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PPAR-γ ligands up-regulate basic fibroblast growth factor-induced VEGF release through amplifying SAPK/JNK activation in osteoblasts

Eisuke Yasuda[a], Haruhiko Tokuda[a,b], Akira Ishisaki[a], Kouseki Hirade[c], Yosuke Kanno[a], Yoshiteru Hanai[b], Norimi Nakamura[b], Takahiro Noda[a], Yoshihiro Katagiri[c,d], Osamu Kozawa[a,*]

[a] *Department of Pharmacology, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan*
[b] *Department of Clinical Laboratory, National Hospital for Geriatric Medicine, National Center for Geriatrics and Gerontology, Obu, Aichi 474-8511, Japan*
[c] *Department of Pharmacy, Gifu University Hospital, Gifu 501-1194, Japan*
[d] *Department of Clinical Pharmacy, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan*

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Abstract

We previously reported that basic fibroblast growth factor (FGF-2) activates stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) and p44/p42 mitogen-activated protein (MAP) kinase resulting in the stimulation of vascular endothelial growth factor (VEGF) release in osteoblast-like MC3T3-E1 cells and that FGF-2-activated p38 MAP kinase negatively regulates the VEGF release. In the present study, we investigated the effects of ciglitazone and pioglitazone, peroxisome proliferator-activated receptor-γ (PPAR-γ) ligands, on the VEGF release by FGF-2 in MC3T3-E1 cells. The FGF-2-induced VEGF release was significantly enhanced by ciglitazone. The amplifying effect of ciglitazone was dose-dependent between 0.1 and 10 μM. Pioglitazone had a similar effect on the VEGF release. GW9662, an antagonist of PPAR-γ, reduced the effects of ciglitazone and pioglitazone. Ciglitazone or pioglitazone markedly enhanced the phosphorylation of SAPK/JNK induced by FGF-2 without affecting both the FGF-2-induced phosphorylation of p44/p42 MAP kinase and p38 MAP kinase. GW9662 markedly reduced the amplification by ciglitazone of the SAPK/JNK phosphorylation. Taken together, these results strongly suggest that PPAR-γ ligands up-regulate FGF-2-stimulated VEGF release resulting from amplifying activation of SAPK/JNK in osteoblasts.

Keywords: PPAR-γ; FGF-2; VEGF; MAP kinase; Osteoblast

Peroxisome proliferator-activated receptor-γ (PPAR-γ) is a member of the nuclear hormone receptor superfamily [1]. PPAR-γ is a ligand-activated transcription factor that binds to specific sequences in the promoters of target genes [1]. Mesenchymal cells differentiate into several types of cells such as adipocytes and osteoblasts. It is well known that PPAR-γ plays a pivotal role in the regulation of adipocyte differentiation. Bone metabolism is regulated by two functional cells, osteoblasts and osteoclasts, the former responsible for bone formation and the latter for bone resorption [2]. It has been shown that PPAR-γ is expressed in osteoblasts and the activation of PPAR-γ modulates osteoblast function [3–5]. However, the exact role of PPAR-γ in osteoblasts has not been fully clarified.

* Corresponding author. Fax: +81 58 230 6215.
E-mail address: okozawa@cc.gifu-u.ac.jp (O. Kozawa).

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Vascular endothelial growth factor (VEGF) has been characterized as a heparin-binding angiogenic growth factor displaying high specificity for vascular endothelial cells [6]. It is well recognized that VEGF, which is produced and secreted from a variety of cell types, increases capillary permeability and stimulates proliferation of endothelial cells [6]. 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 [7]. Accumulating evidence indicates that osteoblasts among bone cells produce and secrete VEGF in response to various humoral factors [6,8–10]. During bone remodeling, capillary endothelial cells provide the microvasculature, and osteoblasts and osteoprogenitor cells, which locally proliferate and differentiate into osteoblasts, migrate into the resorption lacuna. It is currently recognized that the activities of osteoblasts, osteoclasts, and capillary endothelial cells are closely coordinated via humoral factors as well as by direct cell-to-cell contact, and these cells cooperatively regulate bone metabolism [11]. Thus, there is no doubt that VEGF secreted from osteoblasts plays an important role in the regulation of bone metabolism. However, the mechanism behind VEGF synthesis in osteoblasts has not yet been fully clarified.

