Inhibitors of heat shock protein 90 augment endothelin-1-induced heat shock protein 27 through the SAPK/JNK signaling pathway in osteoblasts

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Abstract. It has been previously reported that endothelin-1 (ET-1) stimulates the induction of heat shock protein (HSP) 27 through the activation of p38 mitogen-activated protein (MAP) kinase and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in osteoblast-like MC3T3-E1 cells. The present study investigated whether HSP90, a high-molecular-weight HSP, was implicated in the ET-1-stimulated HSP27 induction in MC3T3-E1 cells. The effects of HSP90 inhibitors on the induction of HSP27 were examined. The HSP90 inhibitors geldanamycin and 17-demethoxygeldanamycin (17-DMAG) significantly amplified HSP27 induction stimulated by ET-1 in a dose-dependent manner. In addition, onalespib (another HSP90 inhibitor) significantly strengthened the ET-1-induced HSP27 protein levels. The ET-1-stimulated phosphorylation of p38 MAP kinase was minimally affected by geldanamycin, 17-DMAG or onalespib. Onalespib and 17-DMAG significantly enhanced the ET-1-induced phosphorylation of SAPK/JNK. In addition, SP600125, a SAPK/JNK inhibitor, notably reduced the amplification by onalespib of the ET-1-induced HSP27. These results suggest that HSP90 limits HSP27 induction in MC3T3-E1 cells. The effects of HSP90 inhibitors on the induction of HSP27 were examined. The HSP90 inhibitors geldanamycin and 17-demethoxygeldanamycin (17-DMAG) significantly amplified HSP27 induction stimulated by ET-1 in a dose-dependent manner. In addition, onalespib (another HSP90 inhibitor) significantly strengthened the ET-1-induced HSP27 protein levels. The ET-1-stimulated phosphorylation of p38 MAP kinase was minimally affected by geldanamycin, 17-DMAG or onalespib. Onalespib and 17-DMAG significantly enhanced the ET-1-induced phosphorylation of SAPK/JNK. In addition, SP600125, a SAPK/JNK inhibitor, notably reduced the amplification by onalespib of the ET-1-induced HSP27. These results suggest that HSP90 limits ET-1-stimulated HSP27 induction at a point upstream of SAPK/JNK in osteoblasts. These results suggest that HSP90 may be a novel clinical target for metabolic bone diseases, including osteoporosis.

Introduction

Heat shock proteins (HSPs) have been first discovered as stress-inducible proteins (1,2). It is generally recognized that HSPs intracellularly act as molecular chaperones and regulate proteostasis under stress conditions (1,2). Based on their molecular masses, HSPs are currently classified into seven families, including HSPA (HSP70), HSPB (small HSPs), HSPC (HSP90) and HSPH (HSP110) (3). Among small HSPs with monomer molecular mass in the range of 12-43 kDa, HSP27, an ATP-independent molecular chaperone, is induced by the heat shock associated with physical and chemical stresses, including radiation, oxidative stress and various chemotherapies (1). HSP27 is able to bind to improperly folded proteins and further transfer them to the ATP-dependent chaperones or to the protein degradation machines including proteasomes or autophagosomes. The functions of HSP27 are regulated by post-translational modifications such as phosphorylation (1). Unphosphorylated HSP27 forms large aggregated oligomers while its phosphorylation results in the conformational changes leading to dissociated small oligomers (1). On the other hand, HSP90, an ATP-dependent molecular chaperone, ubiquitously and abundantly exist in a variety of unstressed cells, represent 1-2% of total cellular proteins (2). In addition to protein folding, it is well known that HSP90 regulates the binding of glucocorticoid to its specific receptor under physiological conditions (2). Evidence is accumulating that HSPs are involved in a variety of pathophysiological cell processes (1,2).

Bone metabolism is strictly regulated by two functional cells, osteoblasts and osteoclasts (4). The former cells contribute to bone formation, whereas the latter cells are responsible for bone resorption. In order to maintain the stability of bone and the integrity, bone tissue is constantly reconstructed by a sequential process consisted of the resorption of old bone and the subsequent formation of new bone, so called bone remodeling (5). Regarding HSP27 in osteoblasts, down-regulation of osteoblast proliferation is reportedly accompanied by a transient increase in the HSP27 mRNA expression (6). In addition, it has been shown that estrogen facilitates the induction of HSP27 stimulated by heat (7). We have previously reported...
that endothelin-1 (ET-1), a bone remodeling agent, stimulates the induction of HSP27 in osteoblast-like MC3T3-E1 cells and that p38 mitogen-activated protein (MAP) kinase and stress activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) play as positive regulators in the HSP27 induction (8,9). In addition, we demonstrated that the mineralization of MC3T3-E1 cells is modulated by the expression level of HSP27 protein and its phosphorylation status (10). On the other hand, as for HSP90 in osteoblasts, the expression of HSP90 protein is reportedly induced by bisphosphonate, the most useful medicine for osteoporosis treatment (11). In addition, it has recently been shown that low-intensity pulsed ultrasound stimulation reportedly upregulates HSP90 level, leading to the formation of mineralized nodule (12). However, the details of both HSP27 and HSP90 in osteoblasts have not yet been clarified.

