EXPERIMENTAL STUDY

Involvement of stress-activated protein kinase/c-Jun N-terminal kinase in endothelin-1-induced heat shock protein 27 in osteoblasts

H Tokuda1,2, M Niwa2, H Ito3, Y Oiso4, K Kato3 and O Kozawa2

1Department of Internal Medicine, Chubu National Hospital, National Institute for Longevity Sciences, Obu, Aichi 474-8511, Japan, 2Department of Pharmacology, Gifu University School of Medicine, Gifu 500-8705, Japan, 3Department of Biochemistry, Institute for Developmental Research, Aichi Human Service Center, Kasugai, Aichi 480-0391, Japan and 4First Department of Internal Medicine, Nagoya University School of Medicine, Nagoya 466-8550, Japan

(Correspondence should be addressed to O Kozawa; Email: okozawa@cc.gifu-u.ac.jp)

Abstract

Objective: We have reported that endothelin-1 (ET-1) activates p38 mitogen-activated protein (MAP) kinase through protein kinase C in osteoblast-like MC3T3-E1 cells, and that p38 MAP kinase plays a role in the ET-1-induced heat shock protein 27 (HSP27). Recently, we found that stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) is activated by ET-1 in these cells. In the present study, we have investigated the involvement of SAPK/JNK in ET-1-induced HSP27 in MC3T3-E1 cells.

Methods: The concentration of HSP27 in soluble extracts of the cells, the expression of mRNA for HSP27, and the phosphorylation of SAPK/JNK were determined by an enzyme immunoassay, Northern blot analysis, and Western blot analysis respectively.

Results: SP600125, a specific inhibitor of SAPK/JNK, markedly reduced ET-1-stimulated HSP27 accumulation. The inhibitory effect of SP600125 was dose dependent in the range between 1 and 50 μM. SP600125 reduced the ET-1-increased level of HSP27 mRNA. Calphostin C and Go 6976, inhibitors of protein kinase C, reduced the ET-1-induced phosphorylation of SAPK/JNK. 12-O-Tetradecanoylphorbol-13-acetate, a direct activator of protein kinase C, induced SAPK/JNK phosphorylation, which was suppressed by SP600125. A combination of SP600125 and p38 MAP kinase inhibitor such as SB203580 and PD169316 additively reduced the ET-1-stimulated accumulation of HSP27.

Conclusions: These results strongly suggest that JNK plays a part in ET-1-induced HSP27 in addition to p38 MAP kinase in osteoblasts.

