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Phosphatidylinositol 3-Kinase/Akt Plays a Role in Sphingosine 1-Phosphate-Stimulated HSP27 Induction in Osteoblasts

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Abstract We previously reported that p38 mitogen-activated protein (MAP) kinase plays a part in sphingosine 1-phosphate-stimulated heat shock protein 27 (HSP27) induction in osteoblast-like MC3T3-E1 cells. In the present study, we investigated whether phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) is involved in the induction of HSP27 in these cells. Sphingosine 1-phosphate time dependently induced the phosphorylation of Akt. Akt inhibitor, 1L-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate, reduced the HSP27 induction stimulated by sphingosine 1-phosphate. The sphingosine 1-phosphate-induced phosphorylation of GSK-3β was suppressed by Akt inhibitor. The sphingosine 1-phosphate-induced HSP27 levels were attenuated by LY294002 or wortmannin, PI3K inhibitors. Furthermore, LY294002 or Akt inhibitor did not affect the sphingosine 1-phosphate-induced phosphorylation of p38 MAP kinase and SB203580, a p38 MAP kinase inhibitor, had little effect on the phosphorylation of Akt. These results suggest that PI3K/Akt plays a part in the sphingosine 1-phosphate-stimulated induction of HSP27, maybe independently of p38 MAP kinase, in osteoblasts. J. Cell. Biochem. 98: 1249–1256, 2006.

Key words: sphingosine 1-phosphate; heat shock protein; protein kinase; osteoblast

Sphingosine 1-phosphate is a metabolite of sphingomyelin. It is generally recognized that sphingomyelin is catalyzed by sphingomyelinase, resulting in the formation of ceramide, which is subsequently metabolized to sphingosine and sphingosine 1-phosphate [Spiegel and Merrill, 1996]. Accumulating evidence indicates that sphingosine 1-phosphate plays an important role in essential cellular functions such as proliferation, differentiation, and migration [Spiegel and Merrill, 1996; Spiegel and Milstein, 2003; Sanchez and Hla, 2004]. Bone metabolism is regulated by two functional cells, osteoblasts and osteoclasts, responsible for bone formation and bone resorption, respectively [Nijweide et al., 1986]. As for osteoblasts, it has been reported that sphingosine 1-phosphate prevents apoptosis via phosphatidylinositol 3-kinase (PI3K) in primary calvaria rat osteoblasts and human osteosarcoma SaOS-2 cells [Grey et al., 2002]. In our study [Kozawa et al., 1997a], we have previously reported that sphingosine 1-phosphate stimulates interleukin-6 synthesis in osteoblast-like MC3T3-E1 cells. However, the exact mechanism of sphingosine 1-phosphate in bone metabolism has not yet been precisely clarified.

Heat shock proteins (HSP) are expressed in both prokaryotic and eukaryotic cells in response to the biological stress such as heat stress and chemical stress [Hendrick and Hartl, 1993]. HSPs are classified into high-molecular-weight

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HSPs and low-molecular-weight HSPs based on apparent molecular sizes. Low-molecular-weight HSPs with molecular masses from 10 to 30 kDa, such as HSP27 and αB-crystallin have high homology in amino acid sequences [Inaguma et al., 1993; Benjamin and McMillan, 1998]. Though the functions of the low-molecular-weight HSPs are known less than those of the high-molecular-weight HSPs, it is generally accepted that they may have chaperoning functions like the high-molecular-weight HSPs [Inaguma et al., 1993; Benjamin and McMillan, 1998]. HSP27 becomes rapidly phosphorylated in response to various stresses, as well as to exposure to cyotokines and mitogens [Gaestel et al., 1991; Landry et al., 1992]. Under unstimulated conditions, HSP27 exists as a high-molecular weight aggregated form. It is rapidly dissociated as a result of phosphorylation [Kato et al., 1994; Rogalla et al., 1999]. The phosphorylation-induced dissociation from the aggregated form correlates with the loss of molecular chaperone activity [Kato et al., 1994; Rogalla et al., 1999]. In a previous study [Kozawa et al., 1999], we have shown that sphingosine 1-phosphate stimulates the induction of HSP27 in osteoblast-like MC3T3-E1 cells and that p38 mitogen-activated protein (MAP) kinase is involved in the HSP27 induction.

