PDGF-BB activates phosphatidylcholine-hydrolyzing phospholipase D via tyrosine kinase activation, resulting in protein kinase C activation in osteoblast-like MC3T3-E1 cells [6]. In addition, we recently showed that PI3-kinase negatively regulates the PDGF-BB-stimulated interleukin-6 synthesis in these cells [7]. However, the exact role of PDGF-BB in osteoblasts has not yet been fully clarified.

Bone remodeling carried out by osteoclasts and osteoblasts is accompanied with new capillaries extending [8,9]. During bone remodeling, capillary endothelial cells provide the microvasculature. Therefore, it is well recognized that the activities of osteoblasts, osteoclasts, and capillary endothelial cells are closely coordinated and regulate bone metabolism [10]. These functional cells are considered to influence one another via humoral factors as well as by direct cell-to-cell contact. Vascular endothelial growth factor (VEGF) is an angiogenic growth factor displaying high specificity for vascular endothelial cells [11]. VEGF that is synthesized and secreted from a variety of cell types such as vascular smooth muscle cells, increases capillary permeability and stimulates proliferation of endothelial cells [11]. As for bone metabolism, it has been shown that inactivation of VEGF causes complete suppression of blood vessel invasion concomitant with impaired trabecular bone formation and expansion of hypertrophic chondrocyte zone in mouse tibial epiphyseal growth plate [12]. Accumulating evidence suggests that osteoblasts among bone cells synthesize and secrete VEGF in response to various physiological agents [11,13–15]. We have previously reported that $PGF_{2\alpha}$ (prostaglandin $F_{2\alpha}$) stimulates VEGF synthesis in MC3T3-E1 cells, and that among the mitogen-activated protein (MAP) kinase superfamily [16], p44/p42 MAP kinase plays as a positive regulator in the VEGF synthesis [17]. Taking these findings into account, it is currently speculated that VEGF secreted from osteoblasts may play an important role in the regulation of bone metabolism [10,18]. However, the mechanism behind VEGF synthesis and its synthesis in osteoblasts is not fully understood.

In the present study, we investigated the effect of PDGF-BB on the $PGF_{2\alpha}$-stimulated VEGF synthesis in osteoblast-like MC3T3-E1 cells. We here show that PDGF-BB up-regulates $PGF_{2\alpha}$-stimulated VEGF synthesis via PI3-kinase in these cells.

2. Materials and methods

2.1. Materials

$PGF_{2\alpha}$ was purchased form Sigma Chemical Co. (St. Louis, MO). PDGF-BB and mouse VEGF enzyme immunoassay kit were purchased from R&D Systems, Inc. (Minneapolis, MN). LY294002, wortmannin and PD98059 were obtained from Calbiochem–Novabiochem Co. (La Jolla, CA). Phospho-specific PI3-kinase antibodies, PI3-kinase antibodies, phosphor-specific p44/p42 MAP kinase antibodies and p44/p42 MAP kinase antibodies were purchased from Cell Signaling Technology (Beverly, MA). ECL Western blotting detection system was purchased from Amersham Biosciences (Piscataway, NJ). Other materials and chemicals were obtained from commercial sources. $PGF_{2\alpha}$, was dissolved in ethanol. LY294002, wortmannin and PD98059 were dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of ethanol or DMSO was 0.1%, which did not affect the assay for VEGF or the Western blotting analysis.

2.2. Cell culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [19] were maintained as previously described [20]. 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 35-mm (5×10^4) or 90-mm (5×10^5) diameter dishes 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.

2.3. VEGF assay

The cultured cells were stimulated by various dose of $PGF_{2\alpha}$ in 1 ml of α-MEM containing 0.3% FCS for the indicated periods. When indicated, the cells were pretreated with LY294002, wortmannin or PD98059 for 60 min. The conditioned medium was collected at the end of the incubation, and the VEGF concentration was measured by ELISA kit.

2.4. Analysis of Western blotting

The cultured cells were stimulated by $PGF_{2\alpha}$ and/or PDGF-BB 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. The cytosolic fraction was collected as a supernatant after centrifugation at $125{,}000 \times g$ for 10 min at 4 °C. SDS-polyacrylamide gel electrophoresis (PAGE) was performed by Laemmli [21] in 10% polyacrylamide gel. A Western blotting analysis was performed as described previously [17] by using phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific PI3-kinase antibodies or PI3-kinase antibodies with

peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second antibodies. The peroxidase activity on PVDG membrane was visualized on X-ray film by means of the ECL Western blotting detection system. When indicated, the cells were pretreated with various doses of LY294002 or wortmannin for 60 min.

2.5. Determination

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

2.6. Statistical analysis

The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between pairs and a $p<0.05$ 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.

3. Results

3.1. Effect of PDGF-BB on $PGF_{2\alpha}$-induced VEGF synthesis in MC3T3-E1 cells

PDGF-BB, which alone had no effect on the VEGF levels, significantly enhanced the $PGF_{2\alpha}$-stimulated VEGF synthesis in a time-dependent manner in osteoblast-like MC3T3-E1 cells (Fig. 1). The effect of PDGF-BB was dose-dependent in the range between 10 and 70 ng/ml (Fig. 2). The maximum effect of PDGF-BB on the VEGF synthesis was observed at 70 ng/ml, which caused about 16 times enhancement of the-$PGF_{2\alpha}$-effect.

3.2. Effect of LY294002 or wortmannin on the amplification by PDGF-BB of the $PGF_{2\alpha}$-induced VEGF synthesis in MC3T3-E1 cells

We have previously reported that PI3-kinase activated by PDGF-BB limits the PDGF-BB-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells [7]. In order to investigate whether the amplifying effect of PDGF-BB on the $PGF_{2\alpha}$-stimulated VEGF synthesis is via activation of PI3-kinase in MC3T3-E1 cells, we next examined the effect of LY294002, a specific inhibitor of PI3-kinase [22], on the synthesis of VEGF. LY294002, which by itself did not suppress the $PGF_{2\alpha}$-induced VEGF synthesis, significantly reduced the enhancement

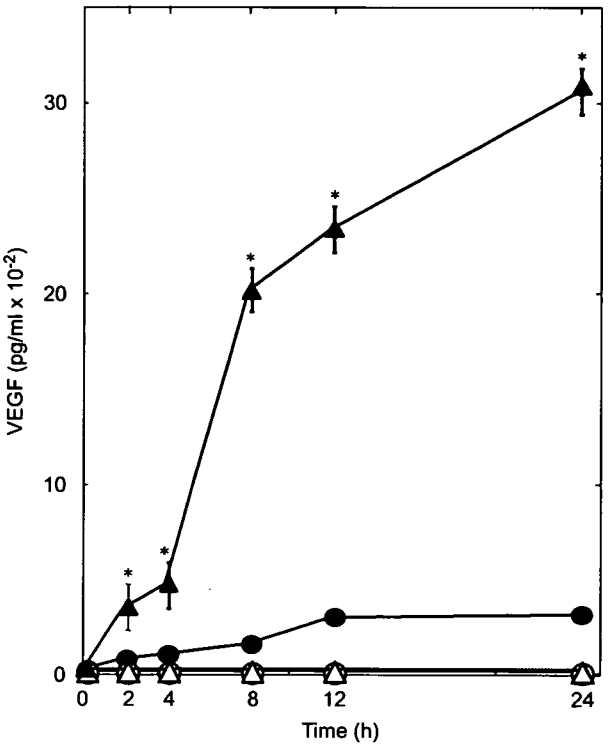

Fig. 1. Effect of PDGF-BB on the $PGF_{2\alpha}$-stimulated VEGF synthesis in MC3T3-E1 cells. The cultured cells were pretreated with 30 ng/ml PDGF-BB (▲, △) or vehicle (●, ○) for 60 min, and then stimulated by 10 μM $PGF_{2\alpha}$ (●, ▲) or vehicle (○, △) for the indicated periods. Each value represents 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 $PGF_{2\alpha}$ alone.

by PDGF-BB of $PGF_{2\alpha}$-induced VEGF synthesis (Table 1). The effect of LY294002 was dose dependent in the range between 1 and 50 μM (data not shown).

Wortmannin, another PI3-kinase inhibitor [23], as well as LY294002, also suppressed the amplification by PDGF-BB of the VEGF synthesis without affecting the $PGF_{2\alpha}$-stimulated synthesis (Table 2). The effect of wortmannin was dose dependent between 10 and 30 μM (data not shown). We also examined the effects of LY294002 or wortmannin on the PDGF-BB-induced phosphorylation of PI3-kinase in these cells. Neither LY294002 nor wortmannin inhibited PDGF-BB-induced phosphorylation of PI3-kinase in these cells (Fig. 3).

3.3. Effect of PD98059 on the amplification by PDGF-BB of the $PGF_{2\alpha}$-induced VEGF synthesis in MC3T3-E1 cells

In a previous study [17], we showed that $PGF_{2\alpha}$ stimulates VEGF synthesis through p44/p42 MAP kinase activation in osteoblast-like MC3T3-E1 cells. In order to clarify whether the up-regulating effect of PDGF-BB on $PGF_{2\alpha}$-induced VEGF synthesis is due to p44/p42 MAP kinase activation in MC3T3-E1 cells, we

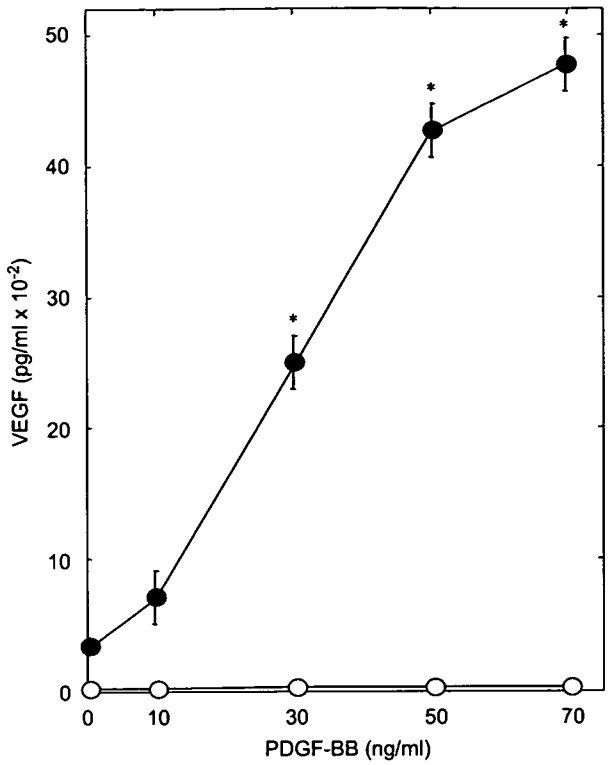

Fig. 2. Dose dependent effect of PDGF-BB on the $PGF_{2\alpha}$-stimulated VEGF synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of PDGF-BB for 60 min, and then stimulated by 10 μM $PGF_{2\alpha}$ (●) or vehicle (○) for 24 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}p<0.05$, compared to the value of $PGF_{2\alpha}$ alone.

Table 1
Effect of LY294002 on the enhancement by PDGF-BB of the $PGF_{2\alpha}$-stimulated VEGF synthesis in MC3T3-E1 cells

LY294002	PDGF-BB	$PGF_{2\alpha}$	VEGF (pg/ml)
–	–	–	15 ± 5
–	–	+	306 ± 27*
–	+	–	20 ± 5
–	+	+	3919 ± 157**
+	–	–	10 ± 5
+	–	+	440 ± 45
+	+	–	23 ± 10
+	+	+	1381 ± 88***

The cultured cells were pretreated with 50 μM LY294002 or vehicle for 60 min and then incubated by 30 ng/ml PDGF-BB or vehicle for 60 min. The cells were stimulated by 10 μM $PGF_{2\alpha}$ or vehicle for 48 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 control.