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Introduction

Cells produce heat shock proteins (HSPs) when exposed to biological stress, such as heat stress (1). HSPs are classified into high and low molecular weight HSPs based on their apparent molecular size. It is well known that high molecular weight HSPs, such as HSP90 and HSP70, act as molecular chaperones in protein folding, oligomerization and translocation (1). Low molecular weight HSPs with molecular masses from 10 to 30 kDa, such as HSP27, have high homology in amino acid sequences (1). It is currently recognized that low molecular weight HSPs may have chaperoning functions like high molecular weight HSPs (1). Bone metabolism is regulated by two functional cells, osteoblasts and osteoclasts, responsible for bone formation and bone resorption respectively (2). The formation of bone structures and bone remodeling results from coupling bone resorption by activated osteoblasts with subsequent deposition of new matrix by osteoblasts. Accumulating evidence indicates that osteoblasts are responsible for bone-resorptive factors such as parathyroid hormone and 1,25-(OH)2 vitamin D3 (2) through the up-regulation of receptor activation of NF-κB ligand (RANKL) expression (3), suggesting that osteoblasts play pivotal roles in the regulation of bone remodeling. In osteoblasts, the expression of HSP27 is induced by heat, which is reportedly facilitated by estrogen (4, 5). In addition, it has been shown that the down-regulation of proliferation is accompanied by a transient increase of the expression of HSP27 mRNA (4, 5). We have shown that physiological agents for bone metabolism, such as prostaglandin E2 and transforming growth factor β, stimulate the induction of HSP27 in osteoblast-like MC3T3-E1 cells (6, 7). However, the exact mechanisms behind the HSP27 induction in osteoblasts and its roles have not yet been precisely clarified.
Endothelin (ET)-1 is a potent vasoconstrictive peptide that is produced and secreted by endothelial cells (8, 9). It is currently recognized that ET has a wide variety of effects on ubiquitous tissues including skeletal tissues through binding to specific receptors (9). During bone remodeling, capillary endothelial cells provide the microvasculature, and osteoblasts and osteoprogenitor cells, which locally proliferate and differentiate into osteoblasts, migrate into the resorption lacuna. Thus, it is currently recognized that the activities of osteoblasts, osteoclasts, and capillary endothelial cells are closely coordinated via humoral factors as well as by direct cell-to-cell contact, and these cells co-operatively regulate bone metabolism (10). ET receptors have been shown to exist on osteoblasts (11). It has been reported that ET-1 stimulates bone resorption and decreases alkaline phosphatase activity in vitro, induces collagen and non-collagen protein synthesis in neonatal mouse calvaria, and stimulates the proliferation of osteoblasts (11). Regarding the intracellular signaling system of ET in osteoblasts, it has been reported that ET-1 stimulates Ca2+ mobilization, and phosphoinositide hydrolysis by phospholipase (PL) C (11). We have shown that ET-1 stimulates phosphatidylycholine hydrolysis by PLD in osteoblast-like MC3T3-E1 cells (12). In addition, we have reported that ET-1 activates both p44/p42 mitogen-activated protein (MAP) kinase and p38 MAP kinase in these cells, and that p38 MAP kinase, but not p44/p42 MAP kinase, plays a role in ET-1-stimulated HSP27 induction (13, 14). Recently, we demonstrated that ET-1 activates stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), another member of the MAP kinase family (15). However, the involvement of SAPK/JNK in osteoblast cell function has not yet been precisely clarified.

In the present study, we investigated the involvement of SAPK/JNK in the ET-1-stimulated HSP27 induction in osteoblast-like MC3T3-E1 cells. We here show that SAPK/JNK, in addition to p38 MAP kinase, acts as a positive regulator in ET-1-induced HSP27 in these cells.

**Materials and methods**

**Materials**

ET-1 was purchased from Peptide Institute, Inc. (Minoh, Japan). SP600125, Go 6976, TPA, SB203580, and PD169316 were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect the assay for HSP27. Northern blot analysis or Western blot analysis.

**Cell culture**

Cloned osteoblast-like MC3T3-E1 cells which were derived from newborn mouse calvaria (16) were maintained as previously described (17). Briefly, 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% CO2/95% air. Cells were then seeded into 35 mm diameter dishes (5 × 10^4/dish) or 90 mm diameter dishes (5 × 10^5/dish) in α-MEM containing 10% FCS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FCS. After 48 h, cells were used for experiments.

**Immunoassay of HSP27**

The concentration of HSP27 in soluble extracts of the cells was determined by means of a sandwich-type enzyme immunoassay, as described previously (18). The source of HSP27 standard was rat hindlimb muscle, and the HSP27 antibodies showed similar affinity to both phosphorylated and unphosphorylated forms of HSP27 (18). Cultured cells were stimulated by ET-1 for the indicated periods in 1 ml serum-free α-MEM. Cells were then washed twice with 1 ml phosphate-buffered saline and frozen at −80°C for a few days before analysis. Frozen cells on each dish were collected and suspended in 0.3 ml phosphate-buffered saline, and then each suspension was sonicated and centrifuged at 125 000 g for 20 min at 4°C. The supernatant was used for an immunoassay employing 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. Incubation was carried out at 30°C for 5 h in a final volume of 0.5 ml 10 mol/l sodium phosphate buffer, pH 7.0, containing 0.3 mmol/l NaCl, 0.5% hydrolyzed gelatin, 0.1% bovine serum albumin (BSA), 1 mmol/l MgCl2, and 0.1% NaN3. After being washed, each ball was incubated at 4°C overnight with 1.5 μM galactosidase-labeled antibodies in a volume of 0.2 ml with 10 mmol/l sodium phosphate buffer, pH 7.0, containing 0.1 mol/l NaCl, 1 mmol/l MgCl2, 0.1% BSA, and 0.1% NaN3. 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