It is well known that Akt, also called protein kinase B, is a serine/threonine protein kinase that plays crucial roles in mediating intracellular signaling of variety of agonists including insulin-like growth factor-I, platelet-derived growth factor (PDGF), and cytokines [Downward, 1995; Franke et al., 1995; Coffer et al., 1998]. It has been shown that Akt regulates biological functions such as gene expression, survival, and oncogenesis [Coffer et al., 1998]. Accumulating evidence suggests that PI3K functions at an upstream from Akt [Chan et al., 1999; Cantley, 2002]. Akt containing a pleckstrin homology domain is recruited to the plasma membrane by the lipid product of PI3K and activated. As for osteoblasts, it has been reported that IGF-I and PDGF induce translocation of Akt to the nucleus [Borgatti et al., 2000]. In addition, recently, Akt is reportedly activated by cyclic stretch or androgen [Danciu et al., 2003; Kang et al., 2004]. We have recently shown that Akt plays an important role in insulin-like growth factor-I-stimulated alkaline phosphatase activity in MC3T3-E1 cells [Noda et al., 2005]. However, the correlation between HSP27 and PI3K/Akt in osteoblasts has not yet been clarified.

In the present study, we investigated whether PI3K/Akt is involved in sphingosine 1-phosphate-stimulated phosphorylation of HSP27 in osteoblast-like MC3T3-E1 cells. We here show that PI3K/Akt pathway is involved in the sphingosine 1-phosphate-stimulated induction of HSP27, maybe independently of p38 MAP kinase, in these cells.

MATERIALS AND METHODS

Materials

Sphingosine 1-phosphate and β-actin antibodies were purchased from Sigma Chemical Co. (St. Louis, MO). Akt inhibitor (1L-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate), LY294002, wortmannin, and SB203580 were obtained from Calbiochem-Novabiochem (La Jolla, CA). Phospho-specific Akt antibodies, Akt antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific GSK-3β antibodies, and GSK-3β antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). HSP27 antibodies were obtained from R&D Systems, Inc. (Minneapolis, MN). An ECL Western blotting detection system was obtained from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. Sphingosine 1-phosphate, Akt inhibitor LY294002, wortmannin, and SB203580 were dissolved in dimethyl sulfoxide (DMSO). All inhibitors became soluble in the cell culture media after once dissolved in DMSO. The maximum concentration of DMSO was 0.1%, which did not affect Western blot analysis.

Cell Culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [Sudo et al., 1983] were maintained as previously described [Kozawa et al., 1997b]. 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 were seeded into 90-mm diameter dishes (25×10^4/dish) in α-MEM containing 10% FCS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h. When indicated, the cells were pretreated with Akt

inhibitor, wortmannin, LY294002, or SB203580 for 60 min prior to stimulation of sphingosine 1-phosphate.

Western Blot Analysis

Cultured cells were stimulated by sphingosine 1-phosphate in serum-free α-MEM for the indicated periods. Cells were washed twice with phosphate-buffered saline and then lysed, homogenized, sonicated, and immediately boiled in a lysis buffer containing 62.5 mM Tris/Cl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol, and 10% glycerol. The sample was used for the analysis by Western blotting. SDS–polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli [Laemmli, 1970] in 10% polyacrylamide gel. Western blot analysis was performed as described previously [Kato et al., 1996], using phospho-specific Akt antibodies, Akt antibodies, HSP27 antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific GSK-3β antibodies, or GSK-3β antibodies with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second antibodies. Peroxidase activity on PVDF membranes was visualized on X-ray film by means of the ECL Western blotting detection system and was quantitated using NIH image software. All of Western blot analyses were repeated at least three times in independent experiments.