Basic fibroblast growth factor (FGF-2) is found in bone matrix, and osteoblasts synthesize FGF-2 [12,13]. FGF-2 expression in osteoblasts is detected during fracture repair [14]. Thus, it is recognized that FGF-2 plays a crucial role in fracture healing and bone remodeling. In our previous studies [15,16], we have previously reported that FGF-2 stimulates VEGF release in MC3T3-E1 cells, and that among the mitogen-activated protein (MAP) kinase superfamily [17], p44/p42 MAP kinase and stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK) participate at least partly in the VEGF release while p38 MAP kinase limits the VEGF release. In the present study, we investigated the effects of PPAR-γ ligands on the FGF-2-induced VEGF release in osteoblast-like MC3T3-E1 cells. We here show that PPAR-γ activation up-regulates FGF-2-stimulated VEGF release via enhancing SAPK/JNK activation in these cells.

Materials and methods

Materials. FGF-2 and mouse VEGF enzyme immunoassay kit were purchased from R&D Systems (Minneapolis, MN). Ciglitazone, pioglitazone, and GW9662 were obtained from Calbiochem–Novabiochem (La Jolla, CA). Phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-specific p38 MAP kinase antibodies, and p38 MAP kinase antibodies were purchased from New England BioLabs (Beverly, MA). ECL Western blotting detection system was purchased from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. Ciglitazone, pioglitazone, and GW9662 were dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of DMSO was 0.1%, which did not affect the assay for VEGF or the analysis of MAP kinases.

Cell culture. Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [18] were maintained as previously described [19]. 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 diameter dishes or 90-mm 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.

VEGF assay. The cultured cells were stimulated by FGF-2 in 1 ml α-MEM containing 0.3% FCS for the indicated periods. When indicated, the cells were pretreated with ciglitazone or pioglitazone for 8 h. The pretreatment of GW9662 was performed for 60 min before the addition of ciglitazone or pioglitazone. The reaction was terminated by collecting the medium, and VEGF in the medium was measured by a VEGF enzyme immunoassay kit.

Analysis of MAP kinases. 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 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*g* for 10 min at 4 °C. SDS–polyacrylamide gel electrophoresis (PAGE) was performed by Laemmli [20] in 10% polyacrylamide gel. Western blotting analysis was performed as described previously [21] by using phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-specific p38 MAP kinase antibodies or p38 MAP kinase antibodies, with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second antibodies. Peroxidase activity on the nitrocellulose sheet was visualized on X-ray film by means of the ECL Western blotting detection system. When indicated, the cells were pretreated with ciglitazone or pioglitazone for 8 h. The pretreatment of GW9662 was performed for 60 min before the addition of ciglitazone or pioglitazone.

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

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 means ± SEM of triplicate determinations. Each experiment was repeated three times with similar results.

Results

Effects of ciglitazone or pioglitazone on the VEGF release by FGF-2 in MC3T3-E1 cells

We have previously shown that FGF-2 stimulates VEGF release in osteoblast-like MC3T3-E1 cells [15]. To clarify whether PPAR-γ ligands' activation affects FGF-2-stimulated VEGF release in osteoblast-like

MC3T3-E1 cells, we first examined the effect of ciglitazone on the VEGF release. Ciglitazone, which alone had little effect on the VEGF levels, significantly enhanced the FGF-2-stimulated release of VEGF (Fig. 1). The amplifying effect of ciglitazone was dose-dependent between 1 and 10 μM (Fig. 1). Ciglitazone at 10 μM caused about 110% enhancement in the FGF-2 alone. Pioglitazone, another PPAR-γ ligand, had a similar enhancing effect on the FGF2-stimulated VEGF release (data not shown).

