J. Biochem. 126, 168-173 (1999)

Ipriflavone Down-Regulates Endothelin Receptor Levels during Differentiation of Rat Calvarial Osteoblast-Like Cells[1]

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Received February 17, 1999; accepted April 27, 1999

Ipriflavone (7-isopropoxy-3-phenyl-4*H*-1-benzopyran-4-one) is a synthetic flavonoid that has been shown to stimulate the activity of osteoblasts. We show here that ipriflavone also promotes the deposition of calcium and the formation of mineralized nodules by newborn rat calvarial osteoblast-like (ROB) cells as well as the activity of alkaline phosphatase. We reported previously that endothelin-1 inhibits the differentiation of ROB cells [Y. Hiruma *et al.* (1998) *J. Cardiovasc. Pharmacol.* 31, S521-S523]. Therefore, we examined the effects of ipriflavone on the expression of endothelin receptors in ROB cells by polymerase chain reaction-Southern blot analysis and in binding assays with ^{125}I-labeled endothelin-1. Ipriflavone reduced levels of endothelin ET_A receptors (to 48% of the control level) in ROB cells around day 7 in our standard cultures, while it had no apparent effect on the expression of the mRNA for the endothelin ET_A receptor. By contrast, treatment with 10^{-7} M endothelin-1 on days 6 through 9 alone suppressed mineralization by ROB cells. Ipriflavone also reduced the ability of endothelin-1 to inhibit mineralization by ROB cells. These results suggest that the acceleration of osteoblastic differentiation by ipriflavone might be due, at least in part, to a time-specific down-regulation of endothelin receptors.

Key words: endothelin-1, endothelin receptor, ipriflavone, mineralization, osteoblast.

Ipriflavone prevents bone loss in several experimental models of osteoporosis (*1-3*) and has been used for treatment of patients with osteoporosis (*4, 5*). The loss of bone mass in osteoporosis results from an imbalance between the formation and resorption of bone (*6*). In osteoclasts, ipriflavone inhibits the differentiation and bone resorption of immature bone marrow cells (*7, 8*). By contrast, ipriflavone also stimulates the differentiation of osteoblastic cells directly (*9-13*). However, the mechanism responsible for the stimulation by ipriflavone on stimulation of bone formation by osteoblastic cells remains poorly understood.

Endothelins are potent vasoconstrictors that were first identified in vascular endothelial cells. They are of three types, namely, endothelin-1, endothelin-2, and endothelin-3 (*14-16*). Two types of receptor for endothelin have been cloned, namely, the endothelin ET_A receptor (*17*) and the endothelin ET_B receptor (*18*). Both receptors belong to the superfamily of G-protein-coupled receptors that have seven transmembrane helices. Endothelin has been shown to regulate the formation of bone both *in vitro* (*19, 20*) and *in vivo* (*21, 22*), and we ourselves reported that endothelin-1 inhibits the deposition of calcium and the formation of mineralized nodules by newborn rat calvarial osteoblast-like (ROB) cells as well as the activity of alkaline phosphatase (*23, 24*). Each of these effects of endothelin-1 involves activation of the endothelin ET_A receptor.

In the present study, we investigated the effects and mechanism of action of ipriflavone during the differentiation of and formation of bone by ROB cells, a well-characterized model system that has been used to study the factors that regulate osteoblastic differentiation (*23, 25, 26*). We found that ipriflavone increased the rate of differentiation and mineralization of ROB cells. We also found that ipriflavone reduced the level of expression of endothelin receptors during the differentiation of ROB cells, as well as the inhibitory effect of endothelin-1 on mineralization by ROB cells.