$^{**}p<0.05$, compared to the value of $PGF_{2\alpha}$ alone.

$^{***}p<0.05$, compared to the value of $PGF_{2\alpha}$ with PDGF-BB pretreatment.

Table 2
Effects of wortmannin or PD98059 on the enhancement by PDGF-BB of the $PGF_{2\alpha}$-stimulated VEGF synthesis in MC3T3-E1 cells

Inhibitor	PDGF-BB	$PGF_{2\alpha}$	VEGF (pg/ml)
–	–	–	20 ± 5
–	–	+	370 ± 28*
–	+	–	15 ± 10
–	+	+	3010 ± 139**
Wortmannin	–	–	20 ± 5
Wortmannin	–	+	462 ± 45
Wortmannin	+	–	18 ± 10
Wortmannin	+	+	406 ± 36***
PD98059	–	–	18 ± 5
PD98059	–	+	114 ± 20**
PD98059	+	–	15 ± 8
PD98059	+	+	122 ± 25***

The cultured cells were pretreated with 30 nM wortmannin, 30 μM PD98059 or vehicle for 60 min and then incubated by 30 ng/ml PDGF-BB or vehicle for 60 min. The cells were stimulated by 10 μM $PGF_{2\alpha}$ or vehicle for 24 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 control.

$^{**}p<0.05$, compared to the value of $PGF_{2\alpha}$ alone.

$^{***}p<0.05$, compared to the value of $PGF_{2\alpha}$ with PDGF-BB pretreatment.

examined the effect of PD98059, a specific inhibitor of the upstream kinase that activates p44/p42 MAP kinase [24], on the enhancement by PDGF-BB. PD98059, which by itself had no effect on the basal levels of VEGF, significantly reduced the enhancement by PDGF-BB of $PGF_{2\alpha}$-induced VEGF synthesis to the levels of the $PGF_{2\alpha}$ with PD98059 (Table 2). The effect of PD98059 was dose dependent in the range between 1 and 30 μM (data not shown).

3.4. Effect of PDGF-BB in combination with $PGF_{2\alpha}$ on the phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells

We further investigated whether PDGF-BB could enhance the phosphorylation of p44/p42 MAP kinase induced by $PGF_{2\alpha}$ in osteoblast-like MC3T3-E1 cells, and whether LY294002 could abolish it in osteoblast-like MC3T3-E1 cells. PDGF-BB itself induced the phosphorylation of p44/p42 MAP kinase in these cells, and that the effects of PDGF-BB and $PGF_{2\alpha}$ on the phosphorylation of p44/p42 MAP kinase were additive (Fig. 4). In addition, LY294002 had little effect on the phosphorylation of p44/p42 MAP kinase induced by $PGF_{2\alpha}$ with PDGF-BB (Fig. 4).

4. Discussion

In the present study, we showed that PDGF-BB, which alone did not affect the levels of VEGF,

significantly amplified the $PGF_{2\alpha}$-stimulated VEGF synthesis in osteoblast-like MC3T3-E1 cells. We next investigated the mechanism of PDGF-BB behind the amplification. In our previous study [7], we reported that PDGF-BB- activated PI3-kinase negatively regulates the interleukin-6 synthesis in MC3T3-E1 cells. We investigated whether or not PI3-kinase functions in the PDGF-BB-enhanced VEGF synthesis in osteoblast-like MC3T3-E1 cells. LY294002, a specific inhibitor of PI3-kinase [22], markedly suppressed the amplification by PDGF-BB of $PGF_{2\alpha}$-induced VEGF synthesis while the $PGF_{2\alpha}$-induced VEGF synthesis was not inhibited by LY294002. Thus, these results suggest that PI3-kinase is implicated in the PDGF-BB-induced potentiation. In addition, wortmannin, another PI3-kinase inhibitor [23], reduced the PDGF-BB-effect in the VEGF synthesis without suppressing the $PGF_{2\alpha}$-induced VEGF synthesis as well as LY294002. We found that neither LY294002 nor wortmannin could inhibit PDGF-BB-induced phosphorylation of PI3-kinase in these cells. Both LY294002 and wortmannin are competitive inhibitors of PI3-kinase [22,23]. It is well recognized that PI3-kinase is activated by its phosphorylation and that LY294002 is a highly selective PI3-kinase inhibitor which blocks PI3-kinase-dependent phosphorylation of its target proteins [22]. Namely, LY294002 and wortmannin are not inhibitors of the phosphorylation of PI3-kinase. Thus, it is reasonable that the effects of these inhibitors on PI3-kinase-mediating PDGF-BB signaling are exerted at a point downstream of the phosphorylation of PI3-kinase in MC3T3-E1 cells. Indeed, we have previously reported that the phosphorylation of Akt induced by PDGF-BB is significantly inhibited by both LY294002 and wortmannin [25]. Therefore, it is sure that these inhibitors attenuate the PDGF-BB-activating PI3-kinase/Akt signaling in these cells. On the other hand, the amplification by PDGF-BB of $PGF_{2\alpha}$ was observed during 4 and 8 h in the time course study. It is possible that PDGF-BB-stimulated other protein synthesis might be involved in the PDGF-BB effect. Therefore, taking our results into account as a whole, it is most likely that PDGF-BB activates the PI3-kinase pathway, resulting in the potentiation of $PGF_{2\alpha}$-induced VEGF synthesis in MC3T3-E1 cells.

It is well recognized that the MAP kinase superfamily mediates intracellular signaling of extracellular agonists and plays a pivotal role in cellular functions including

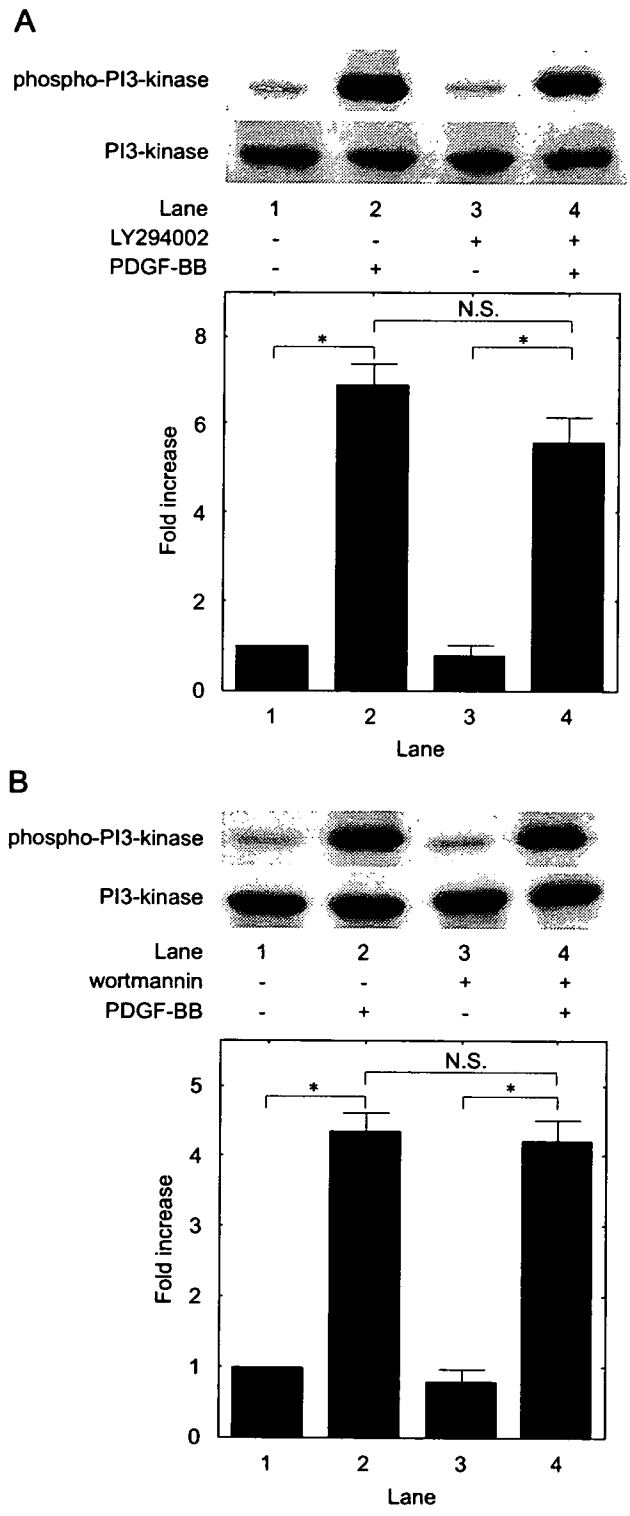

Fig. 3. Effects of LY294002 or wortmannin on the phosphorylation of PI3-kinase induced by PDGF-BB in MC3T3-E1 cells. The cultured cells were pretreated with 50 μM LY294002 (A), 30 nM wortmannin (B) or vehicle for 60 min, and then stimulated by 30 ng/ml PDGF-BB or vehicle for 60 min. The extracts of the cells were subjected to SDS-PAGE with a subsequent Western blotting analysis with antibodies against phospho-specific PI-3 kinase or PI-3 kinase. The histogram shows quantitative representations of the levels of PDGF-BB-induced phosphorylation obtained from a laser densitometric analysis of three independent experiments. 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. N.S., no statistical significance.

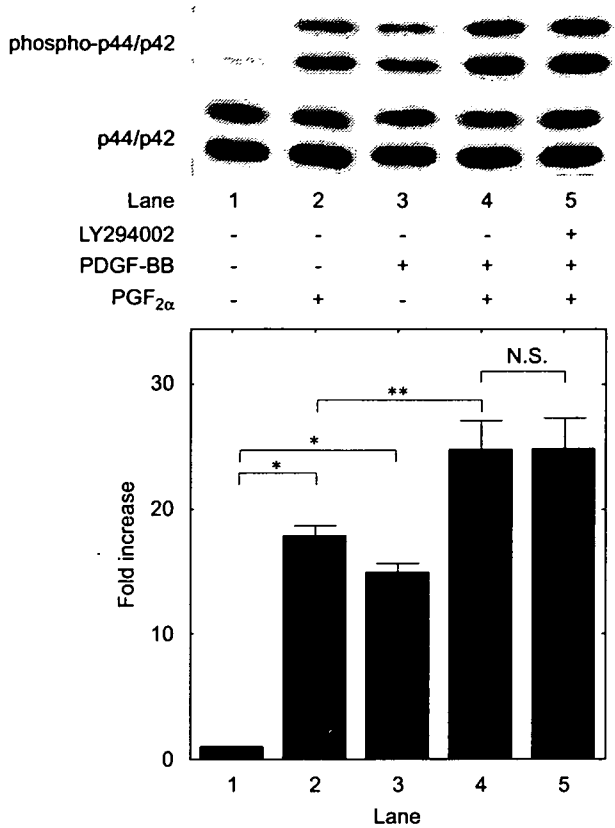

Fig. 4. Effect of PDGF-BB in combination with $PGF_{2\alpha}$ on the phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 50 μM LY294002 or vehicle for 60 min, and then incubated by 30 ng/ml PDGF-BB or vehicle for 60 min. The treated cells were stimulated by 10 μM $PGF_{2\alpha}$ or vehicle for 30 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p44/p42 MAP kinase or p44/p42 MAP kinase. The histogram shows quantitative representations of the levels of PDGF-BB- and/or $PGF_{2\alpha}$-induced phosphorylation obtained from a laser densitometric analysis of three independent experiments. 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. $**p<0.05$, compared to the value of $PGF_{2\alpha}$ alone. N.S., no statistical significance.