In the present study, we investigated the involvement of HSP90 in the ET-1-stimulated HSP27 induction in these cells. We herein show that HSP90 inhibitors amplify the ET-1-induced HSP27 protein levels in MC3T3-E1 cells and that the up-regulating effect of HSP90 inhibitors was exerted via SAPK/JNK.

Materials and methods

Materials. ET-1 was obtained from Peptide Institute Inc. (Minoh, Osaka, Japan). Geldanamycin was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). The inhibitors 17-demethoxygeldanamycin (17-DMAG) and SP600125 were obtained from EMD Millipore (Billerica, MA, USA). Onalespib was purchased from Selleck Chemicals (Houston, TX, USA). HSP27 antibodies and glyceraldehyde 3 phosphate dehydrogenase (GAPDH) antibodies were obtained from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA). Phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies and SAPK/JNK antibodies were purchased from Cell Signaling Technology, Inc., (Danvers, MA, USA). An ECL western blotting detection system was purchased from GE Healthcare Life Sciences (Little Chalfont, UK). Other materials and chemicals were obtained from commercial sources. Geldanamycin, 17-DMAG, onalespib and SP600125 were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect the assay for western blot analysis.

Cell culture. Cloned osteoblast-like cells, MC3T3-E1 cells that have been derived from newborn mouse calvaria (13), were maintained as previously described (14). In brief, 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 were seeded into 90-mm diameter dishes (20x10⁵/dish) for western blot analysis. After 5 days, the medium was exchanged to α-MEM containing 0.3% FBS. The cells were then used for experiments after 48 h.

Western blot analysis. Western blotting was performed as described previously as follows (15). The cultured cells were pretreated with various doses of geldanamycin, 17-DMAG or onalespib for 60 min, and then stimulated by 0.1 μM ET-1 or vehicle in the presence of inhibitors in α-MEM containing 0.3% FCS for the indicated periods. The cells were washed twice with phosphate-buffered saline 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. SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli in 10% polyacrylamide gel (16). The protein was fractionated and transferred onto an Immun-Blot polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked with 5% fat-free dry milk in Tris-buffered saline-Tween (TBS-T; 20 mM Tris/HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20) for 1 h before incubation with primary antibodies. Western blot analysis was performed using HSP27 antibodies, GAPDH antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies and SAPK/JNK antibodies with peroxidase-labeled antibodies raised in goat anti-rabbit IgG which were used as secondary antibodies. Peroxidase activity on PVDF membranes was visualized on X-ray film by means of the ECL western blotting detection system.

Densitometric analysis. A densitometric analysis of the western blots was performed using a scanner and image analysis software program (ImageJ v1.48; National Institutes of Health, Bethesda, MD, USA). HSP27 protein levels or the phosphorylated protein levels were calculated as follows: The background-subtracted intensity was respectively normalized to GAPDH intensity or the total protein intensity, respectively, and plotted as the fold increase in comparison to that of the control cells without stimulation.

Statistical analysis. All data are presented as the mean ± SEM of triplicate determinations from three independent cell preparations. The data were analyzed by ANOVA followed by Bonferroni method for multiple comparisons between pairs. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of geldanamycin, 17-DMAG or onalespib on ET-1-stimulated HSP27 induction in MC3T3-E1 cells. We have previously shown that ET-1 induces the expression of HSP27 protein in osteoblast-like MC3T3-E1 cells (8). In the present study, in order to clarify whether HSP90 is implicated in the ET-1-stimulated HSP27 induction in MC3T3-E1 cells, we examined the effects of HSP90 inhibitors on the HSP27 induction. Geldanamycin, an inhibitor of HSP90 (17), significantly enhanced the ET-1-induced levels of HSP27 protein (Fig. 1A). The effect of geldanamycin on the HSP27 induction was dose-dependent over the range between 10 and 30 nM (Fig. 1A). We also found that 17-DMAG, a less toxic derivative of geldanamycin (18), dose-dependently augmented the ET-1-stimulated HSP27 induction in the range between 10 and 30 nM (Fig. 1B).