EXPERIMENTAL PROCEDURE

Materials—Ipriflavone (7-isopropoxy-3-phenyl-4*H*-1-benzopyran-4-one) was provided by Takeda Chemical Industries, Osaka. Human endothelin-1 was purchased from the Peptide Institute, Osaka. Endothelin-1 was dissolved in 0.1% acetic acid. ^{32}P-labeled nucleotides and ^{125}I-labeled endothelin-1 (74 TBq/mmol) were obtained from Amersham Pharmacia Biotech, Buckinghamshire, UK. α-MEM, fetal bovine serum, and penicillin/strepto-

[1] This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and by grants from the SRF and "Ground Research Announcement for the Space Utilization" promoted by NASDA and Japan Space Forum.
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Abbreviation: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ipriflavone, 7-isopropoxy-3-phenyl-4*H*-1-benzopyran-4-one; ROB cells, osteoblast-like cells from newborn rat calvariae.

mycin antibiotic mixture were obtained from Life Technologies, Grand Island, NY, USA.

Cell Cultures—Cells were isolated enzymatically from calvariae of newborn Wistar rats (day 2) as described previously (*23*). Fourteen calvariae were dissected out and all adhering soft tissue was removed. The calvariae were cut into pieces and subjected to six sequential 20-min digestions (yielding digests 1 through 6) with 2 ml of an enzyme mixture containing 1 mg/ml collagenase (150-250 units/mg; Wako Pure Chemical Industries, Osaka) and 0.5 mg/ml trypsin (Sigma, St. Louis, MO, USA). Cells from a pool of digests 4, 5, and 6 were plated in 75-cm^2 dishes and grown in α-MEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 in air at 37°C. The medium was replaced by fresh medium after 24 h. After reaching confluence, the cells were detached by treatment with 0.05% trypsin, and cells from three dishes (digests 4, 5, and 6) were combined. The cells were replated in 12-well plates (3.8 cm^2/well) at a density of 1×10^4 cells/cm^2 and grown in α-MEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 5 mM β-glycerophosphate, and 50 μg/ml ascorbic acid. During subculture, the medium was replaced every 4 days and ipriflavone was added every 2 days.

Assay of Alkaline Phosphatase Activity—Cells were subcultured in α-MEM containing 10% fetal bovine serum, 5 mM β-glycerophosphate, 50 μg/ml ascorbic acid, and test compounds. The cells were washed twice with 50 mM Tris-HCl, pH 7.2, and sonicated in 1 ml of 50 mM Tris-HCl, pH 7.2, containing 0.1% Triton X-100 and 2 mM $MgCl_2$ for 15 s with a sonicator (Ultrasonic Disruptor UD-201; Tomy, Tokyo). The alkaline phosphatase activity of the sonicate was determined by an established technique with *p*-nitrophenyl phosphate as the substrate. Concentrations of protein were determined with BCA protein assay reagent (Pierce Chemical, Rockford, IL, USA).

Quantitation of Ca^{2+}—Cells were subcultured in α-MEM containing 10% fetal bovine serum, 5 mM β-glycerophosphate, 50 μg/ml ascorbic acid, and endothelins at various concentrations. We measured the amount of calcium in hydroxyapatite in the cell layer. The layers of cells in 12-well plates (3.8 cm^2/well) were washed with phosphate-buffered saline (pH 7.4; PBS; 20 mM sodium phosphate and 130 mM NaCl) and incubated with 1 ml of 2 N HCl overnight with gentle shaking. The Ca^{2+} in the samples was quantitated by the *o*-cresolphthalein complexone method with a Calcium C kit (Wako Pure Chemical Industries). This kit is specific for Ca^{2+} and has a limit of detection of 1 μg/ml. Dilutions of the standard solution of Ca^{2+} (10 mg/dl) in the kit were as used standard solutions.

