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Differential Effects of PD98059 and U0126 on Osteogenesis and Adipogenesis

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Abstract PD98059 and U0126 are considered as specific inhibitors of the p42/44 mitogen-activated protein kinases (MAPK) pathway, which affects osteogenesis and adipogenesis. Here, we show unexpected differential effects of PD98059 and U0126 on osteogenesis and adipogenesis as well as on estrogen (E2)-induced actions in osteoprogenitor KS483 cells. PD98059 dose-dependently inhibited osteogenesis indicated by cellular alkaline phosphatase (ALP) activity and nodule formation, but stimulated adipogenesis shown by the number of adipocytes. In contrast, U0126 slightly decreased osteogenesis but had no effects on adipogenesis, although it inhibited p42/44 MAPK more potently than PD98059. Furthermore, PD98059, but not U0126, counteracted E2-induced osteogenesis and adipogenesis. Transfection experiments showed that PD98059, but not U0126, had estrogenic transcriptional activity. Interestingly, both PD98059 and U0126 potentiated E2-induced estrogenic transcriptional activity in KS483 cells, which is opposite to the response in MCF7 breast cancer cells. Our data indicate that the cross-talk between growth factors and estrogen receptor (ER)-mediated pathways in KS483 cells is different from that in MCF7 cells. In summary, the differential effects of PD98059 and U0126 indicate their actions are not exclusively due to an inhibition of MAPK pathway. Caution should be taken in the interpretation of the results obtained using these inhibitors. J. Cell. Biochem. 92: 525–533, 2004.

Key words: estrogen; adipocyte; osteoblast; MAPKs; PPARγ

Pluripotent bone marrow-derived mesenchymal stem cells can differentiate into osteoblasts, adipocytes, chondrocytes, and myocytes [Nuttall and Gimble, 2000; Bianco et al., 2001]. This differentiation process is controlled by several essential transcription factors. For example, core-binding factor (Cbfa1) and peroxisome proliferator-activate receptor-γ2 (PPARγ2) are critical transcription factors for osteogenesis and adipogenesis, respectively [Yamaguchi et al., 2000; Lazar, 2002; Rosen et al., 2002]. These two transcription factors can be regulated by p44/42 mitogen-activated protein kinases (MAPKs). Phosphorylation of Cbfa1 increases osteogenesis, whereas an inhibition of this phosphorylation decreases osteogenesis [Xiao et al., 2000; Lai et al., 2001]. In contrast, phosphorylation of PPARγ downregulates adipogenesis, whereas an inhibition of the phosphorylation upregulates adipogenesis [Hu et al., 1996; Adams et al., 1997a; Camp and Tafuri, 1997].

Both PD98059 and U0126 have long been used as specific inhibitors of the p42/44 MAPK pathway [Alessi et al., 1995; Cuenda and Alessi, 2000; Davies et al., 2000; Ahn et al., 2001]. However, their effects on osteogenesis and adipogenesis are not consistent. For example, it has been reported that PD98059 blocked osteogenesis in adult human mesenchymal stem cells [Jaiswal et al., 2000] as well as in mouse MC3T3-E1 preosteoblastic cells cultured with osteogenic differentiation medium [Xiao et al., 2000], whereas it promoted the early osteoblastic differentiation and mineralization in BMP-2 treated pluripotent mesenchymal C2C12 cells and in MC3T3-E1 cells [Higuchi et al., 2002]. Furthermore, it has been shown that PD98059 had no effects on adipogenesis of 3T3-L1 preadipocyte cells stimulated by the adipogenic mixture of insulin, dexamethasone, and 1-isobutyl-3-methylxanthine [Qiu et al., 2001].

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It has been also reported that PD98059 stimulated adipogenesis in 3T3-L1 cells treated by TNF-α or transfected by PTHrP [Chan et al., 2001], but inhibited adipogenesis in these cells treated by leukemia inhibitory factor [Aubert et al., 1999]. These inconsistent results may relate to the stimulus used in different systems.