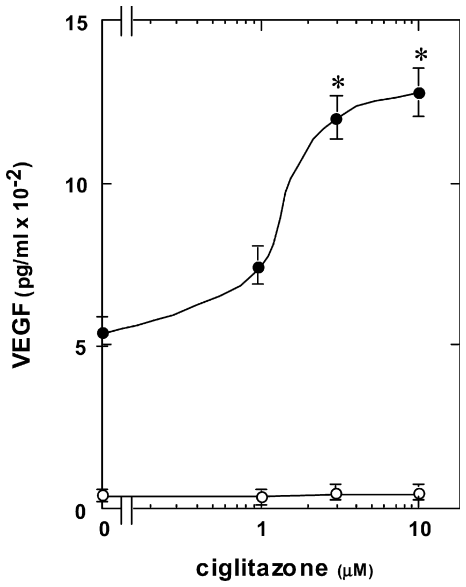

Fig. 1. Effect of ciglitazone on FGF-2-stimulated VEGF release in MC3T3-E1 cells. The cultured cells were pretreated with various doses of ciglitazone for 8 h, and then stimulated by 30 ng/ml FGF-2 (closed circle) or vehicle (open circle) for 24 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$p < 0.05$, compared to the value of FGF-2 alone.

Table 1
Effect of GW9662 on the enhancement by ciglitazone of the FGF-2-stimulated VEGF release in MC3T3-E1 cells

GW9662	Ciglitazone	FGF-2	VEGF (pg/ml)
−	−	−	23 ± 10
−	−	+	565 ± 51
−	+	−	45 ± 10
−	+	+	1170 ± 85*
+	−	−	25 ± 10
+	−	+	523 ± 67
+	+	−	20 ± 10
+	+	+	727 ± 49**

The cultured cells were pretreated with 20 μM GW9662 or vehicle for 60 min and then incubated by 3 μM ciglitazone for 8 h. The cells were stimulated by 30 ng/ml FGF-2 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 FGF-2 alone.

** $p < 0.05$, compared to the value of FGF-2 with ciglitazone pretreatment.

Effect of GW9662 on the enhancement by ciglitazone of FGF-2-stimulated VEGF release in MC3T3-E1 cells

To investigate whether the amplifying effect of ciglitazone or pioglitazone on FGF-2-induced VEGF release is mediated through PPAR-γ in MC3T3-E1 cells, we examined the effect of GW9662, a highly specific antagonist of PPAR-γ [22], on the enhancement by ciglitazone. GW9662, which alone hardly affected the basal level of VEGF or the FGF-2-stimulated VEGF release, significantly reduced the enhancement by ciglitazone of FGF-2-induced VEGF release (Table 1). The pioglitazone-enhanced VEGF release was suppressed by GW9662 as well as ciglitazone (data not shown).

Effects of ciglitazone on the phosphorylation of p44/p42 MAP kinase and SAPK/JNK induced by FGF-2 in MC3T3-E1 cells

We have previously shown that FGF-2 stimulates VEGF release at least in part via p44/p42 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells [15,16]. In order to investigate whether PPAR-γ-effect on the FGF-2-stimulated VEGF release is mediated

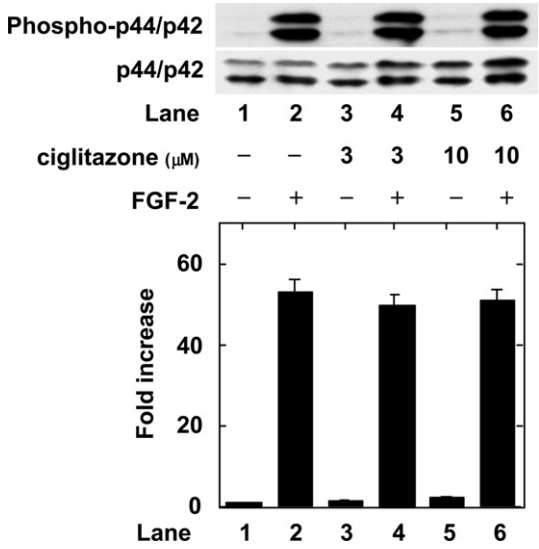

Fig. 2. Effect of ciglitazone on the phosphorylation of p44/p42 MAP kinase induced by FGF-2 in MC3T3-E1 cells. The cultured cells were pretreated with the indicated doses of ciglitazone or vehicle for 8 h, and then stimulated by 30 ng/ml FGF-2 or vehicle for 20 min. The extracts of cells were subjected 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 FGF-2-induced phosphorylation obtained from 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.