von Kossa Staining—Osteoblastic cells in 12-well plates were fixed with 10% formaldehyde for 30 min and washed three times with 10 mM Tris-HCl, pH 7.2. The fixed cells were incubated with 5% silver nitrate for 5 min in daylight, washed twice with H_2O, then treated with 5% sodium thiosulfate. Mineralized nodules were assessed with respect to both number and total area of nodules with an automated imaging system, which consisted of the Mac SCOPE program (Mitani, Fukui), a camera (CCD/ICD-740; Olympus, Tokyo), and a BH microscope (Olympus).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Southern Blotting—RNA was extracted from rat osteoblast-like cells (ROB cells) by the acid guanidinium-phenol-chloroform method (*27*). Total RNA (1 μg) was reverse transcribed by Moloney murine leukemia virus reverse transcriptase, Superscript (200 units; Life Technologies), with oligo(dT) primers (5 nmol) in a 20-μl reaction mixture. The cDNA was amplified by 35 cycles of PCR for endothelin ET_A receptor and 22 cycles for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in 100 μl of *pfu* DNA polymerase mixture (Toyobo, Tokyo) containing 1 μM sense primer, 5′-GGCGCAATCGCTGACAATG-CTGAG-3′, and 1 μM antisense primer, 5′-CCACGTAGA-TAAGGTCTCCAAGGG-3′, for the rat endothelin ET_A receptor (343 bp) (*28*), and 1 μM sense primer, 5′-CGTT-GTGGATCTGACATGCCGCC-3′, and 1 μM antisense primer, 5′-CAGTGTAGCCCAGGATGCC-3′, for rat GAPDH. Each reaction cycle consisted of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min. Products of PCR were subjected to electrophoresis on a 1.5% agarose gel and visualized by staining with ethidium bromide. DNA markers from a kit (Molecular Weight Marker V; Boehringer Mannheim, Tokyo) were used as size markers. For analysis of the endothelin ET_A receptor, products of PCR were blotted onto a nylon membrane (MagnaGraph; Micron Separations, Westborough, MA, USA) after electrophoresis. Southern blots were prehybridized at 37°C for 2 h in 6×sodium chloride/sodium citrate (SSC) solution (1× SSC: 0.15 M NaCl and 15 mM sodium citrate, pH 7.0) that contained 5×Denhardt's solution (1×Denhardt's solution: 0.1% each of bovine serum albumin, polyvinylpyrrolidone, and Ficoll) and 0.1% SDS, then hybridized at 37°C for 16 h in the same solution supplemented with the ^{32}P-labeled probe for the rat endothelin ET_A receptor or rat GAPDH. We prepared cDNAs for the rat endothelin ET_A receptor and GAPDH by PCR using specific primers described above. Each blot was washed twice in 1×SSC plus 1% SDS at 42°C for 1 h, then analyzed with a Bioimage Analyzer (BAS 2000; Fuji Film, Tokyo).

Assay of Binding of ^{125}I-Endothelin-1—Cells, grown in 12-well plates (3.8 cm^2/well), were washed with ice-cold PBS and incubated in 0.5 ml of PBS containing 0.2% (w/v) bovine serum albumin, ^{125}I-labeled endothelin-1 (920 Bq/well), and unlabeled endothelin for 1 h at 4°C. After incubation, cells were washed twice with ice-cold PBS and solubilized in 0.5 ml of 0.1 M NaOH. Radioactivity was measured with a gamma counter (ARC-300; Aloka, Tokyo).

RESULTS

Stimulation of the Differentiation and Mineralization of ROB Cells by Ipriflavone—We examined the effects of ipriflavone on osteoblastic differentiation using ROB cells. Ipriflavone at 10^{-5} M significantly enhanced the activity of alkaline phosphatase (144% of the control activity on day 9), which is a marker of osteoblastic differentiation. Ipriflavone also increased the accumulation of calcium (41.7 ± 2.5 μg/3.8-cm^2 well) to almost twice the control level (22.2±2.5 μg/3.8-cm^2 well) on day 15, as shown in Fig. 1. Figure 2A shows the results of von Kossa staining of mineralized nodules that had been formed by ROB cells during incubation for 12 days in either the continuous presence or in the absence of ipriflavone. Ipriflavone accelerated the formation of mineralized nodules by ROB cells. The nodules were quantitated with an image analyzer,