The calvaria-derived clonal KS483 cell line was initially selected for its commitment towards the osteoblastic lineage and these cells differentiated into only osteoblasts when cultured with fetal bovine serum [Yamashita et al., 1990; Yamashita et al., 1996]. Surprisingly, we observed that KS483 cells can concurrently differentiate into both osteoblasts and adipocytes when they are cultured with charcoal-stripped serum without extra adipogenic stimulus [Dang et al., 2002, 2003]. We further demonstrated that charcoal-stripped serum induced adipogenesis via p42/44 MAPK signaling pathway but not p38 MAPK pathway [Dang and Lowik, unpublished observations]. Using specific inhibitors of the p42/44 MAPK pathway, PD98059 and U0126, we observed unexpected differential effects of these inhibitors on osteogenesis and adipogenesis. These findings suggest that their effects are not exclusively due to an inhibition of p42/44 MAPK pathway and may have fundamental implications regarding inhibitors used.

It has been shown that PD98059 displaced radio-labeled estradiol from estrogen receptor (ER)-α and exert transcriptional estrogenic activity [Long et al., 2001]. It is unknown, however, whether the estrogenic contamination of PD98059 produce biological effects at cellular levels. We showed that responses of osteogenesis and adipogenesis of these cells are sensitive to different treatments including 17β-estradiol (E2), genistein, and PPARγ agonists [Dang et al., 2002, 2003]. In this study, we used KS483 cell culture system to test whether the non-specific estrogenic transcriptional activity of PD98059 could interfere with the differentiation of osteoprogenitor cells into osteoblasts or adipocytes. Moreover, PD98059 has also been used to study the cross-talk between growth factors and ER-mediated pathways. However, the non-specific estrogenic activity of PD98059 may also interfere with estrogen-induced actions.

In the present study, we used KS483 cells, cultured in charcoal-stripped serum without extra adipogenic stimulus, to study the effects of the PD98059 or U0126 on osteogenesis and adipogenesis. Here, we show unexpected differential effects of PD98059 and U0126 on osteogenesis and adipogenesis as well as on E2-induced actions.

MATERIALS AND METHODS

Cell Culture

The methods for cell culture have been described before [Dang et al., 2002]. In brief, KS483 cells were cultured in phenol red free α-minimum essential medium (α-MEM, Invitrogen Corporation, Paisley, Scotland) Corporation) supplemented with 10% non-charcoal stripped foetal bovine serum (FBS, Gibco BrL Life Technologies, GIB, Paisley, Scotland), 50 μg/ml ascorbic acid (Merck, Darmstadt, Germany), and 10 mM β-glycerophosphate (Sigma, St. Louis, MO). Under this condition, these cells differentiate only into osteoblasts. However, when KS483 cells were cultured in the medium supplemented with 10% charcoal-stripped serum, these cells can differentiate into both osteoblasts and adipocytes. Experiments were carried out with the medium supplemented with charcoal-stripped serum. Cells were continuously exposed to PD98059 (BIOMOL, Lot# P53540), U0126 (BIOMOL, Lot# Z21451) either alone or in combination with E2 (Sigma). The compounds were added 1 day after plating of the cells and the experiments were stopped at day 18. Medium was changed every 3–4 days.

Assays

ALP activity of the cells was kinetically determined by a colorimetric assay using *p*-nitrophenylphosphate (Pierce, Rockford, IL) as a substrate at pH 10.5 and reading the optical density at 405 nm [Dang et al., 2002]. DNA was measured by the method of an enhancement of fluorescence using Hoechst 33258 (Sigma) binding to DNA [Labarca and Paigen, 1980]. Numbers of nodules and adipocyte were objectively counted under a light microscope.

Oil Red O Staining

Oil Red O staining is specific for lipid droplets in adipocytes. Oil Red O (4.2 g, Sigma) was dissolved in 1,200 ml of isopropanol (Merck) and then diluted with distilled water at a ratio of 4:3. Cell monolayer was rinsed with PBS and fixed with 4% formalin for 10 min. After washing with

water, cells were stained for 1 h by immersion with Oil Red O solution. The staining was stopped by rinsing with water.

Immunoblot Analysis of p42/44 MAPKs

KS483 cells were seeded in the 6-well plates and cultured in the α-MEM containing 10% non-charcoal stripped serum. After 4 days of cell culture, these cells were cultured in serum free α-MEM overnight and then exposed to different doses of PD98059 or U0126 for 3 h. Stimulation was carried out by adding 10 ng/ml EGF (Sigma) for 20 min. Cells were washed twice in PBS and then collected. Equal amount of proteins of KS483 cells were fractionated by SDS–PAGE with a 12.5% acrylamide (BDH Laboratory, Amsterdam, the Netherlands) separation gel, and the separated proteins were transferred to nitrocellulose membranes (pore size 0.45 μm; Schleicher and Schuell, Dassel, Germany). After blocking the membranes with the mixture of 2% milk powder, 1% BSA, and 0.1% gelatin, the proteins were probed with the p42/p44 MAPK phospho-specfic antibody (Sigma) overnight at 4°C. Anti-mouse IgG-peroxidase conjugates (DAKO, Glostrup, Denmark) were used as a second antibody and the ECL (Pierce) detection system was used.

