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Specific Induction of Heat Shock Protein 27 by Glucocorticoid in Osteoblasts

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Abstract It is generally recognized that osteoporosis is a common complication of patients with glucocorticoid excess and that glucocorticoid receptor is associated with heat shock protein (HSP) 70 and HSP90 in a heterocomplex. In the present study, we investigated whether glucocorticoid induces HSP27, HSP70, and HSP90 in osteoblast-like MC3T3-E1 cells. Dexamethasone time-dependently increased the levels of HSP27, while having no effect on the levels of HSP70 or HSP90. The effect of dexamethasone was dose-dependent in the range between 0.1 nM and 0.1 μM. Dexamethasone induced an increase of the levels of mRNA for HSP27. Dexamethasone induced the phosphorylation of p38 mitogen-activated protein (MAP) kinase. SB203580 and PD169316, inhibitors of p38 MAP kinase, suppressed the HSP27 accumulation by dexamethasone. In addition, SB203580 reduced the dexamethasone-stimulated increase of the mRNA levels for HSP27. The dexamethasone-induced phosphorylation of p38 MAP kinase was reduced by SB203580. These results strongly suggest that glucocorticoid stimulates the induction of neither HSP70 nor HSP90, but HSP27 in osteoblasts, and that p38 MAP kinase is involved in the induction of HSP27. J. Cell. Biochem. 86: 357–364, 2002.

Key words: glucocorticoid; heat shock protein; MAP kinase; osteoblast

Glucocorticoid is well known to have marked in vivo effects on bone metabolism and osteoporosis is a common complication of patients with glucocorticoid excess [Lukert and Kream, 1996; Ishida and Heershe, 1998]. It is recognized that both increased bone resorption and decreased bone formation contribute to the loss of bone mass [Lukert and Kream, 1996; Ishida and Heershe, 1998]. Bone metabolism is regulated mainly by two functional cells, osteoblasts and osteoclasts [Nijweide et al., 1986]. The former cells are responsible for bone formation and the latter for bone resorption. The receptor for glucocorticoid has been shown to exist on osteoblasts [Ishida and Heershe, 1998]. Thus, it is recognized that glucocorticoid physiologically acts as a potent modulator of bone metabolism via osteoblasts. However, the mechanism of glucocorticoid in osteoblasts has not been precisely clarified.

When cells are exposed to a biological stress, such as heat, they produce heat shock proteins (HSPs) [Nover, 1991; Nover and Scharf, 1991]. 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 HSP70, HSP90, and HSP110, are well known to act as molecular chaperones implicated in protein folding, oligomerization, and translocation [Gething and Sambrook, 1992]. Although low-molecular-weight

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HSPs, such as HSP27, are recognized to act as chaperones as well as high-molecular-weight HSPs, the function of them is less understood than that of high-molecular-weight HSPs [Nover, 1991]. In addition, it is well known that the glucocorticoid receptor is associated with HSP70 and HSP90 in a cytosolic heterocomplex [Pratt et al., 1992]. Glucocorticoid promotes dissociation to yield the free-DNA binding form of the receptor [Pratt et al., 1992]. As for HSP70 in bone cells, it has been reported that parathyroid hormone stimulates HSP70 gene expression not mediated by adenylate cyclase or protein kinase C in osteosarcoma SaOS-2 and ROS 17/2.8 cells [Fukuyama et al., 1996]. On the other hand, it has been demonstrated that the expression of HSP27 shows an increase with the downregulation of proliferation in normal osteoblasts [Shakoori et al., 1992]. In addition, estrogen reportedly facilitates the expression of HSP27 in osteoblast-like MC3T3-E1 cells [Cooper and Uoshima, 1994]. In a previous study [Kawamura et al., 1999], we have demonstrated that endothelin-1 (ET-1) stimulates HSP27 induction through activation of p38 mitogen-activated protein (MAP) kinase in osteoblast-like MC3T3-E1 cells. However, the exact mechanism of HSPs induction and their roles in osteoblasts are still poorly understood.

In the present study, we investigated whether glucocorticoid stimulates the induction of HSP90, HSP70, and HSP27 in osteoblast-like MC3T3-E1 cells and the mechanism of the induction. Here, we show that dexamethasone stimulates HSP27 induction while having little effect on the levels of HSP90 or HSP70 in these cells, and that p38 MAP kinase is involved in the HSP27 induction by dexamethasone.