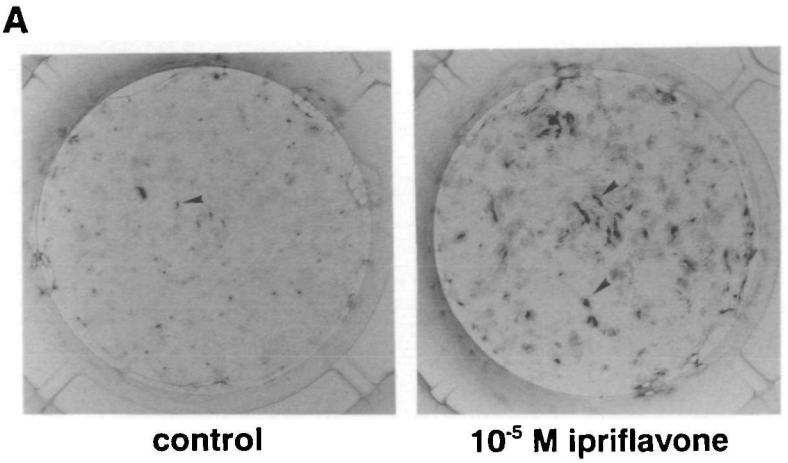
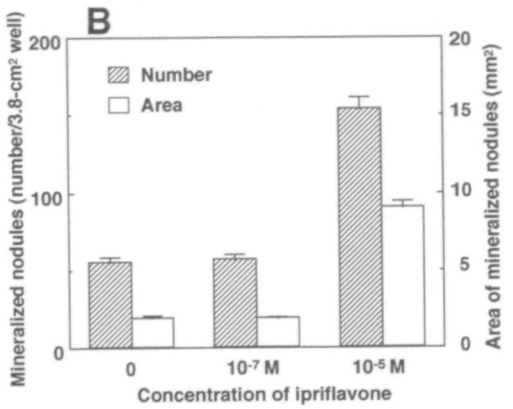


Fig. 2. **Phase-contrast photomicrographs of mineralized nodules in cultures of ROB cells treated with ipriflavone (A) and the results of quantitative analysis (B).** Cells in 12-well plates were cultured for 12 days with α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, 50 μg/ml L-ascorbic acid, and ipriflavone. (A) Mineralized nodules were subjected to von Kossa staining as described in "EXPERIMENTAL PROCEDURES." Arrowheads: stained mineralized nodules. (B) Numbers and areas of mineralized nodules were determined with the Mac SCOPE program. Data are means ± SE of results from four wells. $^*p < 0.001$ *versus* control.



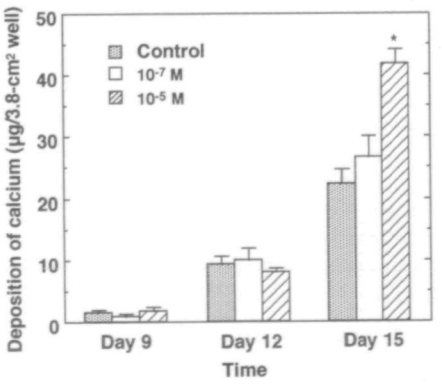


Fig. 1. **Effects of ipriflavone on mineralization by ROB cells.** Cells in 12-well plates (3.8 cm²/well) were cultured with α-MEM containing 10% fetal bovine serum, 5 mM β-glycerophosphate, 50 μg/ml L-ascorbic acid, and ipriflavone or 0.1% ethanol (control). Fresh medium was supplied at 4-day intervals, and ipriflavone was supplied to cells at 2-day intervals. At the times indicated, the deposition of calcium was measured. Ca^{2+} derived from hydroxyapatite was assayed as described in "EXPERIMENTAL PROCEDURES." Data are means ± SE of results from four wells and are typical of results of three separate experiments. $^*p < 0.01$ *versus* control.