Transfection of Cultured Cells and Reporter Assays

KS483 cells were seeded into 24-well plates. After 24 h, they were transfected using a lipid-based FuGENE 6 transfection reagent according to the manufacturer's instructions (Roche, Basel, Switzerland). For each triplicate of sample, 100 ng luciferase reporter and 500 ng β-galactosidase expression vector were applied. The transfection medium was changed after 16 h into the different treatment medium as indicated. After 48 h, cells were washed twice with PBS, lysed in PBS containing 1% Triton X-100 and sonicated. Luciferase activity was measured and expressed as fold induction ± SEM, which was corrected for transfection efficiency using β-galactosidase activity. The estrogen responsive reporter gene construct (2XERE-TATA-luc) containing two copies of a consensus estrogen response element has been described elsewhere [Dang et al., 2003].

Statistics

Data are presented as means ± SEM. Differences between groups were accepted at $P < 0.05$, which were assessed by one-way ANOVA or related test using software Instat (San Diego, CA).

RESULTS

Effects of PD98059 and U0126 on Osteogenesis and Adipogenesis

KS483 cells, cultured in the medium supplemented with charcoal-stripped serum, can concurrently differentiate into both osteoblasts and adipocytes and form mineralized nodules [Dang et al., 2002, 2003]. We used this cell culture system to study the effects of PD98059 and U0126 on osteogenesis and adipogenesis. As shown in Figure 1, PD098059 and U0126 inhibited p42/44 MAPKs in a dose-dependent way. U0126 is more potent than PD98059 in KS483 cells, which is consistent with the reports in many other types of cells [Favata et al., 1998; Davies et al., 2000; Ahn et al., 2001]. Surprisingly, the inhibitory effects of PD98059 on osteogenesis, as shown by cellular ALP activity and nodule formation, were dose-dependent and more potent than those of U0126. Strikingly, PD98059 dose-dependently stimulated adipogenesis, whereas U0126 did not affect adipogenesis. Clearly, our data show that differential effects of PD98059 and U0126 on osteogenesis and adipogenesis cannot be exclusively explained by an inhibition of p42/44 MAPK activity.

Effects of PD98059 and U0126 on Estrogen Action

We have shown that KS483 cells are very sensitive to E2. Maximum stimulation of osteogenesis and inhibition of adipogenesis were found when exposed to 10^{-8} M of E2 [Dang et al., 2002]. PD98059 and U0126 differentially influenced E2-induced osteogenesis and adipogenesis. PD98059 at concentrations of 0.01 and 0.1 μM potentiated E2-increased ALP activity and nodule formation. In contrast, PD98059 at micromolar concentrations (10 and 25 μM) dose-dependently inhibited E2-stimulated ALP activity and nodule formation. U0126 at all tested concentrations, however, did not influence E2-stimulated ALP activity and nodule formation (Fig. 2A,B). PD98059 below micromolar concentrations did not influence E2-inhibited adipogenesis, In contrast, PD98059 at the micromolar concentrations counteracted