Statistical Analysis

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

RESULTS

Time-Dependent Effects of Sphingosine 1-Phosphate on the Phosphorylation of Akt in MC3T3-E1 Cells

Sphingosine 1-phosphate significantly stimulates the phosphorylation of Akt in osteoblast-like MC3T3-E1 cells in a time-dependent manner (Fig. 1). The phosphorylation of Akt was markedly observed at 5 min after the sphingosine 1-phosphate-stimulation. The phosphorylation reached its peak at 15 min after the stimulation and decreased thereafter.

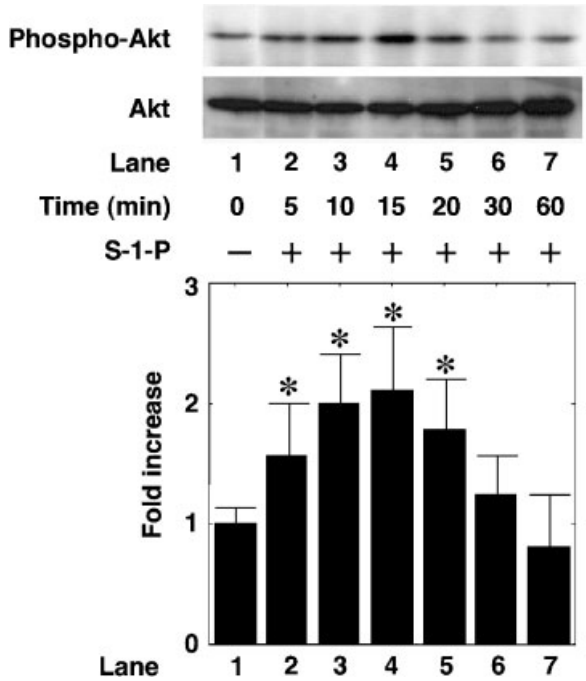

Fig. 1. Effect of sphingosine 1-phosphate (S-1-P) on the phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were stimulated with 30 μM S-1-P for the indicated periods. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific Akt or Akt. The histogram shows quantitative representations of the levels of S-1-P-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Similar results were obtained with two additional and different cell preparations. Each value represents the mean ± SD of triplicate determinations from triplicate independent cell preparations. *$P < 0.05$, compared to the value of control.

Effect of Akt Inhibitor on the Induction of HSP27 in MC3T3-E1 Cells

Then we examined the effect of Akt inhibitor (1L-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate) [Hu et al., 2000] on the sphingosine 1-phosphate-stimulated induction of HSP27. Akt inhibitor partially suppressed the sphingosine 1-phosphate-induced up-regulation of HSP27 levels (Fig. 2). Akt inhibitor (50 μM) caused about 40% reduction in the sphingosine 1-phosphate-effect.

We have previously shown that sphingosine 1-phosphate stimulates HSP27 induction at least in part via p38 MAP kinase in osteoblasts [Kozawa et al., 1999]. However, Akt inhibitor did not influence the sphingosine 1-phosphate-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells (data not shown). It is well known that GSK-3β is one of