Cultured cells were stimulated by ET-1 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 total RNA were subjected to electrophoresis on a 0.9% agarose–2.2 mol/l formaldehyde gel and blotted onto a nitrocellulose membrane. For the Northern blot analysis, the membrane was allowed to hybridize with a cDNA probe that had been labeled with a Multiprime DNA labeling system (Amersham International plc, Amersham, Bucks, UK), as described previously (19). A BamHI-HindIII fragment of cDNA for mouse HSP27 (18) was kindly provided by Dr L F Cooper of the University of North Carolina.

Western blot analysis of HSP27 and SAPK/JNK

Cultured cells were stimulated by ET-1 or TPA in serum-free α-MEM for the indicated periods. Cells were washed twice with phosphate-buffered saline and then lysed, homogenized, and sonicated in a lysis buffer containing 62.5 mmol/l Tris/Cl, pH 6.8, 2% SDS, 50 mmol/l 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-PAGE was performed by the method of Laemmli (20) in 10% polyacrylamide gels. Western blotting was performed as described previously (19) using HSP27 antibodies, phospho-specific SAPK/JNK antibodies, or SAPK/JNK antibodies, with peroxidase-labeled antibodies raised in a goat against rabbit IgG used as second antibodies. Peroxidase activity on the nitrocellulose sheet was visualized on X-ray film by an ECL Western blotting detection system (Amersham Japan, Tokyo, Japan). When indicated, cells were pretreated with calphostin C, Go 6976, or SP600125 for 60 min.

Determinant

The absorbance of enzyme immunoassay samples was measured at 450 nm with an EL 340 Bio Kinetic Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). Densitometric analysis was performed using Molecular Analyst/Macintosh (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Data were analyzed by ANOVA followed by the Bonferroni method for multiple comparison between pairs, and a difference of P < 0.05 was considered significant. All data are presented as the mean ± S.E.M. of triplicate determinations. Each experiment was repeated three times with similar results.

Results

Effect of SP600125 on ET-1-stimulated HSP27 accumulation in MC3T3-E1 cells

We previously reported that ET-1 activates SAPK/JNK in osteoblast-like MC3T3-E1 cells (15). To investigate whether SAPK/JNK is involved in the ET-1-stimulated HSP27 induction in these cells, we examined the effect of SP600125, a highly specific inhibitor of SAPK/JNK (21), on the accumulation of HSP27. SP600125, which alone did not affect the HSP27 accumulation, significantly suppressed ET-1-stimulated HSP27 accumulation (Fig. 1). The inhibitory effect of SP600125 on ET-1-stimulated HSP27 accumulation was dose dependent in the range between 1 and 10 μmol/l (Fig. 2). The maximum effect of SP600125 on HSP27 accumulation was observed at 10 μmol/l, which caused about an 80% reduction in the ET-1 effect. We previously reported that ET-1 increased the levels of HSP27 with a Western blot analysis using the antibodies for HSP27 utilized in the immunoassay (14). We confirmed that the ET-1-increased HSP27 levels were similarly detectable using the commercially obtained antibodies for HSP27 (Fig. 3).

SP600125 effect on ET-1-increased HSP27 mRNA level in MC3T3-E1 cells

We next examined the effect of SP600125 on the ET-1-increased level of mRNA for HSP27 in MC3T3-E1 cells.
SP600125 by itself had little effect on the level of HSP27 mRNA, but markedly reduced the ET-1-increased level of HSP27 mRNA (Fig. 4).