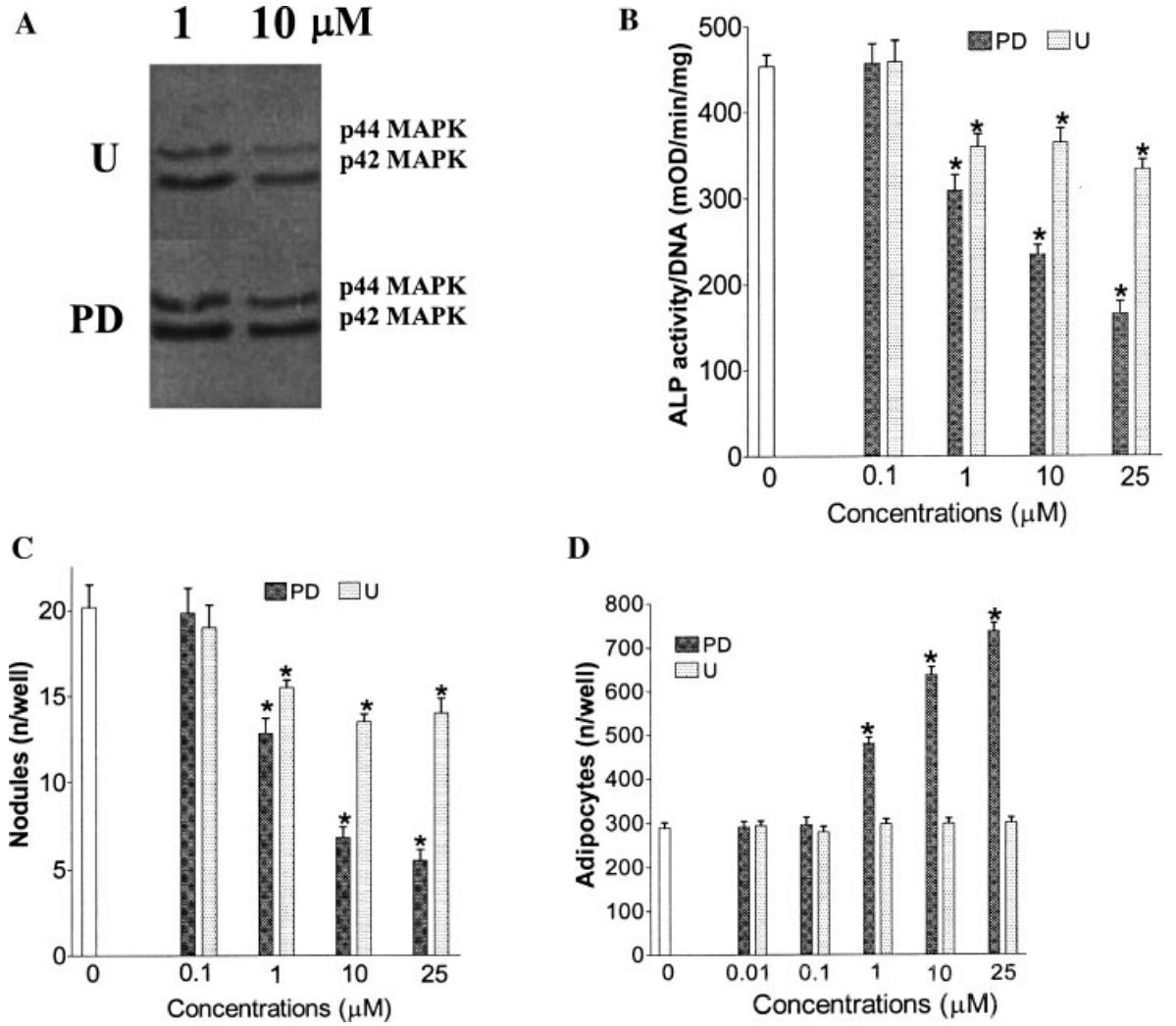

Fig. 1. Effects of PD98059 or U0126 on osteogenesis and adipogenesis. Western blot shows that dually-phosphorylated form of p42/p44 MAPKs were inhibited by PD98059 or U0126 in KS483 cells, but U0126 is more potent than PD98059 (**A**). KS483 cells, cultured in 12-well plates, were continuously exposed to different concentrations of PD98059 or U0126 for 18 days. Cellular ALP activity (**B**), the number of nodules (**C**), and the number of adipocytes (**D**) were quantified. Each value is the mean ± SEM of the results from three different wells and is representative of results from three different experiments. Significant differences ($* < 0.05$) between the control and the treatment were indicated. PD, PD98059; U, U0126.

E2-inhibited adipogenesis in a dose-related way. U0126 at all tested concentrations, however, did not influence E2-inhibited adipogenesis (Fig. 2C,D).

Effects of PD98059 and U0126 on Estrogenic Transcriptional Activity

KS483 cells were transiently transfected with ERE-luc or TATA-luc and exposed to PD98059 or U0126. PD98059 and U0126 increased nonspecific TATA-luc activity, which may result from their MAPK inhibitory effects. Compared to TATA-luc, only PD98059 increased ERE-luc significantly, indicating weak estrogenic activity (Fig. 3A,B).

We then tested whether PD98059 or U0126 could interfere with E2-induced transcriptional activity in KS483 cells. PD98059 and U0126 increased E2-induced transcriptional activity (ERE-luc) in a dose-dependent way. However, PD98059 stimulated E2-induced transcriptional activity to very high levels and was five times more potent than U0126 (Fig. 4A,B).