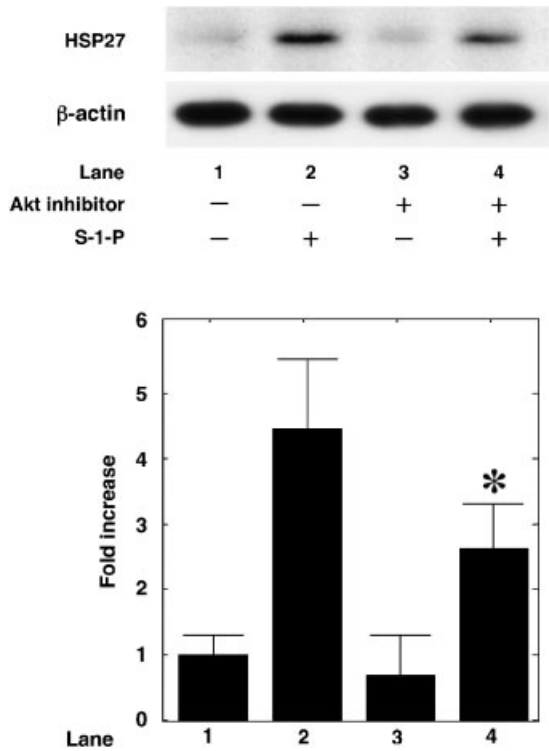

Fig. 2. Effect of Akt inhibitor on the sphingosine 1-phosphate (S-1-P)-induced levels of HSP27 in MC3T3-E1 cells. The cultured cells were pretreated with 50 μM Akt inhibitor for 60 min, and then stimulated by 30 μM of S-1-P or vehicle for 6 h. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against HSP27 or β-actin. The histogram shows quantitative representations of the levels of S-1-P-induced HSP27 after normalization to levels of β-actin. Similar results were obtained with two additional and different cell preparations. Each value represents the mean ± SD of triplicate determinations from triplicate independent cell preparations. $^*P < 0.05$, compared to the value of S-1-P.

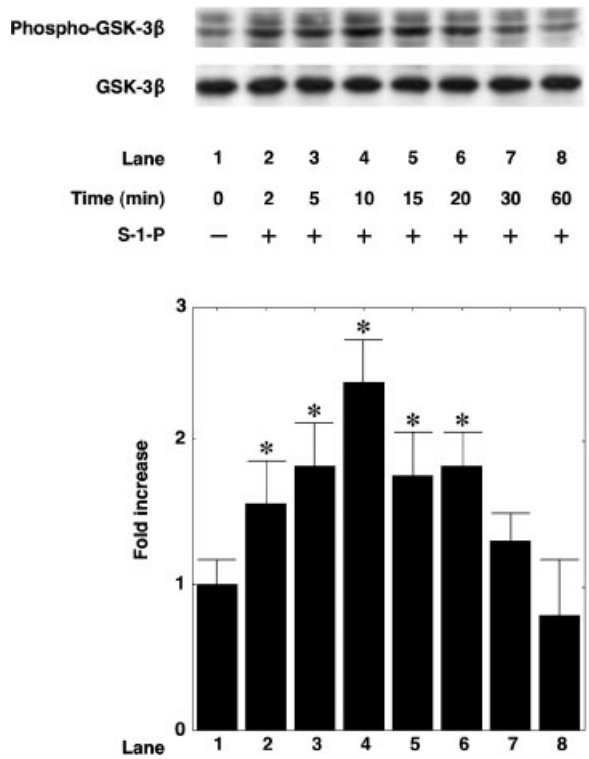

Fig. 3. Effect of sphingosine 1-phosphate (S-1-P) on the phosphorylation of GSK-3β in MC3T3-E1 cells. The cultured cells were stimulated with 30 μM S-1-P for the indicated periods. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific GSK-3β or GSK-3β. The histogram shows quantitative representations of the levels of S-1-P-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Similar results were obtained with two additional and different cell preparations. Each value represents the mean ± SD of triplicate determinations from triplicate independent cell preparations. $^*P < 0.05$, compared to the value of control.

the Akt substrates [Cross et al., 1995]. We found that GSK-3β was time dependently phosphorylated by sphingosine 1-phosphate (Fig. 3). In addition, Akt inhibitor attenuated the sphingosine 1-phosphate-induced phosphorylation of GSK-3β, suggesting that the Akt-mediating pathway actually functions in sphingosine 1-phosphate-stimulated MC3T3-E1 cells (Fig. 4). Akt inhibitor (50 μM) caused about 50% reduction in the sphingosine 1-phosphate-effect.