Effects of calphostin C and Go 6976 on ET-1-stimulated phosphorylation of SAPK/JNK in MC3T3-E1 cells

We have previously shown that ET-1 activates protein kinase C via phosphoinositide-hydrolyzing PLC and phosphatidylcholine-hydrolyzing PLD in osteoblast-like MC3T3-E1 cells (12). In order to investigate whether SAPK/JNK acts at a point downstream of the protein kinase C in the ET-1-stimulated HSP27 induction in MC3T3-E1 cells, we examined the effect of calphostin C, an inhibitor of protein kinase C (22), on the ET-1-induced phosphorylation of SAPK/JNK. Calphostin C significantly attenuated the SAPK/JNK phosphorylation induced by ET-1 (Fig. 5A). Densitometric analysis showed that calphostin C caused a 43% reduction in the ET-1 effect. In addition, the ET-1-induced phosphorylation of SAPK/JNK was suppressed by Go 6976, another inhibitor of protein kinase C (23) (Fig. 5B). Densitometric analysis then showed that Go 6976 elicited a 40% reduction in the ET-1 effect.

Effect of SP600125 on TPA-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells

We further examined the effect of SP600125 on the SAPK/JNK phosphorylation induced by TPA, a protein kinase C-activating phorbol ester (24). TPA alone time-dependently stimulated the phosphorylation of SAPK/JNK (data not shown). SP600125 markedly suppressed the TPA-induced phosphorylation of SAPK/JNK (Fig. 6). Densitometric analysis showed that SP600125 elicited a 70% reduction in the TPA effect.
Combination effect of SP600125 and p38 MAP kinase inhibitor on accumulation of HSP27 stimulated by ET-1 in MC3T3-E1 cells

We have previously shown that SB203580, a specific inhibitor of p38 MAP kinase (25), significantly suppressed ET-1-induced accumulation of HSP27 and the mRNA levels for HSP27 (14). We therefore examined the combination effect of SP600125 and SB203580 on ET-1-stimulated HSP27 accumulation. SP600125 and SB203580 additively suppressed accumulation of HSP27 by ET-1 (Fig. 7). In addition, the ET-1-stimulated accumulation of HSP27 was reduced additively by SP600125 and PD169316, another inhibitor of p38 MAP kinase (26) (Fig. 7).

Discussion

It is well known that the MAP kinase superfamily mediates intracellular signaling of various agonists and plays crucial roles in cellular functions including proliferation, differentiation, and cell death in a variety of cells (27). Three major MAP kinases, p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK, are recognized to transduce signals in mammalian cells (27). We earlier reported that ET-1 activates p44/p42 MAP kinase and p38 MAP kinase, and SAPK/JNK, are recognized to transduce signals in mammalian cells (27). We earlier reported that ET-1 activates p44/p42 MAP kinase and p38 MAP kinase in osteoblast-like MC3T3-E1 cells and that p38 MAP kinase, but not p44/p42 MAP kinase, plays a role in ET-1-stimulated HSP27 induction (13, 14). We have recently demonstrated that ET-1 activates SAPK/JNK in addition to p44/p42 MAP kinase and p38 MAP kinase in osteoblast-like MC3T3-E1 cells (15). We therefore conducted the present study to investigate whether SAPK/JNK plays a role in ET-1-stimulated HSP27 induction in these cells.

In the present study, we showed that SP600125 significantly reduced the ET-1-induced accumulation of HSP27 in MC3T3-E1 cells. We have already found that SP600125 truly reduces the phosphorylation of SAPK/JNK induced by ET-1 in these cells. In addition, the ET-1-increased level of HSP27 mRNA was suppressed by SP600125. Our findings therefore suggest that SAPK/JNK is involved in the ET-1-induced HSP27 as well as p38 MAP kinase in osteoblast-like MC3T3-E1 cells. It is recognized that HSP27 phosphorylation is catalyzed by the MAP kinases such as p38 MAP kinase (1). We previously reported that the phosphorylation of p38 MAP kinase induced by ET-1 occurs within 10 min and returns to the basal level.
of ET-1 alone. **

pretreated with SB203580, PD169316, or SP600125.