In addition, onalespib, a HSP90 inhibitor, which is a different type from geldanamycin (19), as well as geldanamycin and its derivative, markedly upregulated the HSP27 protein levels induced by ET-1 (Fig. 1C).
Effects of geldanamycin, 17-DMAG or onalespib on the ET-1-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. In our previous study (8), we have reported that p38 MAP kinase acts as a positive intracellular molecule in the ET-1-stimulated HSP27 induction in osteoblast-like MC3T3-E1 cells. In order to investigate whether the HSP90 inhibitor-effect on the ET-1-stimulated HSP27 induction is dependent on the activation of p38 MAP kinase in these cells, we examined the effects of geldanamycin, 17-DMAG or onalespib on the ET-1-induced phosphorylation of p38 MAP kinase. However, geldanamycin (Fig. 2A), 17-DMAG (Fig. 2B) or onalespib (Fig. 2C) failed to affect the ET-1-induced phosphorylation levels of p38 MAP kinase.

Effects of onalespib or 17-DMAG on the ET-1-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. We have demonstrated that SAPK/JNK in addition to p38 MAP kinase is involved in the ET-1-stimulated HSP27 induction in MC3T3-E1 cells (9). Thus, we next examined the effect of onalespib on the ET-1-induced phosphorylation of SAPK/JNK. Onalespib, which alone did not affect the SAPK/JNK phosphorylation, significantly strengthened the ET-1-induced phosphorylation levels of SAPK/JNK (Fig. 3A). The amplifying effect of onalespib on the SAPK/JNK phosphorylation was dose-dependent over the range between 0.3 and 1.0 µM (Fig. 3A). Additionally, we showed that 17-DMAG remarkably enhanced the ET-1-induced levels of phosphorylated SAPK/JNK (Fig. 3B).

Effect of SP600125 on the enhancement by onalespib of ET-1-stimulated HSP27 induction in MC3T3-E1 cells. In order to further investigate whether HSP90 inhibitor enhances the ET-1-stimulated HSP27 induction via SAPK/JNK activated by ET-1 in MC3T3-E1 cells, we examined effect of SP600125, an inhibitor of SP600125 (20), on enhancement by onalespib of ET-1-induced HSP27. SP600125 significantly reduced the amplification by onalespib of ET-1-induced levels of HSP27 protein (Fig. 4).

Discussion

In the present study, we showed that geldanamycin and 17-DMAG, a geldanamycin derivative, which belong to HSP90 inhibitors (17,18), significantly potentiated the ET-1-stimulated induction of HSP27 (HSPB1), a small HSP (HSPB), in osteoblast-like MC3T3-E1 cells. Furthermore, onalespib (19), another HSP90 inhibitor different from geldanamycin or its analogues, markedly increased the ET-1-induced HSP27 protein levels. Based on our findings, it seems likely that HSP90 plays a suppressive role in ET-1-stimulated HSP27 induction in osteoblast-like MC3T3-E1 cells.

Regarding the intracellular signaling of ET-1 in osteoblasts, we have demonstrated that ET-1 stimulates the induction of HSP27 via p38 MAP kinase and SAPK/JNK among the MAP kinase superfamily (21), in osteoblast-like MC3T3-E1 cells (8,9). Based on our findings, we investigated whether the amplifying effect of HSP90 inhibitors on the ET-1-stimulated HSP27 induction is due to the modulation of these MAP kinases in MC3T3-E1 cells. However, the
HSP90 inhibitors, geldanamycin, 17-DMAG and onalespib, did not have significant effects on the ET-1-induced levels of phosphorylated p38 MAP kinase. Therefore, it seems unlikely that HSP90 affects the ET-1-stimulated HSP27 induction via the signaling pathway of p38 MAP kinase in osteoblast-like MC3T3-E1 cells. On the other hand, we showed that onalespib significantly augmented the ET-1-induced phosphorylated levels of SAPK/JNK. In addition, the SAPK/JNK phosphorylation induced by ET-1 was markedly enhanced by 17-DMAG. Furthermore, we demonstrated that the enhancement by onalespib of the ET-1-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with various doses of (A) onalespib or (B) 17-DMAG for 60 min and then stimulated by 0.1 µM ET-1 or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE with subsequent western blot analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows the quantitative representations of the levels of phospho-SAPK/JNK following normalization with respect to those of SAPK/JNK obtained from laser densitometric analysis. The levels were expressed as the fold increase to the basal levels presented as lane 1. Each value represents the mean + standard error of the mean of triplicate determinations from three independent cell preparations. *P<0.05 vs. the control (lane 1). **P<0.05 vs. ET-1 alone (lane 2). ET-1, endothelin-1; 17-DMAG, 17-demethoxygeldanamycin; phospho, phosphorylated; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase.