MATERIALS AND METHODS

Dexamethasone was obtained from Sigma Chemical Co. (St. Louis, MO). HSP90 and HSP70 antibodies were purchased from Santa Cruz (Santa Cruz, CA). SB203580 and PD169316 were obtained from Calbiochem-Novabiochem (La Jolla, CA). Phospho-specific p38 MAP kinase antibodies (rabbit polyclonal IgG, affinity purified) and p38 MAP kinase antibodies (rabbit polyclonal IgG, affinity purified) were obtained 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. Dexamethasone was dissolved in ethanol. SB203580 and PD169316 were dissolved in dimethyl sulfoxide. The maximum concentration of ethanol or dimethyl sulfoxide was 0.1%, which did not affect Western blot analysis Northern blot analysis or immunoassay of HSP27.

Cell Culture

Cloned osteoblast-like MC3T3-E1 cells, which have been derived from newborn mouse calvaria [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.

Western Blot Analysis of HSP90, HSP70, HSP27, and p38 MAP Kinase

The cultured cells were stimulated by dexamethasone for the indicated periods. The cells were washed twice with 1 ml of phosphate-buffered saline and then lyzed, 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 was performed as described previously [Kato et al., 1996] by using HSP90 antibodies, HSP70 antibodies, HSP27 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 an ECL Western blotting detection system.

Immunoassay of HSP27

The concentration of HSP27 in soluble extracts of the MC3T3-E1 cells was determined by means of a sandwich-type enzyme immunoassay, as described previously [Kaida et al., 1999]. The cultured cells were stimulated by various

doses of dexamethasone for the indicated periods in 1 ml of serum-free α-MEM. The cells were washed twice with 1 ml of phosphate-buffered saline 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 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 the immunoassay 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-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. The 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 being washed, each ball was incubated at 4°C overnight with 1.5 mU 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.

Isolation of RNA and Northern Blotting Analysis of HSP27

The cultured cells were stimulated by dexamethasone 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 blot analysis, membrane was allowed to hybridize with a cDNA probe that had been labeled with a Multiprime DNA labeling system (Amersham, Buckinghamshire, UK), as described previously [Kato et al., 1996]. A *Bam*HI-*Hind*III fragment of cDNA for mouse HSP27 [Cooper and Uoshima, 1994] was provided by Dr. L.F. Cooper of the University of North Carolina.

Other Methods

Protein concentrations in soluble extracts were determined using a protein assay kit (Bio-Rad, Hercules, CA), 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 [Inaguma et al., 1993]. The densitometric analysis was performed using Molecular Analyst/Macintosh (Bio-Rad Laboratories, Hercules, CA).

Statistical Analysis

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

RESULTS

Effects of Dexamethasone on the Induction of HSP90, HSP70, and HSP27 in MC3T3-E1 Cells

In osteoblast-like MC3T3-E1 cells, HSP90 and HSP70, clearly existed without dexamethasone treatment (Fig. 1). On the other hand, the levels of HSP27 seem to be quite low in these cells. Dexamethasone had little or no effect on the levels of HSP90 or HSP70 in MC3T3-E1 cells (Fig. 1). In contrast, dexamethasone significantly increased the levels of HSP27 in the same sample (Fig. 1). The stimulatory effect of dexamethasone on the HSP27 induction was time-dependent up to 60 h.

Effect of Dexamethasone on the HSP27 Accumulation in MC3T3-E1 Cells

In order to clarify the effect of dexamethasone on the HSP27 induction in MC3T3-E1 cells, we

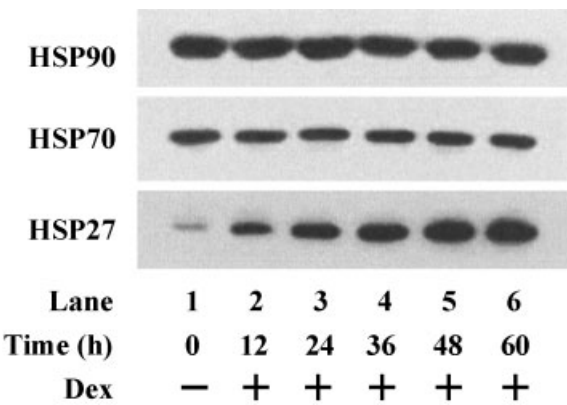

Fig. 1. Western blot analysis of dexamethasone-induced changes in HSP90, HSP70, and HSP27 in MC3T3-E1 cells. The cultured cells were incubated with 0.1 μM dexamethasone for the indicated periods. Extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against HSP90, HSP70, or HSP27.