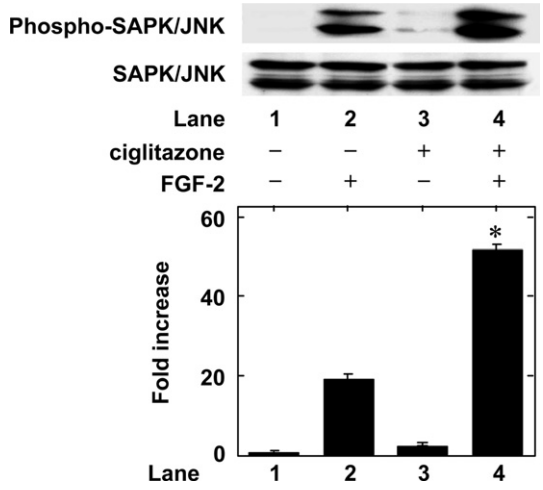

Fig. 3. Effect of ciglitazone on the phosphorylation of SAPK/JNK induced by FGF-2 in MC3T3-E1 cells. The cultured cells were pretreated with 3 μM ciglitazone or vehicle for 8 h, and then stimulated by 30 ng/ml FGF-2 or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE with subsequent 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 ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $*p < 0.05$, compared to the value of FGF-2 alone.

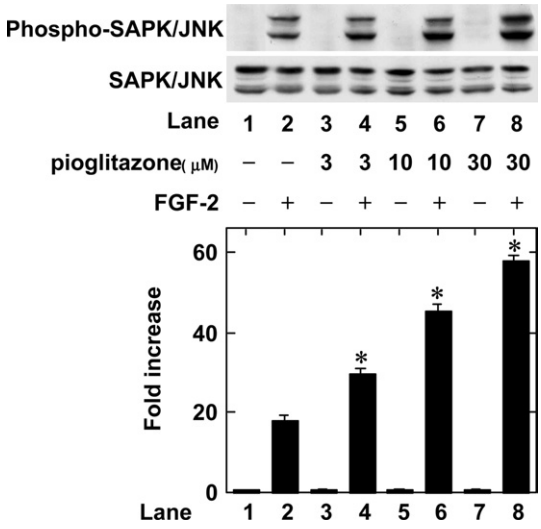

Fig. 4. Effect of pioglitazone on the phosphorylation of SAPK/JNK induced by FGF-2 in MC3T3-E1 cells. The cultured cells were pretreated with various doses of pioglitazone or vehicle for 8 h, and then stimulated by 30 ng/ml FGF-2 or vehicle for 20 min. The extracts of cells were subjected to SDS–PAGE 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 ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $*p < 0.05$, compared to the value of FGF-2 alone.

through p44/p42 MAP kinase activation or SAPK/JNK in MC3T3-E1 cells, we next examined the effect of ciglitazone on the FGF-2-induced phosphorylation of p44/p42 MAP kinase. However, ciglitazone failed to affect the phosphorylation of p44/p42 MAP kinase induced by FGF-2 (Fig. 2). On the other hand, the FGF-2-induced SAPK/JNK phosphorylation was markedly amplified by ciglitazone (Fig. 3). According to the densitometric analysis, ciglitazone (3 μM) caused about 70% enhancement of the FGF-2-effect on the SAPK/JNK phosphorylation. In addition, pioglitazone enhanced the SAPK/JNK phosphorylation in a dose-dependent manner (Fig. 4). According to the densitometric analysis, pioglitazone (30 μM) caused about 80% enhancement of the FGF-2-effect on the SAPK/JNK phosphorylation.