Effects of LY294002 and Wortmannin on the Sphingosine 1-Phosphate-Induced Phosphorylation of Akt in MC3T3-E1 Cells

In order to clarify whether PI3K acts at a point upstream from Akt, we examined the effect of LY294002, a specific inhibitor of PI3K [Vlahos et al., 1994], on the sphingosine 1-phosphate-induced phosphorylation of Akt. LY294002 dose dependently suppressed the sphingosine 1-phosphate-induced Akt phosphorylation (Fig. 5A). LY294002 (10 μM) caused almost complete reduction in the sphingosine 1-phosphate-effect. Wortmannin, another PI3K inhibitor [Arcaro and Wymann, 1993], also suppressed the phosphorylation of Akt (Fig. 5B). Wortmannin (10 μM) caused about 40% reduction in the sphingosine 1-phosphate-effect. However, LY294002 did not affect the sphingosine 1-phosphate-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells (data not shown). In addition, SB203580, a specific inhibitor of p38 MAP kinase [Cuenda et al., 1995] failed to affect the sphingosine 1-phosphate-induced phosphorylation of Akt (data not shown).

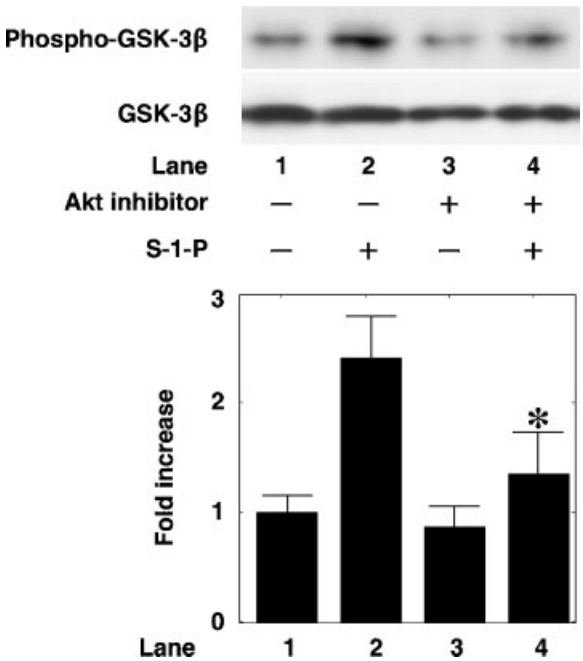

Fig. 4. Effect of Akt inhibitor on the sphingosine 1-phosphate (S-1-P)-induced phosphorylation of GSK-3β in MC3T3-E1 cells. The cultured cells were pretreated with 50 μM Akt inhibitor for 60 min, and then stimulated by 30 μM S-1-P or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific GSK-3β or GSK-3β. The histogram shows quantitative representations of the levels of S-1-P-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Similar results were obtained with two additional and different cell preparations. Each value represents the mean ± SD of triplicate determinations from triplicate independent cell preparations. $*P < 0.05$, compared to the value of S-1-P.

Effects of LY294002 and Wortmannin on the Sphingosine 1-Phosphate-Stimulated Induction of HSP27 in MC3T3-E1 Cells

LY294002 significantly suppressed the sphingosine 1-phosphate-stimulated induction of HSP27 in a dose dependent manner between