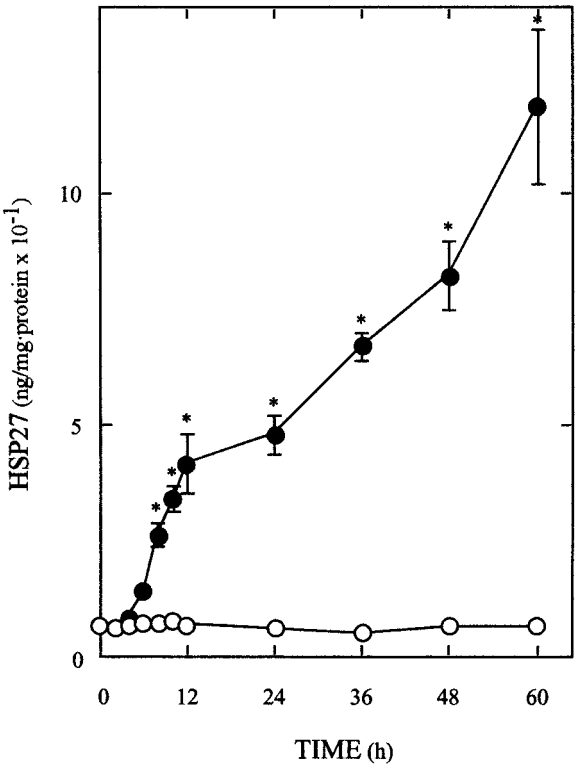

Fig. 2. Effect of dexamethasone on the accumulation of HSP27 in MC3T3-E1 cells. The cultured cells were stimulated by 0.1 μM dexamethasone (closed circles) or vehicle (open circles) for the indicated periods. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, compared to the value of control.

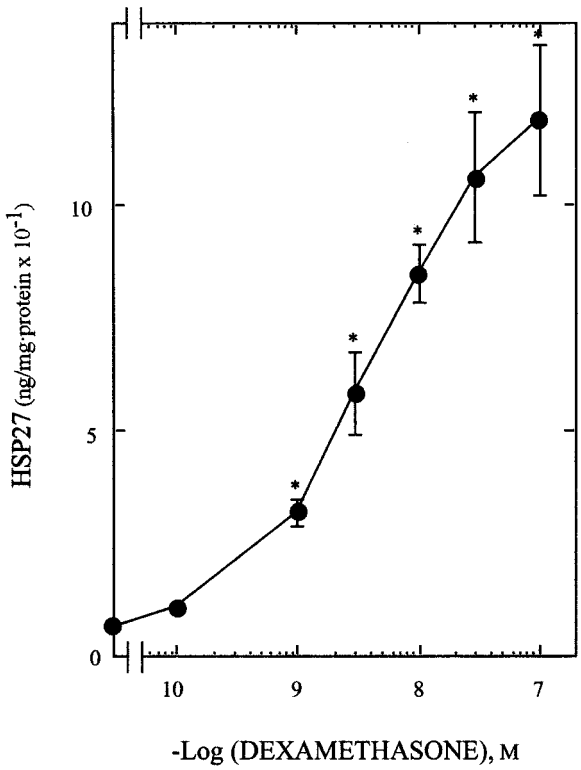

Fig. 3. Dose-dependent effect of dexamethasone on the accumulation of HSP27 in MC3T3-E1 cells. The cultured cells were stimulated by various doses of dexamethasone for 60 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, compared to the value of control.

determined the levels of HSP27 by a specific enzyme immunoassay. Dexamethasone stimulated the accumulation of HSP27 time-dependently up to 60 h (Fig. 2). The stimulatory effect of dexamethasone was dose-dependent in the range between 0.1 nM and 0.1 μM, and the maximum effect of dexamethasone was observed at 0.1 μM (Fig. 3).

Effect of Dexamethasone on the Levels of mRNA for HSP27 in MC3T3-E1 Cells

The expression levels of the mRNA for HSP27 were significantly increased by dexamethasone (Fig. 4). The dexamethasone-induced increase of the mRNA levels was observed at 4 h after the stimulation (Fig. 4). The maximum effect of dexamethasone was observed at 8 h and the levels decreased thereafter (Fig. 4).