Effect of ciglitazone on the phosphorylation of p38 MAP kinase induced by FGF-2 in MC3T3-E1 cells

We have previously reported that the FGF-2-stimulated VEGF release is negatively regulated by FGF-2-activated p38 MAP kinase in MC3T3-E1 cells [15]. In order to investigate whether PPAR-γ-effect on the FGF-2-stimulated VEGF release is mediated through p38 MAP kinase activation in MC3T3-E1 cells, we next

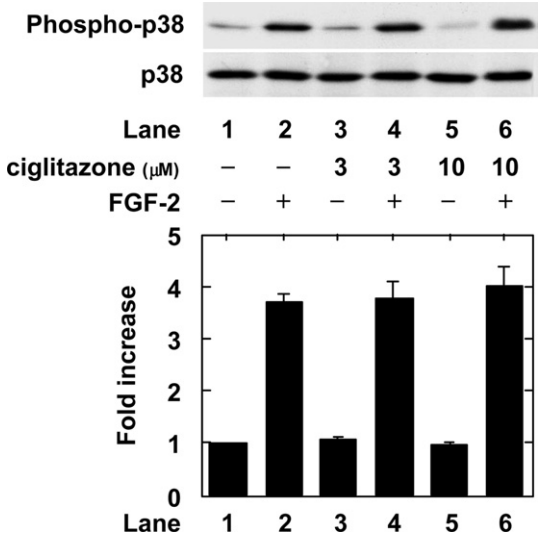

Fig. 5. Effect of ciglitazone on the phosphorylation of p38 MAP kinase induced by FGF-2 in MC3T3-E1 cells. The cultured cells were pretreated with the indicated doses of ciglitazone or vehicle for 8 h, and then stimulated by 30 ng/ml FGF-2 or vehicle for 20 min. The extracts of cells were subjected to 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 ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

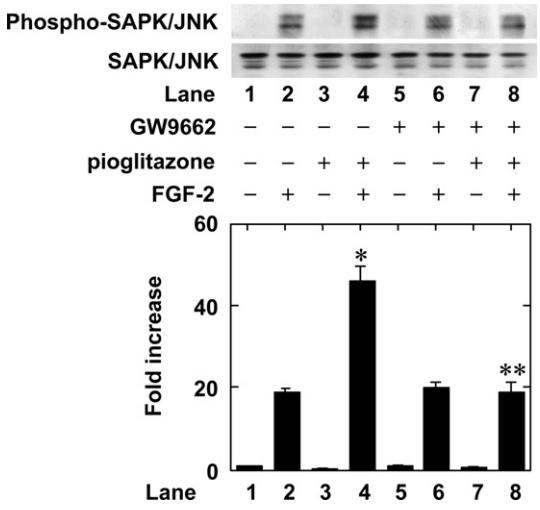

Fig. 6. Effect of GW9662 on the enhancement by pioglitazone of the FGF-2-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM GW9662 or vehicle for 60 min, and then incubated by 10 μM pioglitazone for 8 h. The cells were stimulated by 30 ng/ml FGF-2 or vehicle for 24 h. 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 ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$p < 0.05$, compared to the value of FGF-2 alone. **$p < 0.05$, compared to the value of FGF-2 with pioglitazone pretreatment.

examined the effect of ciglitazone on the FGF-2-induced phosphorylation of p38 MAP kinase. However, ciglitazone hardly affected the FGF-2-induced phosphorylation of p38 MAP kinase (Fig. 5).

Effect of GW9662 on the enhancement by pioglitazone of FGF-2-induced SAPK/JNK phosphorylation in MC3T3-E1 cells

To clarify whether the amplifying effect of ciglitazone or pioglitazone on FGF-2-induced SAPK/JNK phosphorylation is mediated through PPAR-γ in MC3T3-E1 cells, we examined the effect of GW9662 on the enhancement by pioglitazone. GW9662, which alone hardly affected the basal level of VEGF or the FGF-2-induced SAPK/JNK phosphorylation, suppressed the enhancement by pioglitazone almost completely to the levels of FGF-2 alone (Fig. 6).

Discussion

In the present study, we demonstrated that ciglitazone, a PPAR-γ-ligand, which by itself did not affect the levels of VEGF, significantly enhanced the FGF-2-stimulated VEGF release in osteoblast-like MC3T3-E1 cells. In addition, pioglitazone, another ligand of PPAR-γ, amplified the VEGF release as well as ciglitazone. Thus, our findings suggest that the FGF-2-stimulated VEGF release is enhanced via the activation of PPAR-γ. To clarify whether the effects of ciglitazone and pioglitazone are mediated through PPAR-γ activation in MC3T3-E1 cells, we next examined the effect of GW9662, a PPAR-γ antagonist [22], on the amplification by ciglitazone. GW9662 significantly suppressed the ciglitazone-induced enhancement of VEGF release while it failed to affect the FGF-2-stimulated VEGF release. Taking these findings into account, it is most likely that activation of PPAR-γ amplifies the FGF-2-stimulated VEGF release in osteoblast-like MC3T3-E1 cells.