These data are opposite to those reported in MCF7 breast cancer cells exposed to PD98059 or U0126 [Atanaskova et al., 2002; Lu et al., 2002]. To investigate whether these divergent

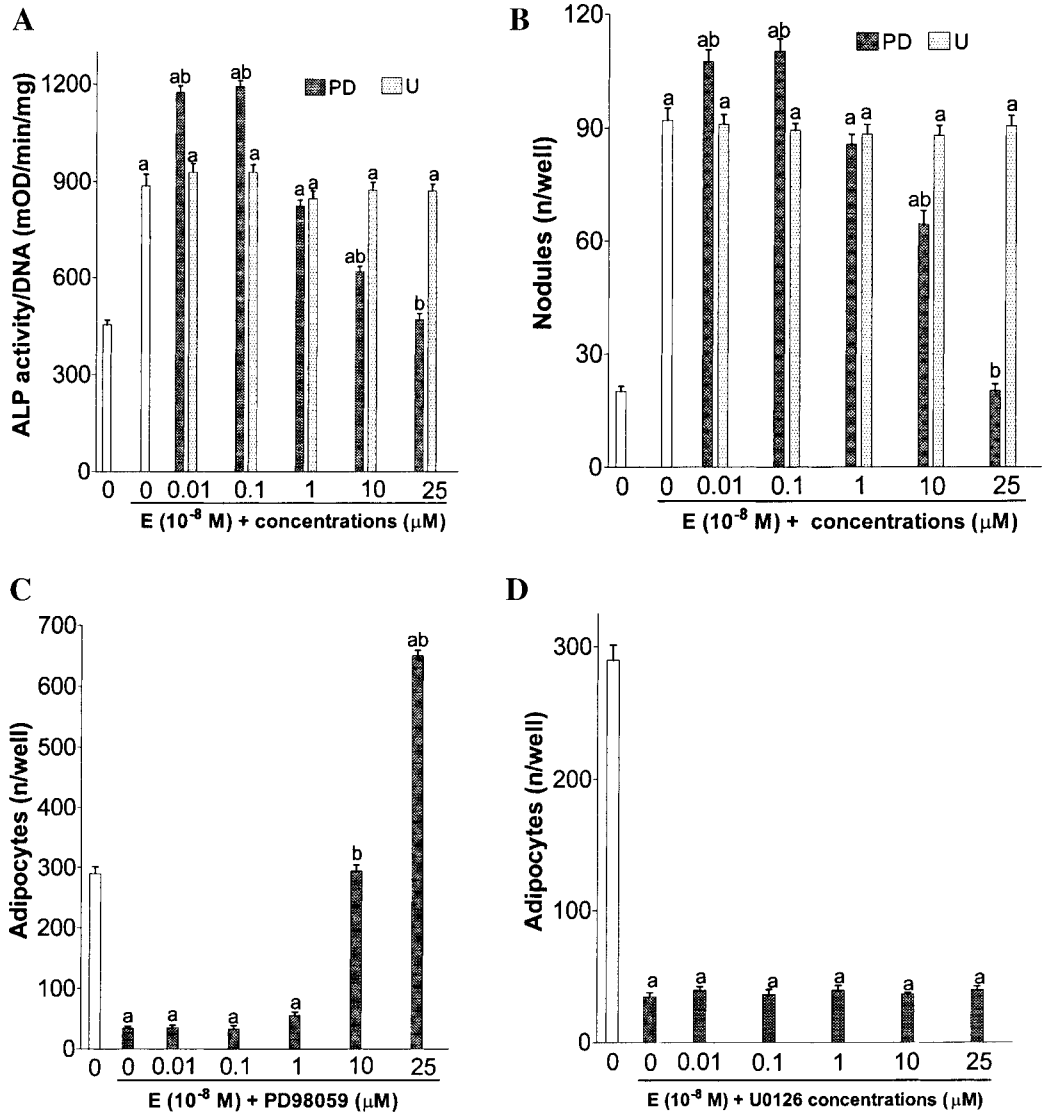

Fig. 2. Differential effects of PD98059 and U0126 on estrogen-induced osteogenesis and adipogenesis. KS483 cells cultured in 12-well plates, were continuously exposed to E (10^{-8} M) or in combination with different concentrations of PD98059 or U0126 for 18 days. Cellular ALP activity (**A**), the number of nodules (**B**), and the number of adipocytes (**C**, **D**) were quantified. Each value is the mean ± SEM of the results from three different wells and is representative of results from three different experiments. Significant differences ($* < 0.05$) between the control and the treatment were indicated as "a", whereas those between E alone and the treatments with E in combination with PD98059 or U0126 as "b". PD, PD98059; U, U0126; E, 17β-estradiol.

