thus our findings suggest that ET-1, as a physiological agonist for osteoblasts, stimulates the induction of HSP 27, a low-molecular-weight HSP, in osteoblast-like MC3T3-E1 cells.

There is an abundant supply of blood in bone tissue, and capillary endothelial cells provide the microvasculature during bone remodeling (7). It has been reported that the activity of bone cells is regulated by a complex network of humoral factors produced within the bone microenvironment, including peptide and nonpeptide molecules (3). Thus the activity of osteoblasts, osteoclasts, and endothelial cells is closely coordinated (7). It is well known that ET-1 is a potent vasoconstrictor produced by endothelial cells (21) and that ET receptors exist on osteoblasts (29). Therefore, it is probable that ET-1, which is produced and secreted from endothelial cells in the bone microenvironment, stimulates the HSP 27 induction through the specific receptors. In addition, osteoclasts in the bone marrow are also biological targets for ET-1 (3). However, it is not known whether ET-1 affects the induction of HSP 27 in osteoclasts.

In a previous study (35), we have reported that ET-1 induces phosphoinositide hydrolysis by PLC and phosphatidylcholine hydrolysis by PLD via independent pathways in osteoblast-like MC3T3-E1 cells. First, to clarify whether the phosphoinositide-hydrolyzing PLC is involved in the ET-1-stimulated induction of HSP 27, we examined the effect of U-73122 (19). U-73122 significantly reduced the ET-1-induced accumulation of HSP 27. In addition, we investigated the involvement of phosphatidylcholine-hydrolyzing PLD in the HSP 27 induction by ET-1. Propranolol, a useful tool for studying the involvement of phosphatidic acid phosphohydrolase (19), markedly inhibited the HSP 27 accumulation by ET-1. Based on our findings, it is most likely that both phosphoinositide-hydrolyzing PLC activation and phosphatidylcholine-hydrolyzing PLD activation are involved in the ET-1-stimulated HSP 27 induction in osteoblast-like MC3T3-E1 cells. It is well recognized that PKC is activated through both hydrolysis of phosphoinositide by PLC and phosphatidylcholine by PLD (26). We next investigated whether PKC activation mediates the effect of ET-1 on HSP 27 induction in osteoblast-like MC3T3-E1 cells. Staurosporine and calphostin C significantly reduced the accumulation of HSP 27 induced by ET-1. These results suggest the involvement of PKC in ET-1-stimulated HSP 27 induction in osteoblast-like MC3T3-E1 cells. In addition, TPA but not 4α-PDD stimulated the HSP 27 accumulation and the expression of mRNA for HSP 27. Furthermore, we demonstrated that the effect of ET-1 on HSP 27 accumulation in PKC-downregulated MC3T3-E1 cells was much weaker than that in the intact cells. Therefore, it is most likely that PKC activation through phosphoinositide-hydrolyzing PLC and phosphatidylcholine-hydrolyzing PLD plays an important role as a positive regulator of ET-1-stimulated HSP 27 induction in osteoblast-like MC3T3-E1 cells.

We previously reported that ET-1 induces phosphoinositide hydrolysis by PLC and phosphatidylcholine hydrolysis by PLD in osteoblast-like MC3T3-E1 cells (16). Thus, to clarify whether p42/p44 MAP kinase activation is involved in the induction of HSP 27 stimulated by ET-1 in these cells, the effect of PD-98059 on the HSP 27 accumulation was first investigated. However, PD-98059 failed to affect the HSP 27-stimulated accumulation of HSP 27. We previously found that PD-98059 suppresses the p42/p44 MAP kinase activation by ET-1 in MC3T3-E1 cells (16). Therefore, it seems unlikely that ET-1 stimulates the induction of HSP 27 through
p42/p44 MAP kinase activation in osteoblast-like MC3T3-E1 cells.