for 12 h. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *P < 0.05 compared with the value of ET-1 alone. **P < 0.05 compared with the value of ET-1 pretreated with SB203580, PD169316, or SP600125.

until 60 min after the ET-1 stimulation, suggesting that the ET-1-activated p38 MAP kinase affects their substrates within 1 h (14). On the other hand, the levels of HSP27 in unstimulated MC3T3-E1 cells were quite low (<1 ng/mg.protein) (14). In addition, we have shown that the ET-1-increased accumulation of HSP27 became significant at 4 h after the stimulation (14). It therefore seems unlikely that ET-1-activated p38 MAP kinase phosphorylates HSP27 in osteoblast-like MC3T3-E1 cells.

We have previously reported that ET-1 stimulates both phosphoinositide-hydrolyzing PLC and phosphatidylycholine-hydrolyzing PLD, resulting in the activation of the protein kinase C in osteoblast-like MC3T3-E1 cells (12). In the present study, TPA, a direct activator of protein kinase C (24), truly phosphorylated SAPK/JNK and SP600125 inhibited the phosphorylation. It is well recognized that MAP kinases are activated by phosphorylation of threonine and tyrosine residues by dual-specificity MAP kinase kinase (27, 28). These data thus suggest that SAPK/JNK functions at a point downstream of the protein kinase C in MC3T3-E1 cells. Furthermore, we have demonstrated that the ET-1-induced phosphorylation of SAPK/JNK was attenuated by calphostin C (22) and Go 6976 (23). Based on our findings, it is most likely that ET-1 activates SAPK/JNK via the protein kinase C, which plays a part in the induction of HSP27 in the osteoblast-like MC3T3-E1 cells. However, the inhibitory effect of SP600125 on the ET-1-induced accumulation of HSP27 and mRNA levels for HSP27 was partial. In a previous study (14), we demonstrated that activation of p38 MAP kinase is involved in the ET-1-induced HSP27 in MC3T3-E1 cells, but the inhibitory effect of SB203580, a specific inhibitor of p38 MAP kinase (25), was partial. In the present investigation, we have shown that SP600125 and a p38 MAP kinase inhibitor additively suppresses the accumulation of HSP27 by ET-1. SP600125 hardly affected the ET-1-induced phosphorylation of p38 MAP kinase, and SB203580 had reportedly little effect on the ET-1-induced SAPK/JNK phosphorylation in MC3T3-E1 cells (15). Taking our results as a whole into account, it is most likely that HSP27 induction stimulated by ET-1 is mediated through activation of both p38 MAP kinase and SAPK/JNK in the osteoblast-like MC3T3-E1 cells. It is well known that low molecular weight HSPs, such as HSP27, as well as high molecular weight HSPs, such as HSP90 and HSP70, act as molecular chaperones in protein folding, oligomerization and translocation (1). It is likely that ET-1-stimulated accumulation of HSP27 plays a role in the regulation of osteoblast cell functions, such as secretion, proliferation, or differentiation. To co-ordinate the functions in the osteoblast, SAPK/JNK and p38 MAP kinase seem to regulate the induction of HSP27 co-operatively. It has been reported that HSP27 shows a protective effect against apoptosis in neuronal cells (29). In addition, the up-regulation of HSP27 by ET-1 seems to play a role in the cell survival pathways, resulting in an increase of resistance to apoptosis. Taking these into account, it is most likely that ET-1-induced HSP27 through the activation of SAPK/JNK in addition to p38 MAP kinase, finely co-ordinates the ET-1-provoked cellular events to minimize the damage in osteoblasts. Further investigations are required to clarify the exact roles of HSP27 in osteoblasts.

In conclusion, our present results strongly suggest that ET-1-activated SAPK/JNK, in addition to p38 MAP kinase, plays a part in ET-1-induced HSP27 in osteoblasts.

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