proliferation, differentiation, and apoptosis in a variety of cells [16]. Three major MAP kinase, p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK are known as central elements used by mammalian cells to transducer the diverse messages [16]. In our previous study [17], we have shown that p44/p42 MAP kinase acts as a positive regulator in $PGF_{2\alpha}$-induced VEGF synthesis in MC3T3-E1 cells. Therefore, we tried to clarify the relationship between PDGF-BB-effect and p44/p42 MAP kinase in the $PGF_{2\alpha}$-stimulated VEGF synthesis in these cells. The amplification by PDGF-BB of the $PGF_{2\alpha}$-stimulated VEGF synthesis was suppressed by PD98059, a specific inhibitor of MEK1/2 [23], similar to the levels of $PGF_{2\alpha}$ with PD98059. Moreover, we found that PDGF-BB itself induced the phosphorylation of p44/p42 MAP kinase in these cells, and that the effects of PDGF-BB and $PGF_{2\alpha}$ on the phosphorylation of p44/p42 MAP kinase were additive. These results suggest that PDGF-BB activates p44/p42 MAP kinase independently from $PGF_{2\alpha}$ in these cells. PDGF-BB as well as $PGF_{2\alpha}$ induced the phorphorylation of p44/p42 MAP kinase, however, VEGF synthesis was only observed by $PGF_{2\alpha}$, not by PDGF-BB. Our results indicate that PDGF-BB-induced p44/p42 MAP kinase activation alone is not sufficient for VEGF synthesis in osteoblasts, which is quite different from $PGF_{2\alpha}$-induced p44/p42 MAP kinase activation. In addition, the increase in VEGF by PDGF-BB and $PGF_{2\alpha}$ was not additive but synergistic. We also found that LY294002 had little effect on the phosphorylation of p44/p42 MAP kinase induced by $PGF_{2\alpha}$ with PDGF-BB. Therefore, it is probable that the enhancement by PDGF of $PGF_{2\alpha}$-induced VEGF synthesis through PI3-kinase is exerted at a point downstream from p44/p42 MAP kinase in these cells. As a whole, it is most likely that PDGF-BB independently activates p44/p42 MAP kinase and PI3-kinase in osteoblasts, and the latter is involved in the enhancement of $PGF_{2\alpha}$-induced VEGF synthesis at a point downstream from p44/p42 MAP kinase.

The expansion of capillary network providing microvasculature is an essential process of bone remodeling [10]. Since VEGF is a specific mitogen of vascular endothelial cells [11], it is recognized that VEGF secreted by osteoblasts plays a crucial role as an intercellular mediator between osteoblasts and vascular endothelial cells in bone metabolism. Moreover, it has been reported that VEGF is involved in trabecular bone formation and expansion of the hypertrophic chondrocyte zone in epiphyseal growth plate of mouse [12], supporting the significance of VEGF in bone metabolism. On the other hand, the mitogenic activities of PDGF and its release by platelets suggest a pivotal role in wound healing and bone fracture repair [26]. Therefore, our present findings lead us to speculate that PDGF-BB-enhanced VEGF synthesis from osteoblasts plays a pivotal role in the process of bone remodeling via up-regulating the proliferation of capillary endothelial cells. Further investigations are required to elucidate the precise role of PDGF-BB in bone metabolism.

In conclusion, our present results strongly suggest that PI3-kinase signaling pathway activated by PDGF-BB amplifies $PGF_{2\alpha}$-induced VEGF synthesis in osteoblasts, and that the effect is exerted at a point downstream from p44/p42 MAP kinase.

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Negative Regulation by p70 S6 Kinase of FGF-2–Stimulated VEGF Release Through Stress-Activated Protein Kinase/c-*Jun* N-Terminal Kinase in Osteoblasts

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ABSTRACT: To clarify the mechanism of VEGF release in osteoblasts, we studied whether p70 S6 kinase is involved in basic FGF-2–stimulated VEGF release in osteoblast-like MC3T3-E1 cells. In this study, we show that p70 S6 kinase activated by FGF-2 negatively regulates VEGF release through SAPK/JNK in osteoblasts.

Introduction: Vascular endothelial growth factor (VEGF) plays an important role in bone metabolism. We have previously reported that fibroblast growth factor-2 (FGF-2) stimulates the release of VEGF through p44/p42 mitogen-activated protein (MAP) kinase and stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) in osteoblast-like MC3T3-E1 cells and that FGF-2–activated p38 MAP kinase negatively regulates VEGF release. However, the mechanism behind VEGF release in osteoblasts is not precisely known.
Materials and Methods: The levels of VEGF released from MC3T3-E1 cells were measured by enzyme immunoassay. The phosphorylation of each protein kinase was analyzed by Western blotting. To knock down p70 S6 kinase in MC3T3-E1 cells, the cells were transfected with siRNA to target p70 S6 kinase.
Results: FGF-2 time-dependently induced the phosphorylation of p70 S6 kinase. Rapamycin significantly enhanced the FGF-2–stimulated VEGF release and VEGF mRNA expression. The FGF-2–induced phosphorylation of p70 S6 kinase was suppressed by rapamycin. Rapamycin markedly enhanced the FGF-2–induced phosphorylation of SAPK/JNK without affecting the phosphorylation of p44/p42 MAP kinase or p38 MAP kinase. SP600125, a specific inhibitor of SAPK/JNK, suppressed the amplification by rapamycin of the FGF-2–stimulated VEGF release similar to the levels of FGF-2 with SP600125. Finally, downregulation of p70 S6 kinase by siRNA significantly enhanced the FGF-2–stimulated VEGF release and phosphorylation of SAPK/JNK.
Conclusions: These results strongly suggest that p70 S6 kinase limits FGF-2–stimulated VEGF release through self-regulation of SAPK/JNK, composing a negative feedback loop, in osteoblasts.
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Key words: p70 S6 kinase, fibroblast growth factor-2, vascular endothelial growth factor, osteoblasts, mitogen-activated protein kinase

INTRODUCTION

It is well known that osteoblasts synthesize basic fibroblast growth factor (FGF)-2, and FGF-2 is embedded in bone matrix.[1,2] FGF-2 expression in osteoblasts is detected during fracture repair.[3] Bone metabolism is strictly regulated by osteoblasts and osteoclasts, which are responsible for bone formation and bone resorption, respectively.[4] Therefore, it is thought that FGF-2 plays a pivotal role in fracture healing, bone remodeling, and osteogenesis.[5] We have previously reported that FGF-2 autophosphorylates FGF receptors 1 and 2 among four structurally related high affinity receptors in osteoblast-like MC3T3-E1 cells.[6] In addition, we reported that FGF-2 stimulates induction of heat shock protein 27 in these cells.[7]

Bone remodeling carried out by osteoclasts and osteoblasts is accompanied by angiogenesis and capillary outgrowth.[8,9] During bone remodeling, capillary endothelial cells provide the microvasculature. It is well recognized that the activities of osteoblasts, osteoclasts, and capillary endothelial cells are closely coordinated and regulate bone metabolism.[10] These functional cells are considered to influence one another through humoral factors and by direct cell-to-cell contact. Vascular endothelial growth factor

The authors state that they have no conflicts of interest.

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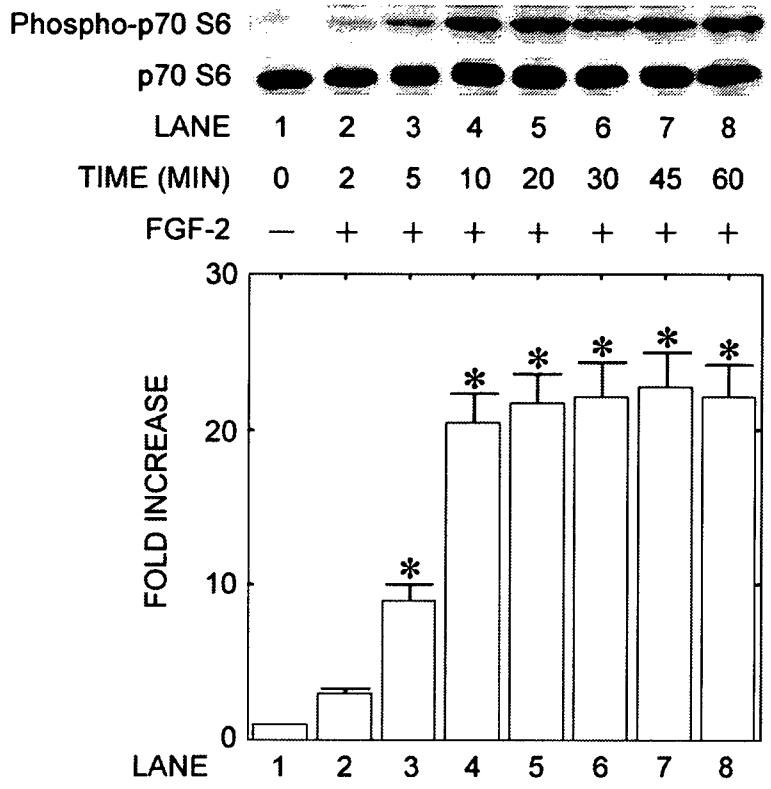

FIG. 1. Effect of FGF-2 on the phosphorylation of p70 S6 kinase in MC3T3-E1 cells. The cultured cells were stimulated by 70 ng/ml FGF-2 for the indicated periods. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p70 S6 kinase or p70 S6 kinase. The histogram shows quantitative representations of the levels of FGF-2–induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SE of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $*p < 0.05$ compared with the value of control.

(VEGF) is an angiogenic growth factor displaying high specificity for vascular endothelial cells.[11] VEGF, produced and secreted from a variety of cell types, increases capillary permeability and stimulates proliferation of endothelial cells.[11] As for bone metabolism, an inactivation of VEGF reportedly causes complete suppression of blood vessel invasion concomitant with impaired trabecular bone formation and expansion of hypertrophic chondrocyte zone in mouse tibial epiphyseal growth plate.[12] Evidence is accumulating that osteoblasts among bone cells produce and secrete VEGF in response to various physiological agents.[11,13–15] We have previously reported that FGF-2 stimulates VEGF release in MC3T3-E1 cells and that the release is positively regulated by p44/p42 mitogen-activated protein (MAP) kinase and stress-activated protein kinase/c-*Jun* *N*-terminal kinase (SAPK/JNK)[16,17] among the MAP kinase superfamily.[18] Based on these findings, it is currently recognized that VEGF secreted from osteoblasts may play an important role in the regulation of bone metabolism.[10,19] However, the mechanism behind VEGF synthesis in osteoblasts and its release from these cells is not fully understood.

p70 S6 kinase is a mitogen-activated serine/threonine kinase required for cell proliferation and G1 cell cycle progression.[20] As for osteoblasts, it has been shown that fluoroaluminate induces an increase in p70 S6 kinase phosphorylation.[21] In our previous study,[22] we reported that p70 S6 kinase plays as a positive regulator in bone morphogenetic protein-4–stimulated release of VEGF in osteoblast-like MC3T3-E1 cells. In addition, we recently showed that p38 MAP kinase, a member of the MAP kinase superfamily, functions at a point upstream from p70 S6 kinase in the release of VEGF in these cells.[23] However, the exact role of p70 S6 kinase in osteoblasts still remains unclear.