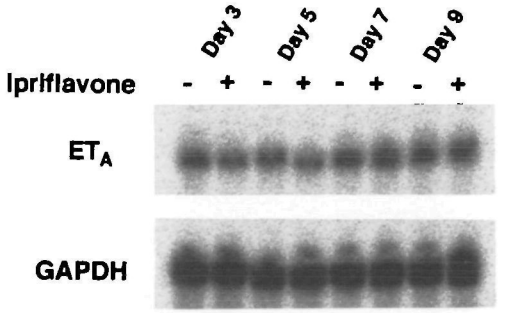


Fig. 3. **Detection of mRNA for endothelin ET_A receptor in ROB cells treated with ipriflavone.** ROB cells were cultured with 10^{-5} M ipriflavone for the indicated periods. RT-PCR and Southern blot analysis was performed as described in "EXPERIMENTAL PROCEDURES." PCR products for the endothelin ET_A receptor and GAPDH were subjected to electrophoresis on an agarose gel and allowed to hybridize with ^{32}P-labeled cDNA for rat endothelin ET_A receptor or GAPDH.

and numbers and total areas of mineralized nodules were recorded. In the presence of 10^{-5} M ipriflavone, there was a 2.8-fold increase in the number of mineralized nodules formed, and the total area of mineralized nodules was 4.8 times greater than that in control (Fig. 2B).

Reduction in the Expression of the Endothelin ET_A Receptors in ROB Cells by Ipriflavone—We reported previously that endothelin-1 inhibits the differentiation of ROB cells and mineralization (*23, 24*). Therefore, using PCR-Southern blot analysis and a binding assay, we investigated the effects of ipriflavone on the expression of endothelin receptors in ROB cells. PCR-Southern blot analysis revealed that ipriflavone had no effect on the expression of the mRNA for the endothelin ET_A receptor from day 3 to day 9 (Fig. 3). By contrast, binding assays with ^{125}I-labeled endothelin-1 revealed that ipriflavone at 10^{-5} M decreased the level of endothelin receptors (1.74 ± 0.14 fmol/10^6 cells) to 48% of the control level (3.61 ± 0.34 fmol/10^6 cells) on day 7, while ipriflavone had no effect on endothelin receptors on days 4 or 13 (Fig. 4). We confirmed that the endothelin receptors expressed in ROB cells were predominantly endothelin ET_A receptors by using a specific ligand for endothelin ET_A receptors, BQ-123 [cyclo(D-Trp-D-Asp-Pro-D-Val-Leu)], and endothelin-3 (data not shown). To determine whether some aspect of the ipriflavone-induced differentiation of osteoblasts is mediated by decreased production of endothelin receptors, we examined the effects of the pulsed administration of endo-