Effect of Dexamethasone on the Phosphorylation of p38 MAP Kinase in MC3T3-E1 Cells

It is well recognized that MAP kinases play important roles in intracellular signaling of a variety of agonists [Nishida and Gotoh, 1993]. We previously demonstrated that p38 MAP kinase is involved in ET-1 induced HSP27 accumulation in MC3T3-E1 cells [Kawamura et al., 1999]. In order to investigate whether

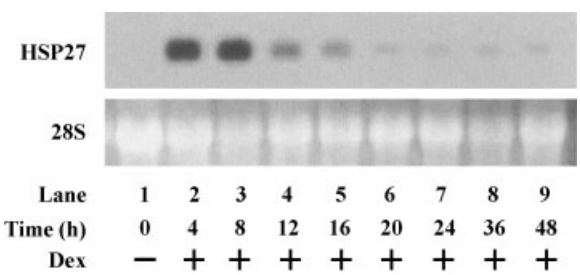

Fig. 4. Effect of dexamethasone on the levels of mRNA for HSP27 in MC3T3-E1 cells. The cultured cells were stimulated by 0.1 μM dexamethasone for the indicated periods. The cells were harvested and total RNA was isolated. Twenty micrograms of RNA from each sample was subjected to electrophoresis and blotted onto a nitrocellulose membrane. The membrane was then allowed to hybridize with cDNA probe for HSP27. Bands of 28S are shown for reference.

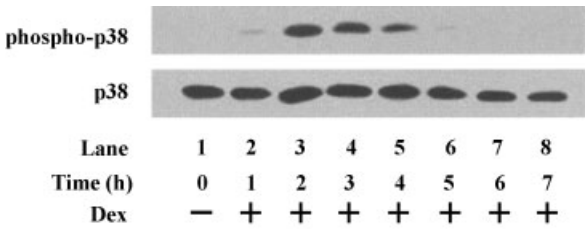

Fig. 5. Effect of dexamethasone on the phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were stimulated by 0.1 μM dexamethasone for the indicated periods. Extracts of cells were subjected to SDS–PAGE against phospho-specific p38 MAP kinase antibodies or p38 MAP kinase antibodies.

glucocorticoid activates p38 MAP kinase in MC3T3-E1 cells, we examined the effect of dexamethasone on the phosphorylation of p38 MAP kinase. Dexamethasone time-dependently induced the phosphorylation of p38 MAP kinase (Fig. 5).

Effects of SB203580 or PD169316 on the Accumulation of HSP27 by Dexamethasone in MC3T3-E1 Cells

To know whether the activation of p38 MAP kinase is involved in the glucocorticoid-induced accumulation of HSP27, we tested the effects of SB203580, a specific inhibitor of p38 MAP kinase [Cuenda et al., 1995], or PD169316, another inhibitor of p38 MAP kinase [Kummer et al., 1997], in MC3T3-E1 cells. SB203580 significantly suppressed the accumulation of HSP27 stimulated by dexamethasone (Fig. 6). The inhibitory effect of SB203580 was dose-dependent in the range between 1 and 30 μM. In addition, the HSP27 accumulation was dose-dependently reduced by PD169316 in the range between 1 and 30 μM (Fig. 7). Furthermore, we investigated the effect of SB203580 on the dexamethasone-increased levels of the mRNA for HSP27. SB203580 significantly suppressed the levels of the mRNA for HSP27 by dexamethasone (Fig. 8). According to the densitometric analysis, SB203580 reduced the dexamethasone-increased levels of mRNA for HSP27 from 2.9- to 1.7-fold (mean of triplicate independent experiments).

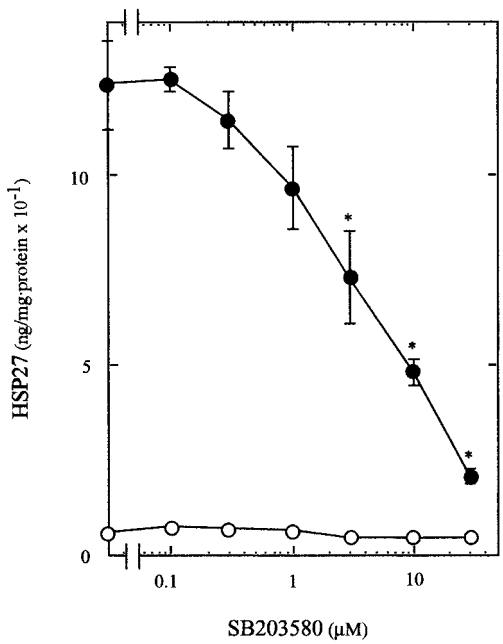

Fig. 6. Effect of SB203580 on the accumulation of HSP27 by dexamethasone in MC3T3-E1 cells. The cultured cells were pretreated with various doses of SB203580 for 60 min, and then stimulated by 0.1 μM dexamethasone (closed circles) or vehicle (open circles) for 60 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^*P < 0.05$, compared to the value of dexamethasone alone.