In this study, we investigated the involvement of p70 S6 kinase in the FGF-2–stimulated VEGF release in osteoblast-like MC3T3-E1 cells. We show that p70 S6 kinase activated by FGF-2 negatively regulates VEGF release through SAPK/JNK in these cells.

MATERIALS AND METHODS

Materials

FGF-2 and mouse VEGF enzyme immunoassay kits were purchased from R&D Systems (Minneapolis, MN, USA). Rapamycin and SP600125 were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA, USA). Phospho-specific p70 S6 kinase antibodies, p70 S6 kinase antibodies, phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies, and SAPK/JNK antibodies were purchased from Cell Signaling (Beverly, MA, USA). ECL Western blotting detection system was purchased from Amersham Biosciences (Piscataway, NJ, USA). Control short interfering RNA (siRNA; Silencer Negative Control no. 1 siRNA) or p70 S6 kinase siRNA (Silencer Predesigned siRNA, 75849, 75755, and 75942) was purchased from Ambion (Austin, TX, USA). siLentFect was pur-

chased from Bio-Rad (Hercules, CA, USA). Trizol reagent was purchased from Invitrogen (Carlsbad, CA, USA). Omniscript Reverse Transcriptase Kit was purchased from QIAGEN (Hilden, Germany). Fast-Start DNA Master SYBR Green I was purchased from Roche Diagnostics (Mannheim, Germany). Other materials and chemicals were obtained from commercial sources. Rapamycin or SP600125 was dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of DMSO was 0.1%, which did not affect the assay for VEGF or Western blot analysis.

Cell culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria[24] were maintained as previously described.[25] Briefly, the cells were cultured in α-MEM containing 10% FCS at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded into 35- (5×10^4) or 90-mm-diameter (5×10^5) dishes 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.

siRNA transfection

To knock down p70 S6 kinse in MC3T3-E1 cells, the cells were transfected with control siRNA (Silencer Negative Control no. 1 siRNA) or p70 S6 kinase siRNA (Silencer Predesigned siRNA, 75849, 75755, and 75942; Ambion) using the siLentFect (Bio-Rad) according to the manufacturer's protocol. In brief, the cells were seeded in a 35-mm-diameter (1×10^5) dish in α-MEM containing 10% FCS and subcultured for 48 h. After that, the cells were incubated at 37°C for 48 h with 250 nM siRNA-siLentFect complexes.

VEGF assay

The cultured cells were stimulated by various doses of FGF-2 in 1 ml of α-MEM containing 0.3% FCS for the indicated periods. When indicated, the cells were pretreated with rapamycin or SP600125 for 60 minutes. The conditioned medium was collected at the end of the incubation, and the VEGF concentration was measured by ELISA kit.

Western blot analysis

The cultured cells were stimulated by FGF-2 in α-MEM containing 0.3% FCS for the indicated periods. The cells were washed twice with PBS, lysed, homogenized, and sonicated in a lysis buffer containing 62.5 mM Tris/HCl, pH 6.8, 2% SDS, 50 mM dithiothreitol, and 10% glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at 125,000*g* for 10 minutes at 4°C. SDS-PAGE was performed according to Laemmli[26] in 10% polyacrylamide gel. Western blotting analysis was performed as described previously[27] using phospho-specific p70 S6 kinase antibodies, p70 S6 kinase antibodies, phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies, or SAPK/JNK antibodies, with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second

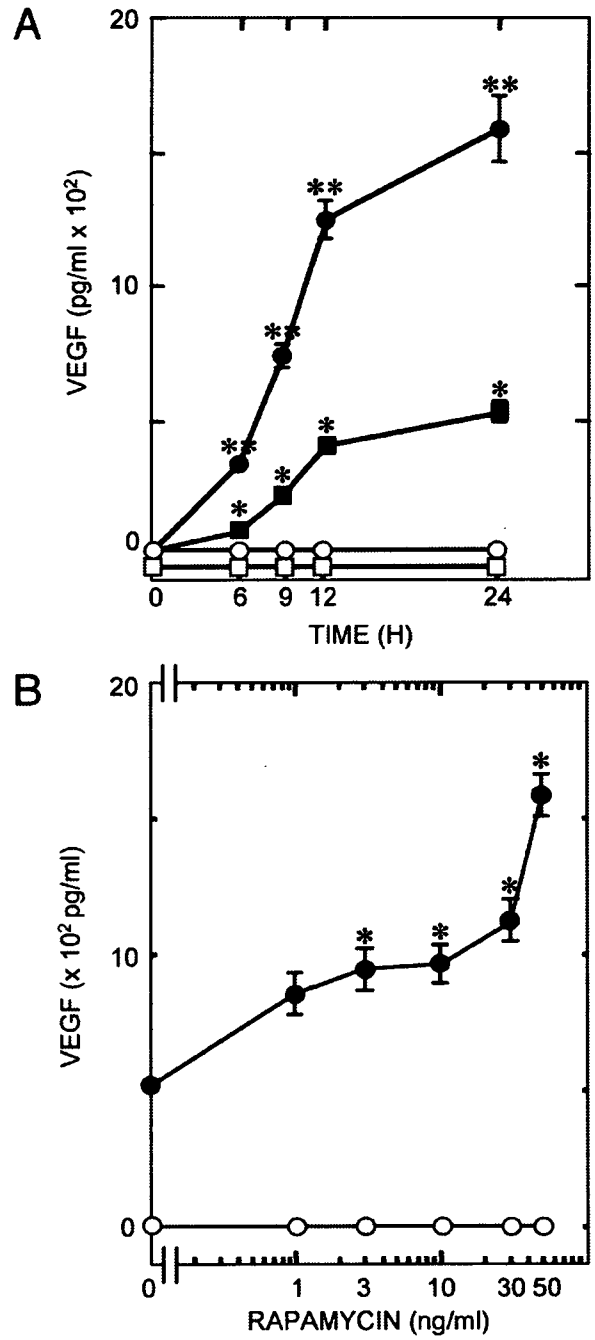

FIG. 2. Effect of rapamycin on the FGF-2–stimulated VEGF release in MC3T3-E1 cells. (A) The cultured cells were pretreated with 10 ng/ml rapamycin (circle symbols) or vehicle (square symbols) for 60 minutes and stimulated by 70 ng/ml FGF-2 (solid symbols) or vehicle (open symbols) for the indicated periods. $*p < 0.05$ compared with the control. $**p < 0.05$ compared with the value of FGF-2 alone. (B) The cultured cells were pretreated with various doses of rapamycin for 60 minutes and stimulated by 70 ng/ml FGF-2 (●) or vehicle (○) for 24 h. $*p < 0.05$ compared with the value of FGF-2 alone. Each value represents the mean ± SE of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

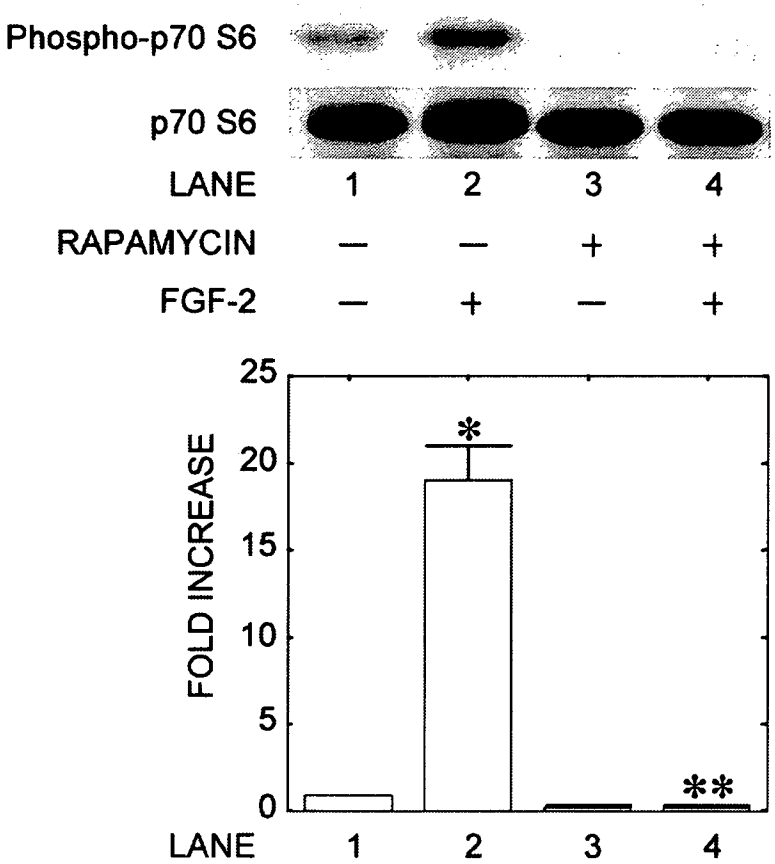

FIG. 3. Effect of rapamycin on the FGF-2–induced phosphorylation of p70 S6 kinase in MC3T3-E1 cells. The cultured cells were pretreated with 10 ng/ml rapamycin or vehicle for 60 minutes and stimulated by 30 ng/ml FGF-2 or vehicle for 10 minutes. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p70 S6 kinase or p70 S6 kinase. The histogram shows quantitative representations of the levels of FGF-2–induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SE of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $*p < 0.05$ compared with the control. $**p < 0.05$ compared with the value of FGF-2 alone.

antibodies. Peroxidase activity on the polyvinylidene difluoride (PVDF) sheet was visualized on X-ray film by means of the electrochemiluminescence (ECL) Western blotting detection system.

Determination

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

Real-time RT-PCR

The cultured cells were pretreated with rapamycin and/or SP600125 and stimulated by FGF-2 for the indicated time period. Total RNA was isolated and transcribed into cDNA using Trizol reagent and Omniscript Reverse Transcriptase Kit. Real-time PCR was performed using a Light Cycler system (Roche Diagnostics) in capillaries and Fast-Start DNA Master SYBR Green I provided with the kit. Sense and antisense primers were synthesized based on the report of Simpson et al.[28] for mouse VEGF mRNA and GAPDH mRNA. The amplified products were determined by melting curve analysis and agarose electrophoresis. VEGF mRNA levels were normalized with those of GAPDH mRNA.

Statistical analysis

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 the mean ± SE of triplicate determinations. Each experiment was repeated three times with similar results.

RESULTS

Effect of FGF-2 on the phosphorylation of p70 S6 kinase in MC3T3-E1 cells

To study whether FGF-2 activates p70 S6 kinase in osteoblast-like MC3T3-E1 cells, we examined the effect of FGF-2 on the phosphorylation of p70 S6 kinase. The stimulation of FGF-2 time-dependently induced the phosphorylation of p70 S6 kinase (Fig. 1). The maximum effect was observed at 20 minutes after the stimulation of FGF-2.