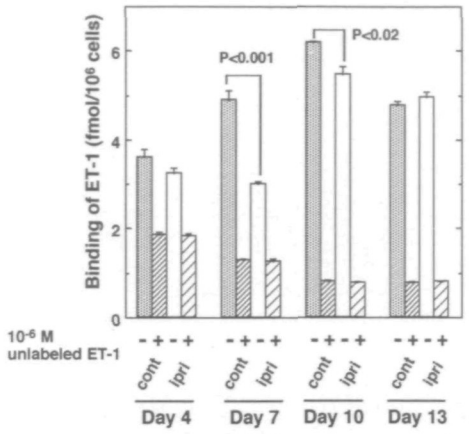


Fig. 4. **Reductions in the level of expression of endothelin receptors by ipriflavone.** ROB cells in 12-well plates were cultured with α-MEM containing 10% fetal bovine serum, 5 mM β-glycerophosphate, 50 μg/ml ascorbic acid, and 10^{-5} M ipriflavone (ipri). Ipriflavone was supplied to cells at 2-day intervals. For binding assays, cells were incubated at 4°C for 1 h with ^{125}I-endothelin-1 (920 Bq) in the presence (nonspecific binding) or absence (total binding) of 10^{-6} M unlabeled endothelin-1. Specific binding was defined as the difference between the amounts of radioligand bound in the presence and the absence of 1 mM unlabeled endothelin-1. Subsequent steps for quantitation of the extent of binding of the radiolabeled ligand are described in "EXPERIMENTAL PROCEDURES." The expression of receptors during a 13-day culture period was examined. Values represent the means ± SE of results from three wells. Data are representative of results from three separate experiments that yielded similar results.

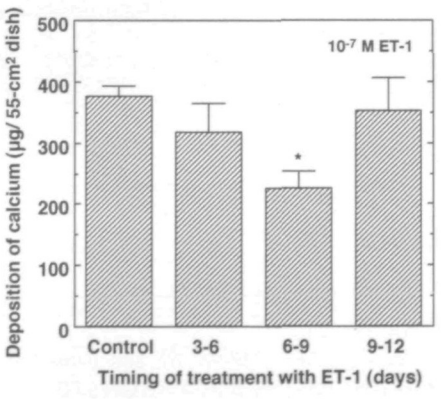


Fig. 5. **Effects of pulsed treatment with endothelin-1 on the mineralization by ROB cells.** Cells in 12-well plates were cultured with α-MEM containing 10% fetal bovine serum, 5 mM β-glycerophosphate, and 50 μg/ml L-ascorbic acid, and they were treated with 10^{-7} M endothelin-1 during the indicated periods. Fresh medium was supplied every 3 days, and endothelin-1 was added at 3-day intervals as indicated. On day 12, Ca^{2+} derived from hydroxyapatite was assayed as described in "EXPERIMENTAL PROCEDURES." Data represent the means ± SE of results from three wells. $^*p < 0.01$ *versus* control.

thelin-1 on the deposition of calcium by ROB cells. As shown in Fig. 5, the presence of 10^{-7} M endothelin-1 from day 6 through day 9 exclusively suppressed mineralization by ROB cells. Furthermore, ipriflavone inhibited the inhibitory effects of endothelin-1 on the deposition of calcium by ROB cells, as shown in Fig. 6.

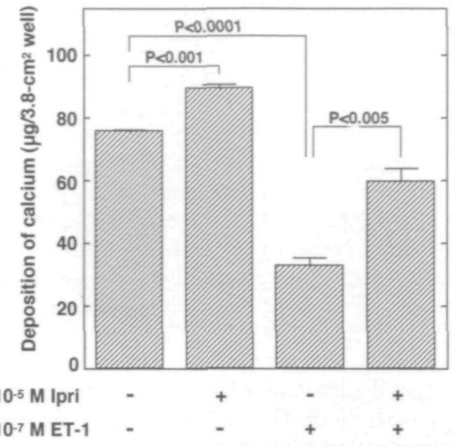


Fig. 6. **Interference by ipriflavone with the inhibitory effects of endothelin-1 on mineralization.** Cells in 12-well plates were cultured with α-MEM containing 10% fetal bovine serum, 5 mM β-glycerophosphate, and 50 μg/ml L-ascorbic acid and treated with 10^{-5} M ipriflavone (ipri) and/or 10^{-7} M endothelin-1 as indicated. Fresh medium was supplied every 4 days, and ipriflavone and endothelin-1, as indicated, were added to cultures at 2-day intervals. On day 14, Ca^{2+} derived from hydroxyapatite was assayed as described in "EXPERIMENTAL PROCEDURES." Data represent the means ± SE of results from three wells.