DISCUSSION

In the present study, we showed that dexamethasone stimulated the levels of HSP27 by means of Western blotting analysis in osteoblast-like MC3T3-E1 cells. In addition, dexamethasone induced the accumulation of HSP27 time- and dose-dependently as detected by a specific immunoassay and stimulated the levels of mRNA for HSP27. Therefore, our results suggest that glucocorticoid stimulates the induction of HSP27 in MC3T3-E1 cells. The stimulatory effects of glucocorticoid on the HSP27 induction have recently been reported in rat cerebellum and mouse skin [Barr and Dokas, 1999; Tuckermann et al., 1999]. To the best of our knowledge, our present study represents the first report showing the induction of HSP27, a low-molecular-weight HSP, by dexamethasone in osteoblasts. The levels of HSP27 increased up to 60 h after the stimulation by dexamethasone, whereas the levels of mRNA peaked after 8 h and declined thereafter. It is possible that the discrepancy between the transient effect of dexamethasone on mRNA for HSP27 and the more prolonged effect on the protein levels is due to the post-transcriptional stabilization of this protein.

We next showed that dexamethasone induced the phosphorylation of p38 MAP kinase in

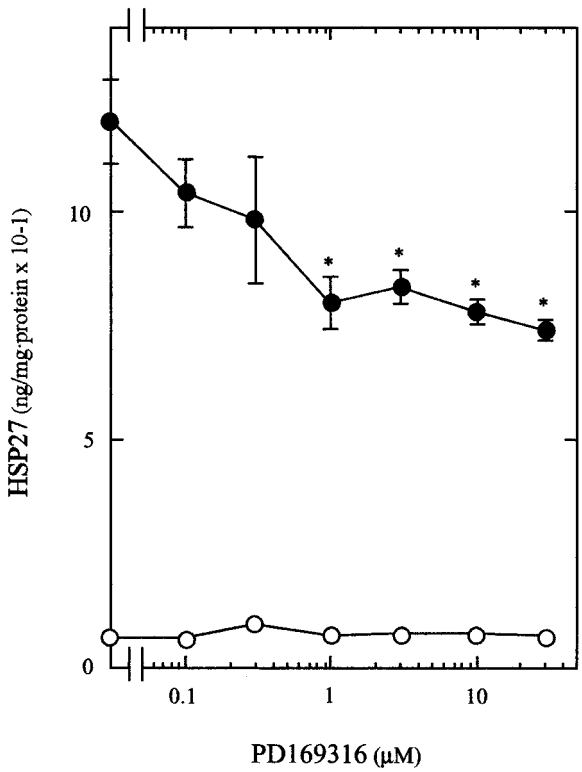

Fig. 7. Effect of PD169316 on the accumulation of HSP27 by dexamethasone in MC3T3-E1 cells. The cultured cells were pretreated with various doses of PD169316 for 60 min, and then stimulated by 0.1 μM dexamethasone (closed circles) or vehicle (open circles) for 60 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $*P < 0.05$, compared to the value of dexamethasone alone.

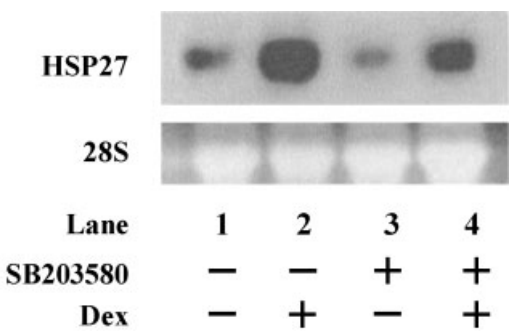

Fig. 8. Effect of SB203580 on the dexamethasone-increased levels of the mRNA for in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM SB203580 or vehicle for 60 min, and then stimulated by 0.1 μM dexamethasone or vehicle for 4 h. The cells were harvested and total RNA was isolated. Twenty micrograms of RNA from each sample was subjected to electrophoresis and blotted onto a nitrocellulose membrane. The membrane was then allowed to hybridize with cDNA probe for HSP27. Bands of 28S are shown for reference.