Effect of rapamycin on the FGF-2–stimulated VEGF release in MC3T3-E1 cells

To clarify whether p70 S6 kinase is involved in the FGF-2–induced release of VEGF in MC3T3-E1 cells, we examined the effect of rapamycin, a specific inhibitor of p70 S6

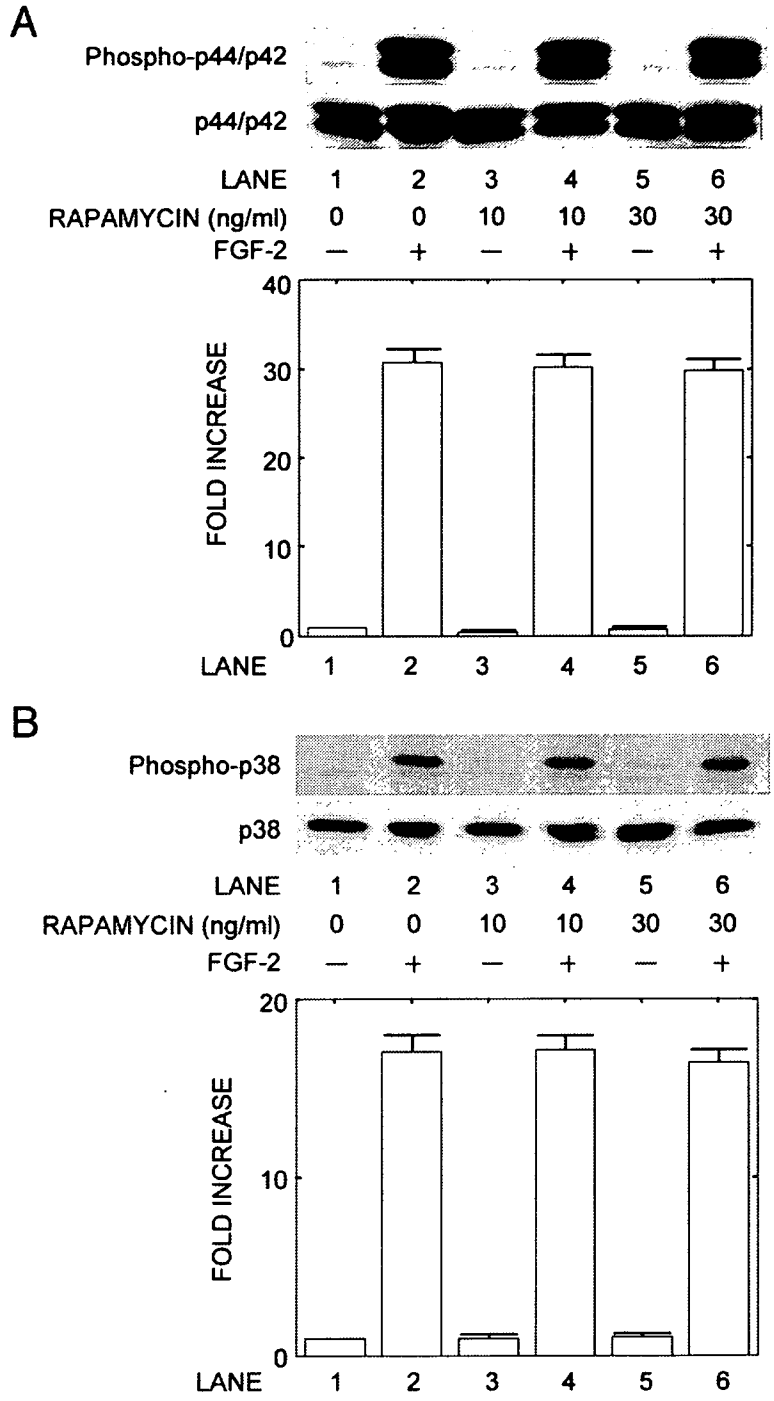

FIG. 4. Effects of rapamycin on the FGF-2–induced phosphorylation of p44/p42 MAP kinase or p38 MAP kinase in MC3T3-E1 cells. (A) The cultured cells were pretreated with various doses of rapamycin for 60 minutes and stimulated by 30 ng/ml FGF-2 or vehicle for 20 minutes. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p44/p42 MAP kinase or p44/p42 MAP kinase. (B) The cultured cells were pretreated with various doses of rapamycin for 60 minutes and stimulated by 30 ng/ml FGF-2 or vehicle for 10 minutes. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. The histogram shows quantitative representations of the levels of FGF-2–induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SE of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

kinase,[29,30] on the FGF-2–induced release of VEGF. Rapamycin, which alone did not affect the basal levels of VEGF, significantly enhanced the FGF-2–induced release of VEGF in a time-dependent manner (Fig. 2A). The amplifying effect of rapamycin was dose dependent in a range between 1 and 50 ng/ml (Fig. 2B). Rapamycin at 50 ng/ml caused ~200% enhancement in the FGF-2 effect.

Effect of rapamycin on the FGF-2–stimulated VEGF mRNA expression in MC3T3-E1 cells

To clarify whether the enhancement of FGF-2–stimulated VEGF release by rapamycin is mediated through transcriptional events, we examined the effect of rapamycin on the FGF-2–induced VEGF mRNA expres-

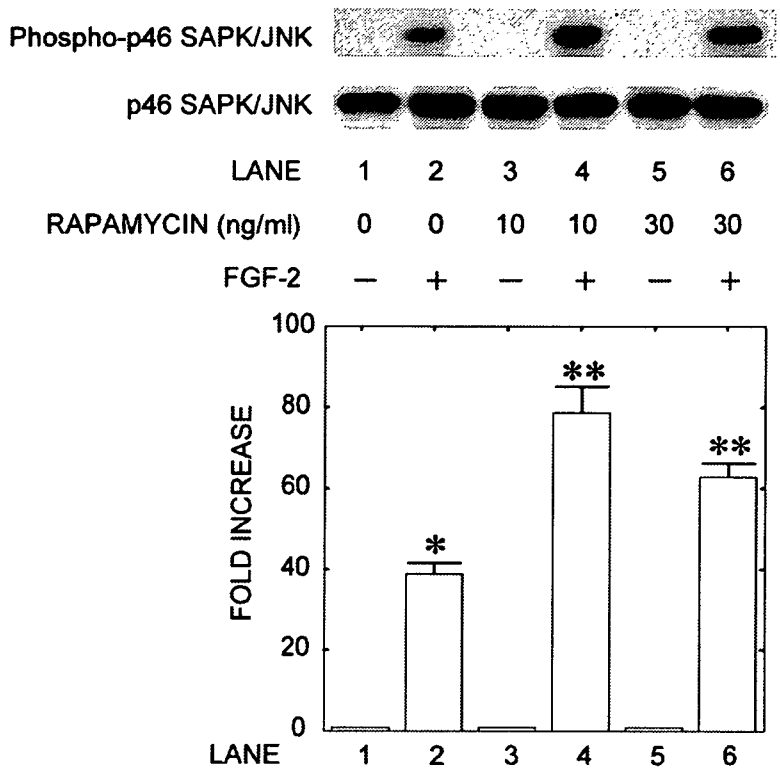

FIG. 5. Effect of rapamycin on the FGF-2–induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with various doses of rapamycin for 60 minutes and stimulated by 30 ng/ml FGF-2 or vehicle for 20 minutes. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the levels of FGF-2-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SE of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$p < 0.05$ compared with the control. **$p < 0.05$ compared with the value of FGF-2 alone.

TABLE 1. EFFECT OF SP600125 ON THE ENHANCEMENT BY RAPAMYCIN OF THE FGF-2–STIMULATED VEGF RELEASE IN MC3T3-E1 CELLS

SP600125	*Rapamycin*	*FGF-2*	*VEGF (pg/ml)*
–	–	–	<7.8
–	–	+	523 ± 40*
–	+	–	<7.8
–	+	+	1586 ± 121†
+	–	–	<7.8
+	–	+	111 ± 12†
+	+	–	<7.8
±	±	±	120 ± 15‡

The cultured cells were pretreated with 50 μM SP600125 or vehicle for 60 minutes and incubated with 50 ng/ml rapamycin or vehicle for 60 minutes. The cells were stimulated by 70 ng/ml FGF-2 or vehicle for 24 h thereafter. Each value represents the mean ± SE of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

*$p < 0.05$ compared with the control.

†$p < 0.05$ compared with the value of FGF-2 alone.

‡$p < 0.05$ compared with the value of FGF-2 with rapamycin pretreatment.

sion by real-time PCR. We found that rapamycin significantly upregulated the FGF-2–induced VEGF mRNA expression 6 h after stimulation (3.5 ± 0.3-fold for 70 ng/ml FGF-2 alone compared with the control; 5.2 ± 0.3-fold for 70 ng/ml FGF-2 with 50 ng/ml rapamycin compared the control). These results suggest that the amplifying effect of rapamycin is mediated, at least in part, by upregulation of VEGF mRNA expression in osteoblast-like MC3T3-E1 cells.

Effects of rapamycin on the FGF-2–induced phosphorylation of p70 S6 kinase in MC3T3-E1 cells

We next examined the effect of rapamycin on the FGF-2–induced phosphorylation of p70 S6 kinase. Rapamycin almost completely attenuated the FGF-2–induced phosphorylation of p70 S6 kinase in a range between 10 and 50 ng/ml (Fig. 3).

Effects of rapamycin on the FGF-2–induced phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, or SAPK/JNK in MC3T3-E1 cells

To study whether the amplifying effect of rapamycin on the FGF-2–stimulated VEGF release was through activation of p44/p42 MAP kinase, p38 MAP kinase, or SAPK/JNK in MC3T3-E1 cells, we examined the effect of rapamycin on the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, or SAPK/JNK induced by FGF-2. Rapamycin failed to affect the FGF-2–induced phosphorylation of p44/p42 MAP kinase (Fig. 4A) or p38 MAP kinase (Fig. 4B). However, the FGF-2–induced phosphorylation of SAPK/JNK was markedly enhanced by rapamycin (Fig. 5). Rapamycin at 10 ng/ml caused ~100% enhancement in the FGF-2 effect.

Effects of SP600125 on the amplification by rapamycin of the FGF-2–induced VEGF release and phosphorylation of SAPK/JNK in MC3T3-E1 cells

SP600125, a specific SAPK/JNK inhibitor,[31] which by itself did not affect the basal levels of VEGF, significantly

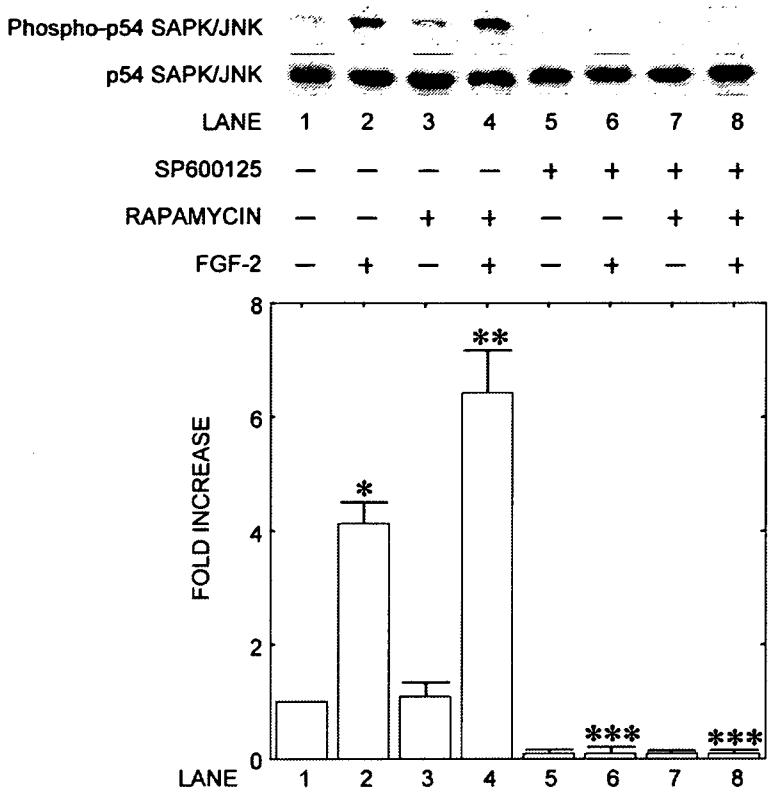

FIG. 6. Effect of SP600125 on the enhancement by rapamycin of the FGF-2–induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with 50 μM SP600125 or vehicle for 60 minutes and incubated by 30 ng/ml rapamycin or vehicle for 60 minutes. The cells were stimulated by 30 ng/ml FGF-2 or vehicle for 20 minutes. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the levels of FGF-2–induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SE of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$p < 0.05$ compared with the control. **$p < 0.05$ compared with the value of FGF-2 alone. ***$p < 0.05$ compared with the value of FGF-2 with rapamycin pretreatment.

reduced the enhancement by rapamycin of FGF-2–induced VEGF release (Table 1). The enhanced levels by rapamycin of FGF-2–induced VEGF release were reduced by SP600125 similar to the levels by FGF-2 with SP600125 treatment. In addition, SP600125 almost completely suppressed both the FGF-2–induced phosphorylation of SAPK/JNK and its enhancement by rapamycin (Fig. 6).