effects are cell line dependent or concentration-related, we transiently transfected MCF7 cells with the same ERE-luc construct as used in KS483 cells and then exposed these cells to PD098059 or U0126, either alone or in combination with E2. Different from KS483 cells, PD98059, and U0126 did not increased non-specific TATA-luc activity in MCF7 cells. PD98059, but not U0126, increased ERE-luc in a dose-dependent way, which confirm our observations in KS483 cells. Moreover, PD98059 slightly increased E2-induced ERE-luc activity at concentrations of 1 and 10 μM, but decreased E2-induced ERE-luc activity at concentrations of 25 μM or higher. U0126 decreased E2-induced ERE-luc activity (Fig. 4C,D). These data obtained from MCF7 cells are in line with the earlier reports [Kato et al., 2000; Atanaskova et al., 2002; Lu et al., 2002], but differ from those obtained from KS483 cells.

DISCUSSION

PD98059 and U0126 are considered to be specific inhibitors and have long been used to study the involvement of the p42/44 MAPK

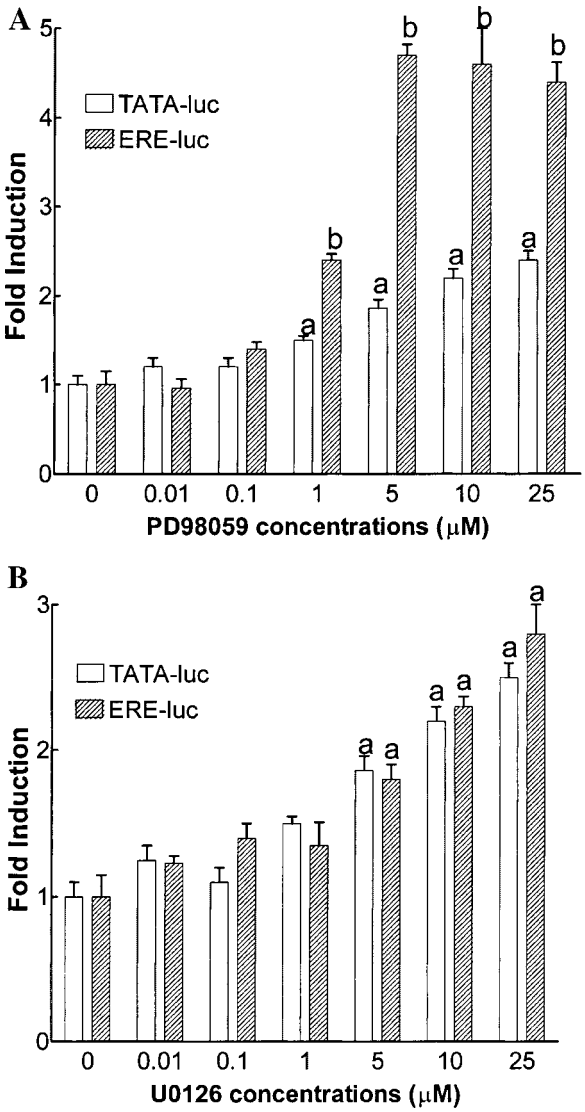

Fig. 3. Effects of PD98059 or U0126 on estrogenic transcriptional activity. KS483 cells were transiently transfected with reporter plasmids and exposed to different concentrations of PD98059 (**A**) or U0126 (**B**) for 2 days. Luciferase reporter activity was determined and expressed as fold induction, which was corrected for transfection efficiency using β-galactosidase activity. Significant differences ($* < 0.05$) between the control and the treatment were indicated. as "a", whereas those between TATA-luc and ERE-luc at the same concentrations as "b".

pathway in cellular events such as growth and differentiation [Alessi et al., 1995; Ahn et al., 2001]. They have also been used as a tool to study the involvement of MAPK in osteogenesis and adipogenesis. It is suggested to use both inhibitors in cell-based assays to minimize the risk of non-specific effects [Davies et al., 2000]. However, our results show that PD98059 and U0126 differentially affected osteogenesis, adipogenesis, and E2-induced actions, which cannot be exclusively explained by inhibition of the MAPK pathway.