MC3T3-E1 cells. It is well known that the MAP kinase superfamily mediates intracellular signaling of various agonists and plays pivotal roles in cellular functions [Nishida and Gotoh, 1993], and p38 MAP kinase is a member of the MAP kinase superfamily [Nishida and Gotoh, 1993]. It is well recognized that p38 MAP kinase is activated by phosphorylation on tyrosine and threonine by dual-specificity MAP kinase kinase [Raingeaud et al., 1995]. Taking these findings into account, it is most likely that glucocorticoid stimulates the activation of p38 MAP kinase in osteoblast-like MC3T3-E1 cells. In addition, we demonstrated that SB203580 and PD169316, specific inhibitors of p38 MAP kinase [Cuenda et al., 1995; Kummer et al., 1997], significantly reduced the HSP27 accumulation induced by dexamethasone. SB203580 was much more effective than PD169316 in the inhibition of the accumulation of HSP27 by dexamethasone. As our experiments were performed using whole cells, it is possible that the intracellular concentrations of these inhibitors depend upon their permeability of the plasma membrane. Furthermore, we found that the increase of mRNA for HSP27 by dexamethasone was inhibited by SB203580. Therefore, our results strongly suggest that the activation of p38 MAP kinase is involved in the glucocorticoid-stimulated HSP27 induction in osteoblast-like MC3T3-E1 cells. As for the relationship between glucocorticoid and p38 MAP kinase, it has recently been reported that dexamethasone destabilizes cyclo-oxygenase 2 mRNA by inhibiting p38 MAP kinase in HeLa Tet-off cells [Lasa et al., 2001]. In vascular endothelial cells, dexamethasone reportedly fails to affect p38 MAP kinase activation [Gonzalez et al., 1999]. Thus, the response of p38 MAP kinase to glucocorticoid seems to be different among each cell types.

It has been reported that a stress, such as heat, stimulates the induction of HSP90, HSP70, and HSP27 in osteoblasts [Shakoori et al., 1992]. In the present study, we demonstrated that dexamethasone failed to affect the levels of HSP90 or HSP70 in MC3T3-E1 cells while stimulating the HSP27 induction as described above. It is probable that the induction of HSP27 plays a crucial role in glucocorticoid signaling in osteoblasts. Parathyroid hormone, a physiological agent, reportedly upregulates HSP70 transcription in osteosarcoma SaOS-2 and ROS17/2.8 cells [Fukuyama et al., 1996]. It is recognized that high-molecular-weight HSPs,

such as HSP90 and HSP70, act as molecular chaperones and protect cells under potentially hazardous conditions [Nover and Scharf, 1991]. In addition, it is well known that HSP70 and HSP90 bind directly to glucocorticoid receptor and exist in a cytosolic heterocomplex [Pratt et al., 1992]. Glucocorticoid promotes dissociation of the complex, resulting in the formation of the free-DNA binding form of the receptor [Pratt et al., 1992]. On the contrary, the exact roles of low-molecular-weight HSPs, such as HSP27, are still poorly understood, although these low-molecular-weight HSPs are speculated to act as chaperones as well. Taking into account of our present findings, it is possible that the different molecular weight HSPs skillfully share the roles in response to a variety of physiological stimuli.

In human patients with osteosarcoma, it has been shown that overexpression of HSP27 in the specimens of biopsy is associated with poor prognosis [Uozaki et al., 1997]. Additionally, HSP70-positive osteosarcomas reportedly respond better to chemotherapy than HSP70-negative cases [Trieb et al., 1998]. These findings suggest that HSPs are involved in the regulation of cell function, such as proliferation. Therefore, our present results may indicate that the induction of neither HSP90 nor HSP70, but HSP27 by glucocorticoid in osteoblasts is involved in glucocorticoid-induced modulation of cellular function of osteoblasts, resulting in the alteration of bone metabolism. Further investigations would be necessary to clarify the roles of HSP27 in osteoblast cell functions.

In conclusion, these results strongly suggest that glucocorticoid stimulates the induction of neither HSP90 nor HSP70, but of HSP27 in osteoblasts.

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