Effects of SP600125 on the upregulation by rapamycin of the FGF-2–stimulated VEGF mRNA expression in MC3T3-E1 cells

To clarify whether the suppressive effect by SP600125 of FGF-2–stimulated VEGF release is mediated through transcriptional events, we examined the effect of SP600125 in the presence or absence of rapamycin on the FGF-2–stimulated VEGF mRNA expression by real-time PCR. We found that SP600125 significantly downregulated the FGF-2–induced VEGF mRNA expression 2 h after stimulation (5.0 ± 0.9-fold for 70 ng/ml FGF-2 alone compared with the control; 3.2 ± 0.4-fold for 70 ng/ml FGF-2 with 10 μM SP600125 compared with the control; 3.0 ± 0.3-fold for 70 ng/ml FGF-2 with 10 μM SP600125 and 50 ng/ml rapamycin compared with the control). These results suggest that the suppressive effect of SP600125 is mediated, at least in part, by downregulation of VEGF mRNA expression in osteoblast-like MC3T3-E1 cells.

Effect of p70 S6 kinase-siRNA on the FGF-2–stimulated VEGF release in MC3T3-E1 cells

To confirm the results from rapamycin, we examined the effects of p70 S6 kinase downregulation by p70 S6 kinase siRNA on the VEGF release and the phosphorylation of SAPK/JNK induced by FGF-2 in osteoblast-like MC3T3-E1 cells. We found that p70 S6 kinase siRNA (250 nM) successfully reduced the p70 S6 kinase levels compared with those of control siRNA determined by the band intensity of Western blot analysis (Fig. 7A). The FGF-2–induced levels of VEGF release in p70 S6 kinase–downregulated cells were truly enhanced compared with those in control siRNA-transfected cells. Downregulation of p70 S6 kinase caused ~180% enhancement in the FGF-2 effect (Table 2). FGF-2 significantly enhanced the phosphorylation levels of SAPK/JNK in the p70 S6 kinase–downregulated cells (Fig. 7B).

DISCUSSION

In this study, we found that FGF-2 time-dependently induced the phosphorylation of p70 S6 kinase in osteoblast-like MC3T3-E1 cells, using phospho-specific p70 S6 kinase (Thr389) antibodies. It is generally recognized that the activity of p70 S6 kinase is regulated by multiple phosphorylation events.[20] It has been shown that phosphorylation at Thr389 among the phosphorylation most strongly correlates with p70 S6 kinase activity.[20] Thus, it is probable that

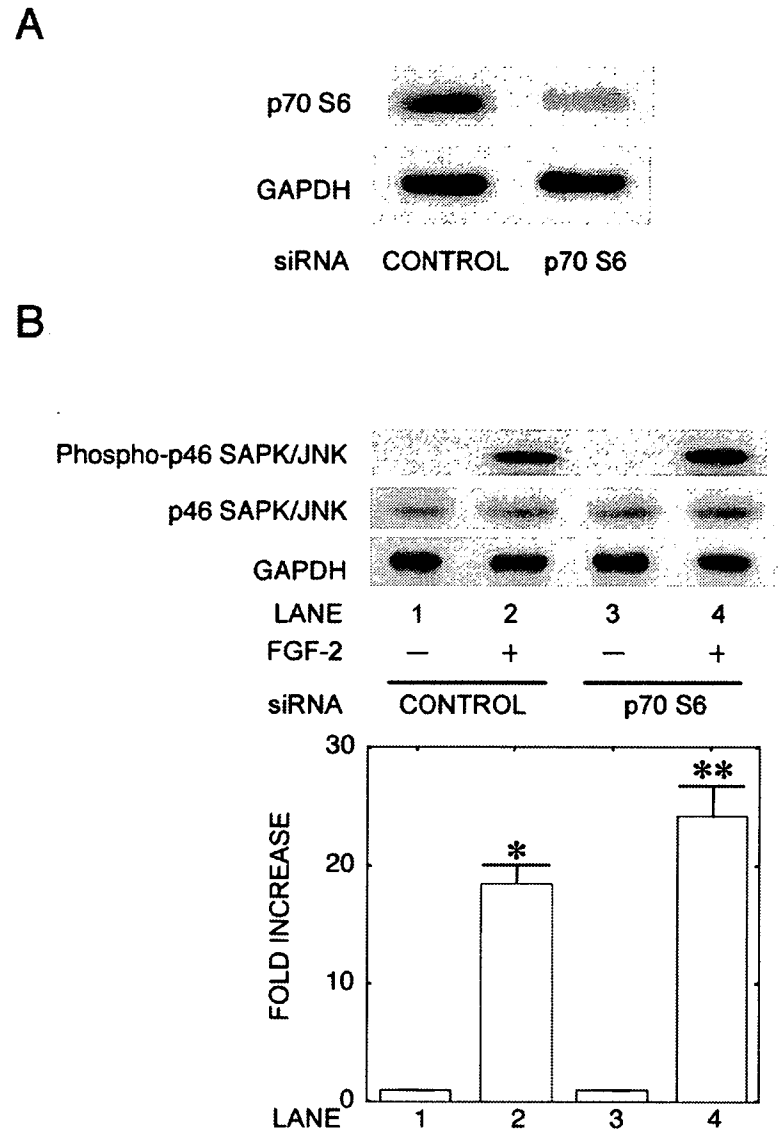

FIG. 7. Effect of p70 S6 kinase-siRNA in MC3T3-E1 cells and effect of p70 S6 kinase downregulation on the FGF-2–induced-phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cells were transfected with 250 nM of control siRNA or p70 S6 kinase siRNA by using the siLentFect. (A) Cells were incubated at 37°C for 48 h with siRNA-siLentFect complexes and subsequently harvested for preparation of Western blot analysis. (B) The cultured cells transfected with control siRNA or p70 S6 kinase siRNA were stimulated by 70 ng/ml FGF-2 or vehicle for 20 minutes and subsequently harvested for preparation of Western blot analysis. The histogram shows quantitative representations of the levels of FGF-2–induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SE of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $*p < 0.05$ compared with the value of vehicle. $**p < 0.05$ compared with the value of FGF-2 with control siRNA transfection.

FGF-2 induces the activation of p70 S6 kinase in osteoblast-like MC3T3-E1 cells. To the best of our knowledge, this is probably the first report showing the FGF-2–induced p70 S6 kinase activation in osteoblasts.

We have previously reported that FGF-2 stimulates the release of VEGF in osteoblast-like MC3T3-E1 cells.[16] Therefore, we studied whether p70 S6 kinase functions in FGF-2–stimulated VEGF release in osteoblast-like MC3T3-E1 cells. Rapamycin, a specific inhibitor of p70 S6 kinase,[29,30] markedly enhanced the FGF-2–stimulated release of VEGF and expression of VEGF mRNA in MC3T3-E1 cells. We confirmed that the FGF-2–induced phosphorylation of p70 S6 kinase was truly attenuated by rapamycin. In addition, downregulation of p70 S6 kinase by siRNA markedly enhanced FGF-2–stimulated VEGF release in these cells. These results strongly suggest that FGF-2–stimulated VEGF release is reduced by the activation of p70 S6 kinase. Therefore, it is probable that FGF-2 activates the p70 S6 kinase pathway, resulting in negatively regulating the release of VEGF. We speculate that p70 S6 kinase signaling activated by FGF-2 limits the FGF-2–induced over-release of VEGF in osteoblast-like MC3T3-E1 cells. As far as we know, this finding is the first report to show that the activation of p70 S6 kinase leads to negative feedback regulation of VEGF release in osteoblasts.

We have previously shown that FGF-2 induces the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK in MC3T3-E1 cells.[16,17] It is generally recognized that the MAP kinase superfamily mediates intracellular signaling of extracellular agonists and plays a crucial role in cellular functions including proliferation, differentiation, and apoptosis in a variety of cells.[18] Three major MAP kinases, p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK, are known as central elements used by mam-

TABLE 2. EFFECT OF p70 S6 KINASE (S6K) siRNA ON THE FGF-2-STIMULATED VEGF RELEASE IN MC3T3-E1 CELLS

siRNA	*FGF-2*	*Time (h)*	*VEGF (pg/ml)*
Control	–	4	<7.8
Control	+	4	244 ± 32*
S6K	–	4	<7.8
S6K	+	4	558 ± 73†
Control	–	9	<7.8
Control	+	9	681 ± 106*
S6K	–	9	<7.8
S6K	+	9	1269 ± 226†

The cultured cells were transfected with control siRNA or p70 S6 kinase siRNA by using the siLentFect according to the manufacturer's protocol. The cells were stimulated by 70 ng/ml FGF-2 or vehicle for the indicated time periods. Each value represents the mean ± SE of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

* $p < 0.05$ compared with the value of vehicle.

† $p < 0.05$ compared with the value of FGF-2 with control siRNA transfection.

malian cells to transducer the diverse messages.[18] In our previous studies,[16,17] we showed that p44/p42 MAP kinase and SAPK/JNK act as positive regulators in FGF-2–induced VEGF release. On the other hand, FGF-2–activated p38 MAP kinase negatively regulates VEGF release. These findings lead us to speculate that there is cross-talk regulation between p70 S6 kinase and these MAP kinases in FGF-2–stimulated VEGF release in these cells. However, rapamycin[29,30] failed to affect the phosphorylation of p44/p42 MAP kinase and p38 MAP kinase. Therefore, it seems unlikely that the p70 S6 kinase signaling pathway affects FGF-2–stimulated release of VEGF through the amplification of activities of p44/p42 MAP kinase and p38 MAP kinase in osteoblast-like MC3T3-E1 cells. On the contrary, we showed that the phosphorylation levels of FGF-2–induced SAPK/JNK were strengthened by rapamycin and p70 S6 kinase siRNA transfection. In addition, the amplification by rapamycin of the FGF-2–stimulated VEGF release and mRNA expression was suppressed by SP600125, a specific inhibitor of SAPK/JNK,[31] similar to the levels of FGF-2 with SP600125. Furthermore, we showed that SP600125 almost completely reduced the rapamycin-enhanced phosphorylation of SAPK/JNK and FGF-2–induced phosphorylation of SAPK/JNK. Taking our findings into account, it is probable that the p70 S6 kinase signal pathway affects the FGF-2–stimulated VEGF release through upregulation of SAPK/JNK in osteoblast-like MC3T3-E1 cells.