Figure 2. Effects of geldanamycin, 17-DMAG or onalespib on ET-1-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of (A) geldanamycin, (B) 17-DMAG or (C) onalespib for 60 min and then stimulated by 0.1 µM ET-1 or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE with subsequent western blot analysis with antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. The histogram shows the quantitative representations of the levels of phosphorylated p38 MAP kinase after normalization with respect to those of p38 MAP kinase obtained from laser densitometric analysis. The levels were expressed as the fold increase to the basal levels presented in lane 1. Each value represents the mean + standard error of the mean of triplicate determinations from three independent cell preparations. *P<0.05 vs. the control (lane 1). N.S, no significant difference; ET-1, endothelin-1; 17-DMAG, 17-demethoxygeldanamycin; phospho, phosphorylated; MAP, mitogen-activated protein.

Figure 3. Effects of onalespib or 17-DMAG on the ET-1-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with various doses of (A) onalespib or (B) 17-DMAG for 60 min and then stimulated by 0.1 µM ET-1 or vehicle for 30 min. The extracts of cells were subjected to SDS-PAGE with subsequent western blot analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows the quantitative representations of the levels of phospho-SAPK/JNK following normalization with respect to those of SAPK/JNK obtained from laser densitometric analysis. The levels were expressed as the fold increase to the basal levels presented as lane 1. Each value represents the mean + standard error of the mean of triplicate determinations from three independent cell preparations. *P<0.05 vs. the control (lane 1). **P<0.05 vs. ET-1 alone (lane 2). ET-1, endothelin-1; 17-DMAG, 17-demethoxygeldanamycin; phospho, phosphorylated; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase.
ET-1-stimulated HSP27 induction was reduced by an inhibitor of SAPK/JNK, SP600125 (20). Taking our findings into account as a whole, it is most likely that HSP90 acts at a point upstream from SAPK/JNK and negatively regulates the ET-1-stimulated HSP27 induction in osteoblast-like MC3T3-E1 cells. The potential mechanism of HSP90 in the ET-1-induced HSP27 in osteoblasts shown here is summarized in Fig. 5.

It is firmly established that HSP90, a major molecular chaperone, plays as a central regulator of proteostasis such as protein folding under stress conditions (2). In addition to protein folding, accumulating evidence indicates that HSP90 is implicated in a variety of physiological and pathological cell processes including responses to steroid hormones, and neurodegenerative diseases (2). Regarding HSP90 in osteoblasts, it has been shown that bisphosphonate, the most useful medicine for osteoporosis, stimulates expression of HSP90 in osteoblasts (11). In addition, low-intensity pulsed ultrasound stimulation reportedly enhances osteoblasts proliferation and up-regulates HSP90, leading to dense mineralization (12). We have previously demonstrated that phosphorylated HSP27 acts as a negative regulator of calcification in osteoblast-like MC3T3-E1 cells whereas un-phosphorylated HSP27 has a stimulatory effect on the mineralization (10). It is well known that the functions of small HSPs are regulated by post-translational modifications including phosphorylation, indicating that phosphorylated status of HSP27 has a switching role in cellular functions (1). Our present results show that HSP90 inhibitors limits the ET-1-stimulated HSP27 induction in osteoblast-like MC3T3-E1 cells. Based on these findings, it seems likely that HSP90 regulates the mineralization of osteoblasts through modulating the HSP27 protein levels. Our present findings regarding about the regulation by HSP90 inhibitors of the HSP27 induction in osteoblasts might provide a new aspect of HSP90 as a therapeutic target for metabolic bone diseases such as osteoporosis. However, the physiological significance of HSP27 in osteoblasts remains unclear. Further investigations using another human osteoblast cells such as USO2 or MG63 would be required to clarify the exact roles of HSP90 and HSP27 in bone metabolism.

In conclusion, our results strongly suggest that HSP90 negatively regulates ET-1-stimulated HSP27 induction at a point upstream of SAPK/JNK in osteoblasts.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.
Authors’ contributions

KF, TO and OK conceived and designed the experiments. KF, TK, GS and RMN performed the experiments. KF, RMN, OK and HT analyzed the data. KF, TO, OK and HT wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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