Consensus exists that inhibition of phosphorylation of Cbfa1 decreases osteogenesis [Xiao et al., 2000; Lai et al., 2001], whereas inhibition of phosphorylation of PPARγ increases adipogenesis [Hu et al., 1996; Adams et al., 1997b; Camp and Tafuri, 1997]. Consistent with this concept, both PD98059 and U0126 downregulated osteogenesis of KS483 cells, which is in line with observations in adult human mesenchymal stem cells [Jaiswal et al., 2000] and in mouse MC3T3-E1 cells [Xiao et al., 2000]. Furthermore, our observations that PD98059 stimulated adipogenesis are consistent with this consensus and in line with those reported in 3T3-L1 cells [Chan et al., 2001].

Similar to PD98059, U0126 inhibited osteogenesis and MAPK activity in KS483 cells although the effect on osteogenesis was not really dose-dependent. Surprisingly, U0126 did not influence adipogenesis, although U0126 inhibited MAPK activity more potently than PD98059. Furthermore, the U0126 did not influence E2-induced osteogenesis and adipogenesis. Taken together, these data suggest that the effects of U0126 may not be exclusively due to its inhibition of MAPK activity.

It has been suggested that contaminating estrogenic activity of PD98059 may confound interpretation of experimental results in estrogen-responsive cells [Long et al., 2001]. Although PD98059 has weak estrogenic effects, our data suggest that these are most probably overruled by its more dominant MAPK inhibitory effects. Following observations supports this conclusion. First, PD98059 and U0126 increased non-specific transcriptional activity in KS483 cells but not in MCF7 cells. However, only PD98059 increased ERE-luc activity in KS483 cells as well as in MCF7 cells. Inspite of this estrogenic activity, PD98059 inhibited p42/44 MAPK activity. Second, KS483 cells are very sensitive to E2, with the lowest effective concentration of 10^{-13} M [Dang et al., 2002], which is lower than the detection limit (E2, 10^{-12} M) of activating ERE-luc (unpublished results). So changes in osteogenesis and adipogenesis are sensitive enough to detect pure estrogenic effects. However, the effects of PD98059 on osteogenesis and adipogenesis were opposite to those of E2, indicating a dominant MAPK inhibitory effects. Third, PD98059 in-