The p70 S6 kinase pathway is recognized to play a crucial role in various cellular functions, especially cell cycle progression.[20] These results indicate that the p70 S6 kinase pathway in osteoblasts has an important role in the control of the production of VEGF, one of the key regulators of bone metabolism. Bone remodeling carried out by osteoclasts and osteoblasts is accompanied by angiogenesis and capillary outgrowth.[10] Because VEGF is a specific mitogen of vascular endothelial cells,[11] these results lead us to speculate that FGF-2–activated p70 S6 kinase signaling acts as a negative regulator of the microvasculature development in bone. Thus, the p70 S6 kinase pathway in osteoblasts might be considered to be a new candidate as a molecular target of bone resorption concurrent with various bone diseases. On the contrary, we have previously shown that p70 S6 kinase acts as a positive regulator in bone morphogenetic protein-4–stimulated release of VEGF in MC3T3-E1 cells.[22] The physiological significance of a regulatory mechanism by p70 S6 kinase in osteoblasts still remains unclear. Further study is required to clarify the exact roles of p70 S6 kinase in osteoblasts and bone metabolism.

In conclusion, these results strongly suggest that p70 S6 kinase plays an important role in the regulation of FGF-2–stimulated VEGF release in osteoblasts and may serve as a negative feedback mechanism to prevent oversynthesizing of VEGF through SAPK/JNK in these cells.

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Protein kinase C δ regulates the phosphorylation of heat shock protein 27 in human hepatocellular carcinoma

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Abstract

We have recently reported that attenuated phosphorylation of heat shock protein (HSP) 27 correlates with tumor progression in patients with hepatocellular carcinoma (HCC). In the present study, we investigated what kind of kinase regulates phosphorylation of HSP27 in human HCC-derived HuH7 cells. 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and 1-oleoyl-2-acetylglycerol, direct activators of protein kinase C (PKC), markedly strengthened the phosphorylation of HSP27. Bisindorylmaleimide I, an inhibitor of PKC, suppressed the TPA-induced levels of HSP27 phosphorylation in addition to its basal levels. Knock down of PKCδ suppressed HSP27 phosphorylation, as well as p38 mitogen-activated protein kinase (MAPK) phosphorylation. SB203580, an inhibitor of p38 MAPK, suppressed the TPA-induced HSP27 phosphorylation. Our results strongly suggest that activation of PKCδ regulates the phosphorylation of HSP27 via p38 MAPK in human HCC.
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Keywords: PKCδ; HSP27; Phosphorylation; Protein kinase; Hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is characterized by its high incidence in hepatitis virus-associated liver disease, reaching approximately 3% in hepatitis B virus-infected cirrhotic patients and 7% in hepatitis C virus-infected cirrhotic patients (Ikeda et al., 1993; Shiratori et al., 2001). Moreover, the incidence of post-therapeutic recurrence is approximately 20% to 25% a year in cirrhotic patients who have already undergone curative treatment of the primary HCC (Kumada et al., 1997; Koda et al., 2000). Thus, overall survival of patients with HCC is still unsatisfactory even after hepatectomy. Therefore, it is required to clarify the further exact mechanism of HCC carcinogenesis.

Heat shock proteins (HSP) are produced by cells exposed to biological stressors such as heat and chemicals (Shimada et al., 1998). HSPs are classified as high-molecular-weight HSPs, such as HSP70, HSP90, and HSP110, or low-molecular-weight HSPs, which have molecular masses from 10 to 30 kDa. High-molecular-weight HSPs act as molecular chaperones in protein folding, oligomerization, and translocation (Benjamin and McMillan, 1998). Though the functions of low-molecular-weight HSPs, such as HSP27 and αB-crystallin, are not as well-characterized as those of the high-molecular-weight HSPs, it is thought that they may also have chaperone functions (Benjamin and McMillan, 1998). It is recognized that HSP27 activity is regulated by post-translational modifications such as phosphorylation (Welch, 1985; Benjamin and McMillan, 1998). Mouse HSP27 is phosphorylated at two serine residues (Ser-15 and Ser-82), whereas human HSP27 is phosphorylated at three serine residues (Ser-15, Ser-78, and Ser-82) (Benjamin and McMillan, 1998). It was also reported that phosphorylated HSP27 is translocated from the cytosol to the nucleus in hippocampal progenitor cells, and prevents apoptosis (Geum et al., 2002). In a recent study (Yasuda et al., 2005), we have shown

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that the levels of phosphorylated HSP27 were correlated inversely with tumor stage by TNM classification in patients with HCC. It has been reported that HSP27 phosphorylation is catalyzed by the mitogen-activated protein kinase (MAPK) superfamily (p38 MAPK, phospho-stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), and p44/p42 MAPK) (Kyriakis and Avruch, 1996; Guay et al., 1997; Benjamin and McMillan, 1998). In addition, it has been reported that p38 MAPK and p44/p42 MAPK are activated in HCC and contribute to the acceleration of the cell cycle (Ito et al., 1998; Iyoda et al., 2003).

Protein kinase C (PKC) is reportedly an upstream regulator of MAPK superfamily cascade (Noguchi et al., 1993; Tanaka et al., 2003; Tokuda et al., 2003). PKC is a Ser/Thr protein kinase family with multiple isoforms, its isoforms have been classified into three groups, classical PKC (α, β, γ), novel PKC (δ, ε, η, θ), and atypical PKC (ζ, ι/λ) (Saito et al., 2001). To date, these PKC isotypes are believed to play distinct regulatory roles. Regarding about the low-molecular-weight HSPs, PKC-dependent phosphorylation of low-molecular-weight HSPs by phorbol-esters has been previously described in Hela cells and MCF-7 cells (Arrigo, 1990; Faucher et al., 1993). In addition, it has been demonstrated that Ca^{2+}-independent PKCδ is superior in its ability to phosphorylate low-molecular-weight HSPs compared with a panel of other PKC isoforms in vitro (Maizels et al., 1998). Furthermore, the detection of phosphorylated low-molecular-weight HSPs in the rat corpora lutea of late pregnancy is reportedly associated with abundant and activated PKCδ (Maizels et al., 1998). However, the kinases that regulate phosphorylation of HSP27 in human HCC have not yet been clarified. In the present study, we investigated what kind of kinase regulates phosphorylation of HSP27 in human HCC. Our results strongly suggest that activation of PKCδ regulates the phosphorylation of HSP27 via p38 MAPK in human HCC.

Materials and methods

Materials

12-*O*-tetradecanoylphorbol-13-acetate (TPA) was purchased from Sigma Chemical Co. (St. Louis, MO). 1-Oleoyl-2-acetylglycerol (OAG) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). PD98059, bisindolylmaleimide I, and SB203580 were obtained from Calbiochem-Novabiochem (La Jolla, CA). HSP27 antibodies, phospho-HSP27 (Ser-15) antibodies, and phospho-HSP27 (Ser-78) antibodies were purchased from Stressgen Biotechnologies. (Victoria, British Columbia, Canada). Phospho-HSP27 (Ser-82) was purchased from Biomol Research Laboratories. (Plymouth Meeting, PA). β-actin antibodies were purchased from Sigma. Phospho-Raf-1 antibodies, phospho-MEK1/2 antibodies, phospho-p44/p42 MAPK antibodies and p44/p42 MAP kinase antibodies, phospho-p38 MAPK antibodies, p38 MAPK antibodies, phospho-SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-PKC (pan) (βII Ser-660) antibodies, phospho-PKCδ (Thr-505) antibodies, PKCδ antibodies, and phospho-PKCθ (Thr-538) antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). ECL Western blot detection system was purchased from Amersham Japan (Tokyo, Japan). The PKCδ siRNA (Silencer® Validated siRNA), PKCε siRNA (Silencer® Pre-designed siRNA) and non-specific control siRNA (Silencer® Negative control #1 siRNA) were obtained from Ambion, Inc. (Austin, TX). siLentFect was obtained from Bio-Rad Laboratories, Inc. (Hercules, CA). Other materials and chemicals were obtained from commercial sources. TPA, PD98059, bisindolylmaleimide I, and SB203580 were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect assay for HSP27 phosphorylation.

Cell culture

Human HCC-derived HuH7, which were originated from well-differentiated HCC tissues, were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). HuH7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) 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 in DMEM containing 10% FCS. After 7 days, the medium was exchanged for FCS-free DMEM. The cells were immediately used for experiments. When indicated, the cells were pre-treated with respective inhibitors and then stimulated with TPA or OAG for the indicated periods. Cell viability was estimated by the trypan blue dye exclusion method. Experiments were performed in triplicate.

Western blotting analysis

The cultured cells were washed twice with phosphate-buffered saline. The cultured cells were then lysed, homogenized and sonicated in 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. The linear range of loading volume in Western blotting analysis was tested with serially diluted protein samples. Protein samples (10 μg) were loaded equally to SDS-polyacrylamide gel electrophoresis (PAGE) in respective experiments (except for total HSP27). For the detection of total HSP27, 2.5 μg of proteins were subjected to the each well of the gel. SDS-PAGE was performed by Laemmli (1970) in polyacrylamide gel. Western blotting analysis was performed as described previously (Kato et al., 1996). Band intensities visualized on X-ray film were determined by integrating the optical density over the band area (band volume) with NIH image software.

siRNA protocol

Transfection was performed according to the manufacturer's protocol (Bio-Rad). Briefly, 5 μl of siLentFect and finally 10 nM siRNA were diluted with FCS-free DMEM, pre-incubated at room temperature for 20 min and then added to the culture medium containing 10% FCS. Cells were incubated at 37 °C for 48 h with siRNA–siLentFect complexes and subsequently harvested for preparation of Western blotting analysis.

Statistical analysis

The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between pairs, and a $p < 0.05$ was considered significant. All data are presented as the mean+S.E. of triplicate determinations. Each experiment was repeated three times with similar results.

Results

Comparisons between phosphorylated levels of p44/p42 MAPK and HSP27 in HuH7 cells

It is recognized that HSP27 phosphorylation is catalyzed by the MAP kinase superfamily (p38 MAPK, SAPK/JNK, and p44/p42 MAPK) (Kyriakis and Avruch, 1996; Guay et al., 1997; Benjamin and McMillan, 1998). It has been reported that p44/p42 MAPK is constantly activated in HCC (Ito et al., 1998). Therefore, we first examined the relationship between p44/p42 MAPK and HSP27 phosphorylation in HuH7 cells. The expression of HSP27 and its phosphorylated form (Ser-78 and Ser-82) were detectable in HuH7 cells (Fig. 1A). In addition, p44/p42 MAPK were constitutively phosphorylated in HuH7 cells (Fig. 1B). To elucidate whether p44/p42 MAPK is involved in the phosphorylation of HSP27 in HuH7 cells, we examined the effect of PD98059, a specific inhibitor of MEK1/2 (Alessi et al., 1995), on the phosphorylated levels of HSP27. Though PD98059 suppressed the phosphorylation of p44/p42 MAPK dose dependently in the range between 10 and 50 μM, the levels of HSP27 phosphorylation were not affected (Fig. 1C).

Effect of PKC activation on the HSP27 phosphorylation in HuH7 cells

It is well-recognized that PKC is an upstream regulator of Raf-1-MEK1/2-p44/p42 MAPK cascade (Noguchi et al., 1993). Thus, we investigated whether PKC is activated in HuH7 cells. PKC activity is controlled by three distinct phosphorylation events (specifically, the threonine 500 in the activation loop, the threonine 641 autophosphorylation site, and the serine 660 hydrophobic site at the carboxy terminus of PKCβII are phosphorylated in vivo) (Keranen et al., 1995). Since we have preliminary confirmed that phospho-PKC (pan) (βII Ser-660) antibodies can detect PKCα/β and PKCε by using the respective antibodies, we used phospho-PKC (pan) (βII Ser-660) antibodies to detect them. PKCα/β and PKCδ were markedly phosphorylated in HuH7 cells (Fig. 2A). To elucidate whether PKC is involved in the phosphorylation of HSP27 in these cells, we examined the effect of bisindolylmaleimide I, an inhibitor of classical PKC and novel PKC (Toullec et al., 1991), on the basal levels of HSP27 phosphorylation. Bisindolylmaleimide I decreased the phosphorylated levels of HSP27 dose dependently in the range between 10 and 50 μM (Fig. 2B). It is well-known that both classical and novel PKC are activated by phorbol-esters such as TPA (Nishizuka, 1991). We next investigated the effect of TPA (Nishizuka, 1991), a direct activator of PKC on the phosphorylated levels of HSP27 in HuH7 cells.