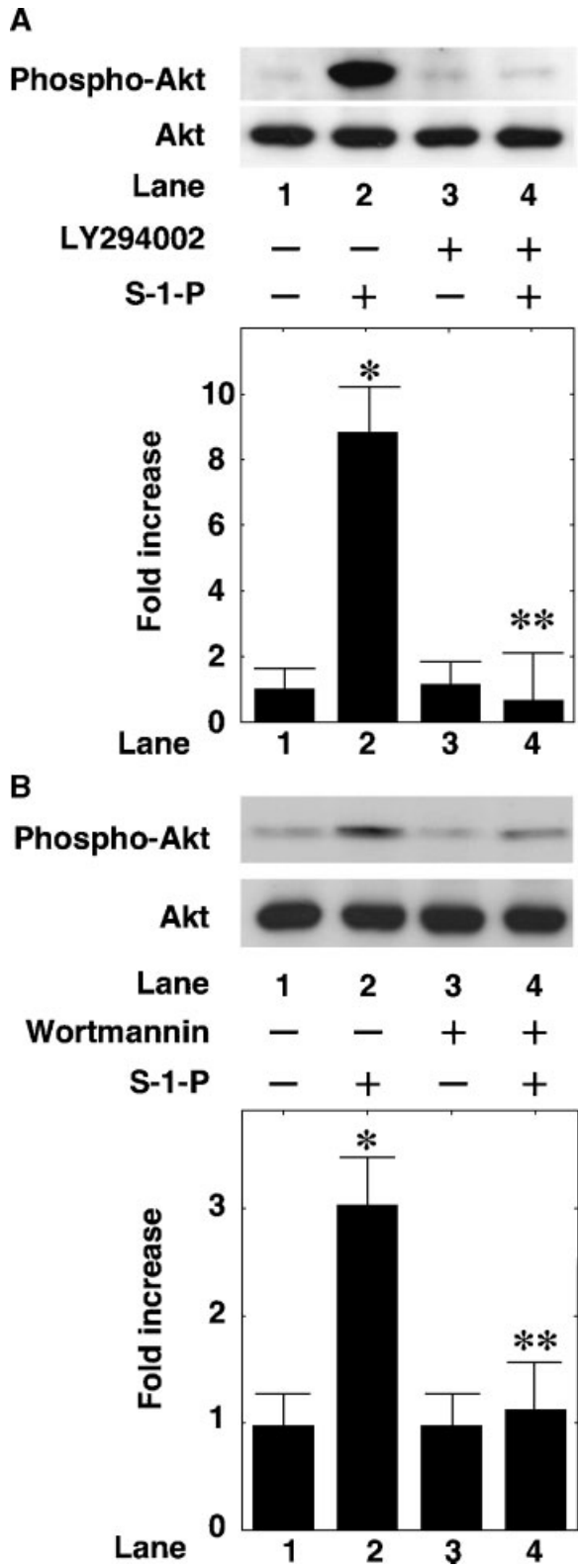

Fig. 5. Effects of LY294002 or wortmannin on the sphingosine 1-phosphate (S-1-P)-induced phosphorylation of Akt in MC3T3-E1 cells. The cultured cells were pretreated with 10 μM LY294002 (**A**) or 10 μM wortmannin (**B**) for 60 min, and then stimulated by 30 μM S-1-P or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against phospho-specific Akt or Akt. The histogram shows quantitative representations of the levels of S-1-P-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Similar results were obtained with two additional and different cell preparations. Each value represents the mean ± SD of triplicate determinations from triplicate independent cell preparations. $*P < 0.05$, compared to the value of control (without agonist and inhibitor). $**P < 0.05$, compared to the value of S-1-P alone.

10 and 50 μM (Fig. 6A). Additionally, wortmannin markedly reduced the induction of HSP27 similarly to LY294002 (Fig. 6B).