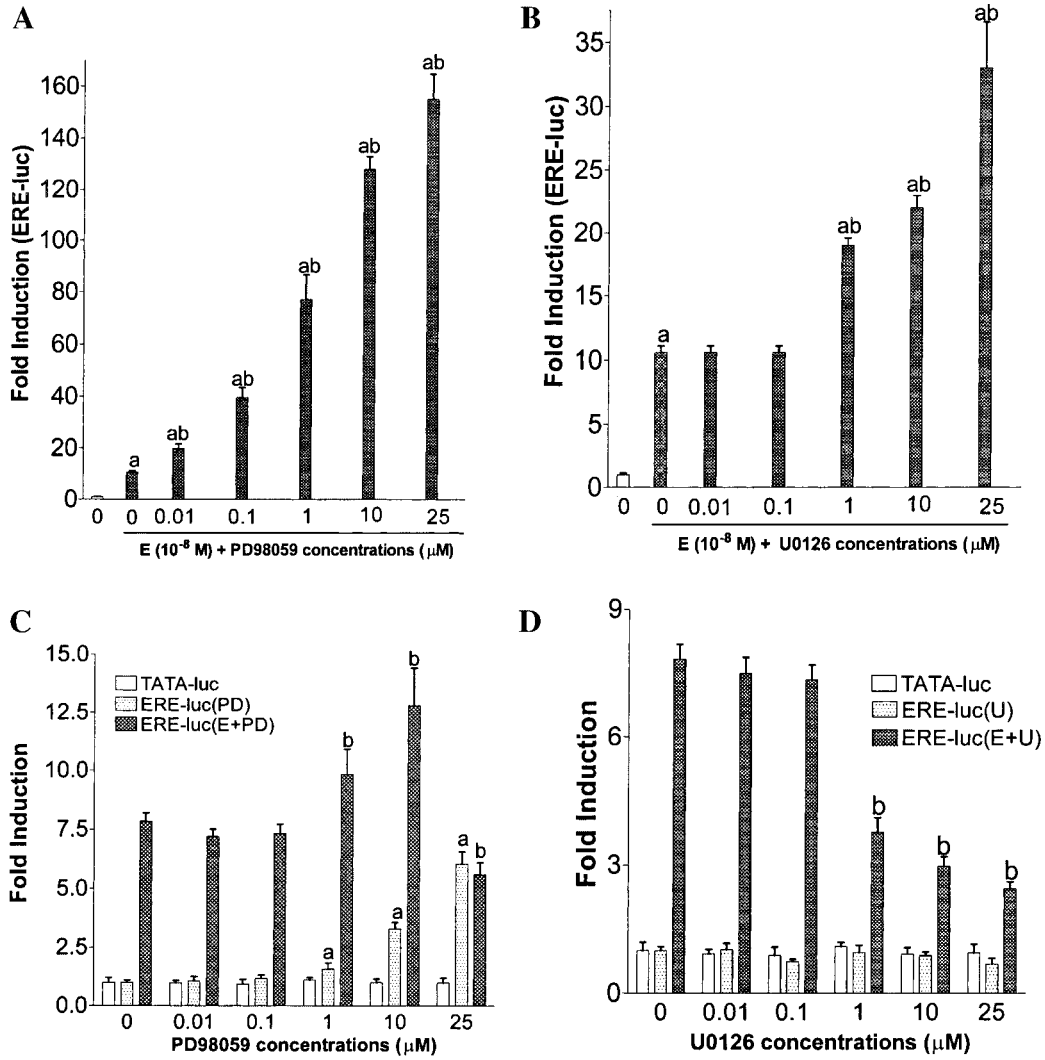

Fig. 4. PD98059 or U0126 influences estrogen-induced transcriptional activity. KS483 cells (**A**, **B**) or MCF7 cells (**C**, **D**) were transiently transfected with ERE-luc and then exposed to E or in combination with different concentrations of PD98059 (A) or U0126 (B) in KS483 cells or PD98059 (C), U0126 (D) in MCF7 cells for 2 days. Luciferase reporter activity was determined and expressed as fold induction, which was corrected for transfection efficiency using β-galactosidase activity. The results are from three different wells and are representative of results from three different experiments. Significant differences ($* < 0.05$) between the control and the treatment were indicated as "a", whereas those between E alone and the treatments with E in combination with PD98059 or U0126 as "b". PD, PD98059; U, U0126; E, 17β-estradiol.

creased E2-induced transcriptional estrogenic activity. However, this increase may not result from the estrogenic activity of PD98059. Instead, it may result from the MAPK inhibitory effects because this increase was observed at PD98059 concentrations that did not have estrogenic activity and also observed in U0126-treated KS483 cells.

Interestingly, PD98059 and U0126 increased E2-induced estrogenic transcriptional activity in KS483 cells. Consistently, genistein, a compound that has both estrogenic and MAPK inhibitory effects, has higher estrogenic transcriptional activity in KS483 cells [Dang et al., 2003] and in human embryonic kidney 293 cells [Pettersson et al., 2000]. Moreover, the finding that PD98059 (0.01 and 0.1 μM) potentiated E2-stimulated osteogenesis is in line with these transcriptional data. In contrast, PD98059 and U0126, as reported by others [Atanaskova et al., 2002; Lu et al., 2002], decreased E2-induced estrogenic transcriptional activity in MCF7 cells. These differential effects in two different cell lines may be due to the differences in cellular context (non-cancerous cells vs. cancer cells, breast cells versus bone cells) or

in species, i.e., mouse versus human. It is also possible that the cross-talk between growth factors and ER-mediated pathways is different in these two types of cells.

In summary, we showed differential effects of PD98059 and U0126 on osteogenesis and adipogenesis as well as on E2-induced actions. Both PD98059 and U0126 inhibited osteogenesis, but only PD98059 in a dose-dependent way. PD98059 inhibited adipogenesis, whereas U0126 did not. PD98059 counteracted E2-induced osteogenesis and adipogenesis, whereas U0126 did not. Our results suggest that the effects of PD98059 and U0126 on osteogenesis and adipogenesis of KS483 cells are not exclusively due to an inhibition of MAPK activity. So caution should be taken in the interpretation of the results obtained using these inhibitors. Finally, our consistent findings concerning the effects of PD98059 or U0126 on E2-induced estrogenic transcriptional activity suggest that the cross-talk between growth factors and ER-mediated pathways in KS483 cells is different from that in MCF7 cells.

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