It is well known that p38 MAP kinase is another member of the MAP kinase superfamily (25). In osteoblasts, it has been reported that the mitogenic response of osteoblastic cells to epidermal growth factor or hypoxia is associated with the selective phosphorylation and activation of not p38 MAP kinase but p42/p44 MAP kinase (22). In cultured neonatal mouse calvaria and Saos-2 osteoblastic cells, SK&F 86002, the cytokine-suppressant anti-inflammatory drug, has been shown to reduce IL-6 synthesis by inhibiting p38 MAP kinase (2). However, the exact role of p38 MAP kinase in osteoblasts has not yet been fully clarified. Thus, we next investigated whether or not ET-1 stimulates the activation of p38 MAP kinase in MC3T3-E1 cells. We demonstrated that the phosphorylation of p38 MAP kinase was significantly stimulated by ET-1. It is well recognized that p38 MAP kinase is activated by phosphorylation on tyrosine and threonine by dual-specificity MAP kinase kinase (30). Therefore, it is probable that ET-1 activates p38 MAP kinase in MC3T3-E1 cells. We next showed that SB-203580 markedly suppressed the HSP 27 accumulation by ET-1. In addition, PD-98059 suppressed the phosphorylation of p42/p44 MAP kinase induced by ET-1 while having no effect on the phosphorylation of p38 MAP kinase. These results suggest that not p42/p44 MAP kinase but p38 MAP kinase is involved in the ET-1-stimulated HSP 27 induction in MC3T3-E1 cells. Furthermore, the ET-1-induced levels of mRNA for HSP 27 were markedly reduced by SB-203580. When our findings are taken into account, it is most likely that not p42/p44 MAP kinase activation but p38 MAP kinase activation is necessary for the ET-1-stimulated HSP 27 induction in osteoblast-like MC3T3-E1 cells. Namely, ET-1 stimulates IL-6 synthesis and HSP 27 induction via activation of distinct MAP kinases, p42/p44 MAP kinase and p38 MAP kinase, in these cells. There are no reports showing that any other humoral factors activate the cascade of p38 MAP kinase to HSP 27 in osteoblasts as far as we know.

It is recognized that p38 MAP kinase is involved in the phosphorylation and translocation of HSP 27 in various biological systems. HSP 27 is present in a significant amount, even in unstimulated cells of several types, such as smooth muscle (13). It has been shown that interleukin-1 activates p38 MAP kinase, which results in the phosphorylation of HSP 27 in human epidermal carcinoma cells, and that the phosphorylation of HSP 27 via p38 MAP kinase is induced by carbobach in airway smooth muscle (9, 20). It is generally recognized that these phenomena are induced within 1 h after the stimulation. In the present study, we demonstrated that ET-1 significantly induced HSP 27 after 4 h of stimulation. When our findings are taken into account, it is most likely that p38 MAP kinase activation is required for the HSP 27 induction by ET-1 in osteoblast-like MC3T3-E1 cells.

We showed that calphostin C suppressed the ET-1-induced phosphorylation of p38 MAP kinase. In addition, TPA by itself induced the phosphorylation of p38 MAP kinase in a time-dependent manner. Thus these findings suggest that the ET-1-stimulated p38 MAP kinase activation is dependent on PKC activation in MC3T3-E1 cells. Furthermore, the ET-1-induced phosphorylation of p38 MAP kinase was reduced by U-73122. Thus our findings strongly suggest that PKC is an important mediator in p38 MAP kinase activation of HSP 27 synthesis induced by ET-1.

It is known that staurosporine is a mixed inhibitor of PKC and protein tyrosine kinases (31). Thus we investigated the role of tyrosine kinases in the ET-1-stimulated HSP 27 induction in MC3T3-E1 cells. We found that genistein significantly suppressed the phosphorylation of p38 MAP kinase induced by ET-1. Thus these findings suggest that tyrosine kinases are involved in the ET-1-stimulated HSP 27 induction in these cells. It is well recognized that p38 MAP kinase is activated by dual phosphorylation on tyrosine and threonine by specific MAP kinase kinase (30). When our findings are taken into account, it is probable that staurosporine reduces the activation of p38 MAP kinase through both the suppression of the activation of PKC and the phosphorylation of tyrosine of p38 MAP kinase, resulting in the inhibition of HSP 27 induction.

As for HSP 27 in osteoblastic function, it has been shown that the downregulation of their proliferation of osteoblasts is accompanied by a transient increase of the expression of HSP 27 mRNA in physiological conditions (4). On the other hand, it has been reported that overexpression of HSP 27 at biopsy in patients with osteosarcoma is associated with poor prognosis (39). However, the precise roles of HSP 27 in osteoblasts have not yet been fully elucidated. Further investigations would be necessary to clarify the roles of HSP 27 in osteoblastic function. In conclusion, these results strongly suggest that ET-1 stimulates HSP 27 induction via p38 MAP kinase activation in osteoblast-like cells.

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E1054 HEAT SHOCK PROTEIN 27 AND OSTEOSTERALS