It is well recognized that the MAP kinase superfamily mediates intracellular signaling of extracellular agonists and plays a crucial role in cellular functions including proliferation, differentiation, and cell death in a variety of cells [17]. Three major MAP kinases, p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK, are known as central elements used by mammalian cells to transduce the diverse messages [17]. In our previous studies [15,16], we have shown that FGF-2 activates three MAP kinases in osteoblast-like MC3T3-E1 cells, and p44/p42 MAP kinase and SAPK/JNK act as positive regulators in FGF-2-induced VEGF release while p38 MAP kinase negatively regulates the VEGF release. In the present study, we showed that ciglitazone did not affect the FGF-2-induced phosphorylation of p44/p42 MAP kinase. Thus, it seems unlikely that ciglitazone amplified the FGF-2-induced VEGF release through up-regulating the activation of p44/p42 MAP kinase in osteoblast-like MC3T3-E1 cells. In addition, ciglitazone had little effect on the FGF-2-induced p38 MAP kinase phosphorylation. Therefore, it seems unlikely that the ciglitazone-induced enhancement of FGF-2-stimulated VEGF release is due to the inhibition of p38 MAP kinase activation.

On the contrary, we showed that the FGF-2-induced SAPK/JNK phosphorylation was markedly amplified by ciglitazone. Furthermore, pioglitazone dose-dependently strengthened the SAPK/JNK phosphorylation as well as ciglitazone. These results suggest that the PPAR-γ activation up-regulates the FGF-2-stimulated activation of SAPK/JNK. We next demonstrated that GW9662 [22] did not affect the SAPK/JNK phosphorylation induced by FGF-2 alone, but markedly suppressed the enhancement by pioglitazone almost to the levels of FGF-2 alone. Therefore, it is probable that the amplification in the SAPK/JNK phosphorylation is mediated through the activation of PPAR-γ. Based on our findings as a whole, it is most likely that PPAR-γ activation up-regulates FGF-2-stimulated VEGF release through enhancing the activation of SAPK/JNK in osteoblast-like MC3T3-E1 cells. Further

investigations are required to elucidate the precise mechanism of PPAR-γ activation in the amplification of VEGF release.

It is generally recognized that the expansion of capillary network providing microvasculature is an essential process of bone remodeling [11]. Since VEGF is a specific mitogen of vascular endothelial cells [6], it is speculated that VEGF synthesized by osteoblasts acts as a crucial intercellular mediator between osteoblasts and vascular endothelial cells. 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 [7], supporting the importance of VEGF in bone metabolism. On the other hand, it is well known that PPAR-γ transcription factor determines the differentiation of progenitors into adipocytes [1]. In addition, a mesenchymal stem cell gives rise to adipocytes, osteoblasts, endothelial cells, and chondrocytes [23]. It has recently been reported that PPAR-γ is expressed also in osteoblasts including MC3T3-E1 cells [3]. Taking our results into account, it is probable that PPAR-γ ligand-enhanced VEGF release in osteoblasts plays an important role in the process of bone remodeling through regulating the capillary endothelial cells proliferation.

As for effects of PPAR-γ ligands on osteoblasts, it has recently been shown that PPAR-γ activators modulate osteoblast maturation such as alkaline phosphatase activity, Cbfa1 activity, and the expression of osteocalcin [3–5]. Therefore, it is probable that activation of PPAR-γ in osteoblasts functions as a pivotal role in bone metabolism. Further investigations are necessary to clarify the exact role of PPAR-γ activation in osteoblasts.

In conclusion, our present results strongly suggest that PPAR-γ activation up-regulates FGF-2-stimulated VEGF release via enhancing activation of SAPK/JNK among the MAP kinase superfamily in osteoblasts.

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