DISCUSSION

We have previously shown that sphingosine 1-phosphate stimulates induction of HSP27 in osteoblast-like MC3T3-E1 cells and that p38 MAP kinase takes a part in the sphingosine 1-phosphate-effect [Kozawa et al., 1999]. In the present study, we first demonstrated that sphingosine 1-phosphate stimulated the phosphorylation of Akt in a time-dependent manner in MC3T3-E1 cells. In addition, we showed that PI3K inhibitors such as LY294002 and wortmannin, suppressed the sphingosine 1-phopshate-induced phosphorylation of Akt, suggesting that Akt functions at a point downstream from PI3K in these cells. PI3K is recruited upon growth factor receptor activation and produces 3′ phosphoinositide lipids [Dudek et al., 1997; Katso et al., 2001]. The lipid products of PI3K act as second messengers by binding to and activating diverse cellular target proteins. These events constitute the start of a complex signaling cascade, which ultimately results in the mediation of cellular activities such as proliferation, differentiation, chemotaxis, and survival. The PI3K/Akt signaling pathway is currently considered to play a critical role in mediating survival signals in a wide range of cell types. The recent identification of a number of substrates for the serine/threonine kinase Akt suggests that it blocks cell death by both impinging on the cytoplasmic cell death machinery and by regulating the expression of genes involved in cell death and survival. In addition, recent experiments suggest that Akt may also use metabolic pathways to regulate cell survival [Brunet et al., 2001; Masuyama et al., 2001].

Therefore, we next examined the correlation between the sphingosine 1-phosphate-stimulated induction of HSP27 and PI3K/Akt in osteoblast-like MC3T3-E1 cells. In the present study, the sphingosine 1-phosphate-stimulated HSP27 induction was reduced by Akt inhibitor. As for Akt inhibitor, 1L-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate [Hu et al., 2000], we found that it blocked the phosphorylation of GSK-3β, one of the Akt substrates [Cross et al., 1995]. In addition, we showed that PI3K inhibitors also suppressed

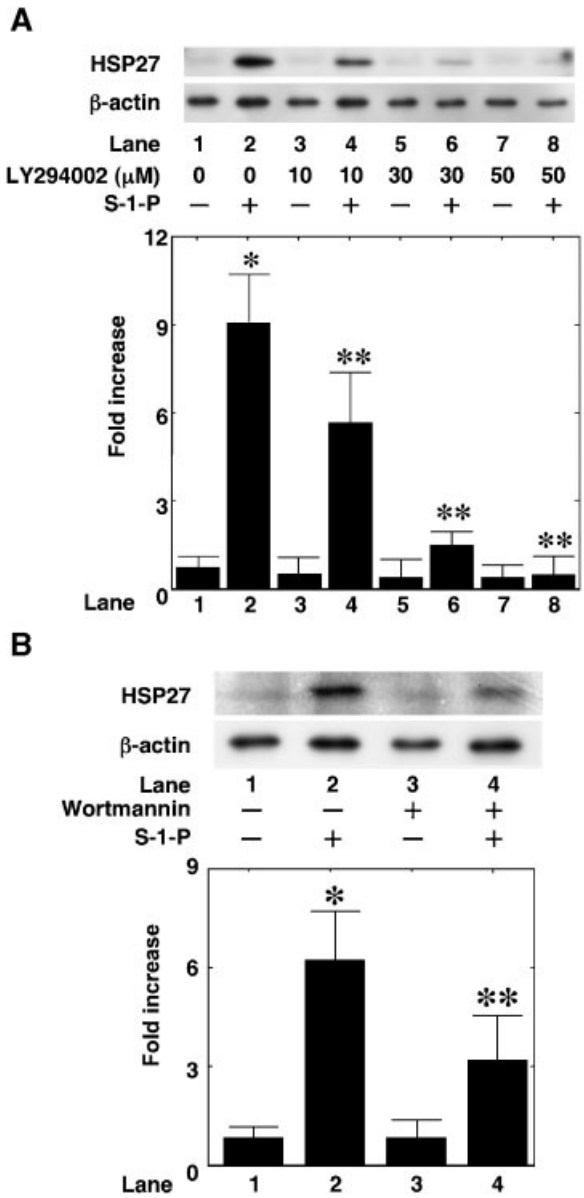

Fig. 6. Effects of LY294002 or wortmannin on the sphingosine 1-phosphate (S-1-P)-induced levels of HSP27 in MC3T3-E1 cells. The cultured cells were pretreated with various doses of LY294002 (**A**) or 50 μM wortmannin (**B**) for 60 min, and then stimulated by 30 μM S-1-P or vehicle for 6 h. The extracts of cells were subjected to SDS–PAGE with subsequent Western blotting analysis with antibodies against HSP27 or β-actin. The histogram shows quantitative representations of the levels of S-1-P-induced HSP27 after normalization to levels of β-actin. Similar results were obtained with two additional and different cell preparations. Each value represents the mean ± SD of triplicate determinations from triplicate independent cell preparations. $^{*}P < 0.05$, compared to the value of control. $^{**}P < 0.05$, compared to the value of S-1-P alone.