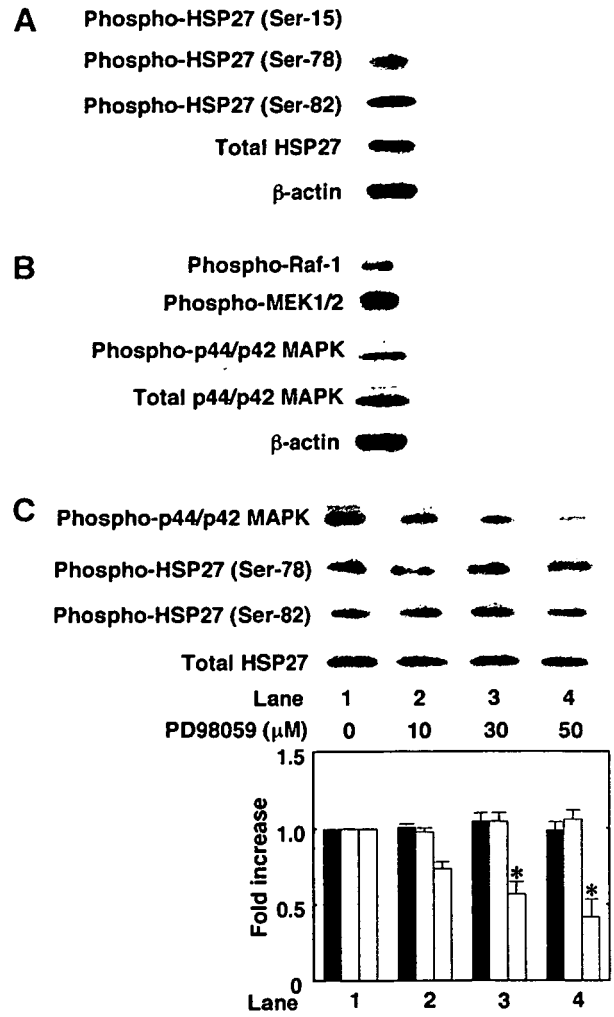

Fig. 1. The levels of HSP27 phosphorylation and the phosphorylated levels of Raf-1-MEK1/2-p44/p42 MAPK cascade, and effect of PD98059 on HSP27 phosphorylation in HuH7 cells. HuH7 were cultured in DMEM containing 10% FCS for 7 days. After 7 days, the medium was exchanged for FCS-free DMEM. The cells were immediately used for experiments. (A) The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-HSP27 (Ser-15), phospho-HSP27 (Ser-78), phospho-HSP27 (Ser-82), total HSP27 and β-actin. (B) The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-Raf-1, phospho-MEK1/2, phospho-p44/p42 MAPK, total p44/p42 MAPK and β-actin. (C) The cultured cells were pre-treated with various doses of PD98059 for 60 min, and then washed twice and collected. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-p44/p42 MAPK, phospho-HSP27 (Ser-78), phospho-HSP27 (Ser-82) and total HSP27. The phosphorylated levels were normalized by the levels of total HSP27. The histogram shows the fold increase of levels of phospho-HSP27 (Ser-82) (black bars), phospho-HSP27 (Ser-78) (white bars) and phospho-p44/p42 MAPK (gray bars) in PD98059-treated cells versus those of PD98059-untreated cells. Each value represents the mean±S.E. of triplicate determinations from three independent experiments. Representative results from triplicate independent experiments with similar results are shown. $^*p<0.05$, compared to the value of control.

TPA significantly strengthened the phosphorylated levels of HSP27 in a time-dependent manner (Fig. 2C). The phosphorylated levels reached a peak at 60 min after the TPA-stimulation. TPA stimulated the phosphorylation of HSP27 dose dependently in the range between 0.01 and 0.1 μM, the maximum effect was

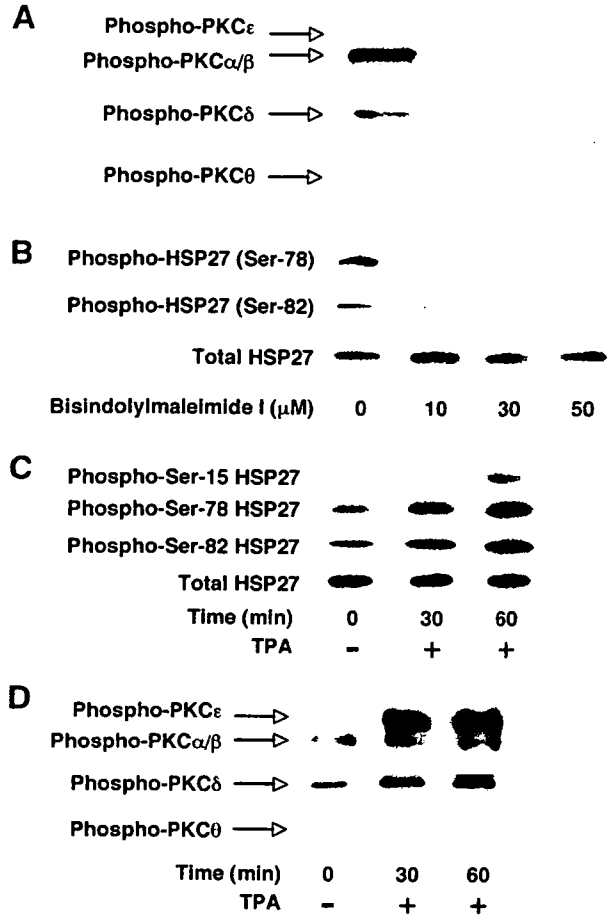

Fig. 2. Effect of bisindorylmaleimide I on HSP27 phosphorylation, and effects of TPA on HSP27 phosphorylation and PKC phosphorylation in HuH7 cells. HuH7 cells were cultured in DMEM containing 10% FCS for 7 days. After 7 days, the medium was exchanged for FCS-free DMEM. The cells were immediately used for experiments. (A) The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-PKC (pan) (βII Ser-660), phospho-PKCδ (Thr-505) and phospho-PKCθ (Thr-538). (B) The cultured cells were pre-treated with various doses of bisindorylmaleimide I for 60 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-HSP27 (Ser-78), phospho-HSP27 (Ser-82) and total HSP27. (C, D) The cultured cells were stimulated with 0.1 μM TPA for the indicated periods. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against (C) phospho-HSP27 (Ser-15), phospho-HSP27 (Ser-78), phospho-HSP27 (Ser-82) and total HSP27, and (D) phospho-PKC (pan) (βII Ser-660), phospho-PKCδ (Thr-505) and phospho-PKCθ (Thr-538). Representative results from triplicate independent experiments with similar results are shown.

observed at a dose of 0.1 μM (data not shown). In addition, TPA markedly enhanced the phosphorylation levels of novel PKC (δ, ε) in a time-dependent manner. On the contrary, the levels of both PKCα/β and PKCθ were not affected by TPA or were at least in part slightly enhanced by TPA (Fig. 2D).

Effects of bisindolylmaleimide I or PKC down-regulation on TPA-induced phosphorylation of HSP27 in HuH7 cells

We examined the effect of bisindolylmaleimide I on the TPA-induced phosphorylation of HSP27. Bisindolylmaleimide I (30 μM) suppressed the TPA-induced phosphorylation of HSP27 (Fig. 3A). TPA-induced novel PKC(δ, ε) phosphorylation was also suppressed by bisindolylmaleimide I (Fig. 3B).

It has been reported that treatment of TPA (0.1 μM) for 24 h down-regulates PKC (Blumberg, 1991). To clarify the role of PKC on the HSP27 phosphorylation in HuH7 cells, we examined the effect of TPA long term pre-treatment on the phosphorylation of HSP27. The effect of TPA on HSP27 phosphorylation was reduced in the PKC down-regulated cells compared with that in the cells without TPA treatment (Fig. 3C).

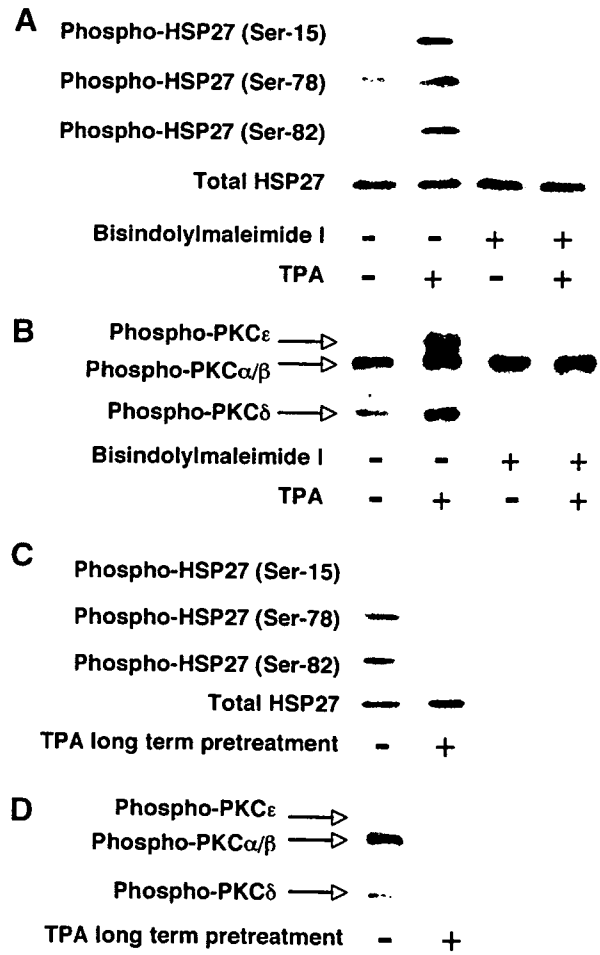

Fig. 3. Effects of bisindorylmaleimide I on TPA-induced HSP27 phosphorylation and PKC phosphorylation, and effect of PKC down-regulation on the phosphorylation of HSP27 in HuH7 cells. HuH7 cells were cultured in DMEM containing 10% FCS for 7 days. After 7 days, the medium was exchanged for FCS-free DMEM. The cells were immediately used for experiments. (A, B) The cultured cells were pre-treated with 30 μM of bisindorylmaleimide I or vehicle for 60 min and then, stimulated by 0.1 μM TPA or vehicle for 60 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against (A) phospho-HSP27 (Ser-15), phospho-HSP27 (Ser-78), phospho-HSP27 (Ser-82) and total HSP27, and (B) phospho-PKC (pan) (βII Ser-660) and phospho-PKCδ (Thr-505). (C, D) The cultured cells were pre-treated with 0.1 μM TPA or vehicle for 24 h. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against (C) phospho-HSP27 (Ser-15), phospho-HSP27 (Ser-78), phospho-HSP27 (Ser-82) and total HSP27, and (D) phospho-PKC (pan) (βII Ser-660) and phospho-PKCδ (Thr-505). Representative results from triplicate independent experiments with similar results are shown.