DISCUSSION

Ipriflavone enhances the activity of alkaline phosphatase, a marker of the differentiation of bone cells, in rat osteosarcoma UMR-106 cells (*9*), in ROB cells (*10*), and in rat bone marrow stromal cells (*11*). Ipriflavone also stimulates the differentiation and mineralization of stromal osteoprogenitor cells in human bone marrow and trabecular bone osteoblasts (*12*). In this study, we showed that ipriflavone stimulated the deposition of calcium and the formation of mineralized nodules by ROB cells, as well as the activity of alkaline phosphatase. Our results indicated that ipriflavone had direct effects on the differentiation and mineralization of rat calvarial osteoblast-like cells. The various observations, when taken together, indicated that ipriflavone stimulated both endochondral ossification and membranous ossification. However, the mechanism whereby ipriflavone stimulated osteoblastic differentiation and mineralization remained to be elucidated.

In our investigation of the mechanism of action of ipriflavone, we noted the possible involvement of the endothelin system. Endothelins evoke a variety of physiological responses: they have potent vasoconstrictive and vasopressor activities (*15*), and they can regulate neuroendocrine functions (*29, 30*) and modulate cell growth (*31, 32*). In addition, endothelin-1 can regulate the differentiation of adipocytes (*33*). The effects of endothelins on bone metabolism have been reported by several groups. In endothelin-1-deficient mice, the formation of bone and of cartilage that is derived from the branchial arch is abnormal (*21*). Moreover, endothelin-1 has been reported to activate (*34*) and to inhibit (*35, 36*) bone resorption by osteoclasts. Endothelins also increase the turnover of inositol phosphate and decrease the activity of alkaline phosphatase in osteoblastic cells (*19, 20, 23*). In previous studies, we

demonstrated that endothelin-1 acts to inhibit both the differentiation of ROB cells and mineralization (*23, 24*). In addition, BQ-123, a specific antagonist for the endothelin ET_A receptor, accelerates the formation of mineralized nodules by ROB cells, indicating that endothelin-1 might act on osteoblast differentiation in an autocrine fashion (unpublished data). Thus, the endothelin system seems to have the potential to inhibit osteoblast differentiation and the formation of bone. In the present study, we examined whether responses to endothelin-1 can be influenced by ipriflavone. We found, as shown in Fig. 4, that ipriflavone down-regulated the endothelin ET_A receptors in ROB cells. This down-regulation might be controlled post-transcriptionally, since ipriflavone had no effect on the level of the mRNA for endothelin ET_A receptors (Fig. 3). Our results suggest that ipriflavone might interfere with the inhibitory effects of endothelin-1 on differentiation and mineralization. In addition to ipriflavone, other factors have been reported to down-regulate the expression of endothelin receptors in osteoblastic cells. Bone morphogenetic protein-7, which stimulates the expression of several features of osteoblasts, down-regulates endothelin ET_A receptors in primary rat osteoblasts (*37*). Moreover, levels of endothelin receptors in rat osteoblastic sarcoma cells can be reduced by 1,25-dihydroxy-vitamin D_3 (*38*). These observations imply that responsiveness to endothelin-1 might be necessary for bone formation *in vivo*. In future, we shall examine the way in which ipriflavone decreases the expression of endothelin receptors in ROB cells.

We examined the effects of ipriflavone on the production of endothelin-1 by ROB cells by use of radioimmunoassay. However, we could not determine the effect of ipriflavone, since the amounts of endothelin-1 that were formed by both control and 10^{-5} M ipriflavone-treated cells were below the limit of detection ($\sim$1 pg/ml).

In conclusion, in this study using ROB cells, we characterized the responses of osteoblasts to ipriflavone and investigated whether responses to the vascular mediator endothelin-1 might be regulated by ipriflavone. Our results demonstrate that ipriflavone stimulates bone formation by ROB cells. Ipriflavone also halved the level of expression of endothelin receptors in ROB cells around day 7, when endothelin-1 is effective in the inhibition of mineralization. These observations suggest that ipriflavone might regulate the formation of bone by ROB cells *via* suppression of the expression of endothelin receptors in ROB cells at a specific time.

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