the sphingosine 1-phosphate-stimulated HSP27 induction thorough the reduction of the sphingosine 1-phosphate-induced Akt phosphorylation. Therefore, based on our findings, it is most likely that the sphingosine 1-phosphate-stimulated induction of HSP27 is regulated by PI3K/Akt in osteoblast-like MC3T3-E1 cells.

We have previously reported that the activation of p38 MAP kinase is involved in HSP27 induction by sphingosine 1-phosphate in osteoblast-like MC3T3-E1 cells [Kozawa et al., 1999]. Therefore, we investigated to clarify the relationship between p38 MAP kinase and PI3K/Akt in these cells. Akt inhibitor and PI3K inhibitor, LY294002 [Vlahos et al., 1994], failed to influence the sphingosine 1-phosphate-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells, and p38 MAP kinase inhibitor, SB203580 [Cuenda et al., 1995], had little effect on the sphingosine 1-phosphate-induced phosphorylation of Akt. In addition, the inhibitory effect of Akt inhibitor or wortmannin on the sphingosine 1-phosphate-stimulated HSP27 induction was partial. We have previously shown that the suppressive effect of SB203580 on the HSP27 induction was partial [Kozawa et al., 1999]. These findings suggest that PI3K/Akt pathway plays a role at least in part in addition to p38 MAP kinase pathway in the sphingosine 1-phosphate-stimulated HSP27 induction in MC3T3-E1 cells. Taking these results into account as a whole, it is most likely that sphingosine 1-phosphate stimulates the induction of HSP27 probably via two independent pathways, PI3K/Akt and p38 MAP kinase, in osteoblast-like MC3T3-E1 cells.

It is recognized that HSP27 is present at two forms, an aggregated form and a dissociated small form in unstressed conditions [Benjamin and McMillan, 1998]. It has been shown that HSP27 is constitutively expressed at high levels in various tissues and cells, especially in skeletal muscle cells and smooth muscle cells [Benjamin and McMillan, 1998]. Post-translational modifications such as phosphorylation and oligomerization are crucial regulators of its functions [Benjamin and McMillan, 1998]. In our previous study [Kato et al., 1994], we have reported that HSP27 is dissociated concomitantly with the phosphorylation of the aggregated form of HSP27. In addition, we have shown that conversion from the non-phosphorylated, aggregated form of HSP27 to the phosphorylated, dissociated form results in decreased tolerance to heat stress [Kato et al., 1994]. It has been shown that estrogen-induced resistance to osteoblast apoptosis is associated with increased HSP27 expression [Cooper et al., 2000]. We speculate that expression of HSP27 via p38 MAP kinase and PI3K/Akt in osteoblasts might be related to the maintenance of the number of viable osteoblasts in bone tissue. Interestingly, sphingosine 1-phosphate reportedly prevents apoptosis in primary rat osteoblasts and human osteosarcoma SaOS-2 cells [Grey et al., 2002]. Taking our findings into account, it is probable that sphingosine 1-phosphate directly affects osteoblasts through the induction of HSP27 through PI3K/Akt and p38 MAP kinase. However, the physiological significance of HSP27 in osteoblasts has not yet been precisely clarified. Further investigations are necessary to clarify the exact roles of HSP27 in osteoblasts.

In conclusion, these results strongly suggest that sphingosine 1-phosphate stimulates the induction of HSP27 via PI3K/Akt pathway in addition to p38 MAP kinase in osteoblasts.

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