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Identification and Characterization of Endothelin Receptors on Rat Osteoblastic Osteosarcoma Cells: Down-regulation by 1,25-Dihydroxy-vitamin D_3

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SUMMARY

Endothelins (ETs) (ET-1, ET-2, and ET-3), a family of 21-amino acid peptides, mediate a host of biological responses by binding to specific cell surface receptors termed ET_A and ET_B. Because a role for ET in bone remodeling has been suggested, the present study was undertaken (a) to characterize ET receptors and their responses in the rat osteosarcoma cell line ROS 17/2.8 and (b) to study their regulation by 1,25-dihydroxy-vitamin D_3. Binding studies using ^{125}I-ET-1 (a nonselective agonist) and ^{125}I-IRL-1620 (an ET_B receptor-selective agonist) indicated that these cells display high affinity ET_A and ET_B receptors in the ratio of 3:1. Addition of ET-1 or sarafotoxin 6c to *myo*-[^{3}H]inositol-labeled cells resulted in an increase in inositol phosphate accumulation as well as in intracellular Ca^{2+} release, suggesting that these receptors are coupled to phospholipase C. In addition, ET-1 but not sarafotoxin 6c induced a modest increase in the expression of osteocalcin protein that was completely blocked by BQ123 (an ET_A receptor-selective antagonist), indicating that activation of ET_A receptors plays a role in the induction of osteocalcin. Treatment of ROS osteoblasts with 10 nM 1,25-dihydroxy-vitamin D_3 for 14 hr resulted in a significant (>50%) decrease in ^{125}I-ET-1 and ^{125}I-IRL-1620 binding. This decrease in binding was shown to be due to a decrease in the number of ET receptors, with no change in affinity. Although both ET_A and ET_B receptors were down-regulated in response to 1,25-dihydroxy-vitamin D_3, only ET_A receptor mRNA levels were significantly decreased, with very little change in ET_B mRNA levels. These data indicate that ROS osteoblasts display both ET_A and ET_B receptors that are functional. Induction of osteocalcin was primarily mediated by ET_A receptors, and these receptors were also down-regulated at the mRNA level by 1,25-dihydroxy-vitamin D_3.

ET-1 is a vasoactive hormone synthesized and released by vascular endothelial cells (1). After the initial isolation of ET-1, subsequent gene cloning and peptide isolation studies revealed the presence of two related peptides, ET-2 and ET-3, and a series of snake venom peptides (sarafotoxins) (2). Only ET-1 is produced by endothelial cells, whereas the sites of ET-2 and ET-3 are presently unknown. Although initially identified for potent vasoconstrictor activity (1), ET peptides are now known to exert diverse biological effects through specific receptors present on a wide variety of tissues and cell types (3–6).

The biological responses to ET peptides are mediated by two distinct receptors, namely ET_A and ET_B, that have been cloned, sequenced, and expressed from many species including humans (7–9). There are some reports in the literature suggesting binding sites for ET on cultured osteoblasts derived from rat calvaria (8, 10) and the rat osteoblast-like osteosarcoma cell line ROS 17/2.8 (11). The profiles of the receptor composition reported in these studies are varied and, furthermore, appear to be dependent upon the stage of differentiation or the type of osteoblast-like cell used. Nevertheless, the presence of ET receptors on osteoblasts and the presence of ET receptor mRNA in ROS 17/2.8 cells (8), the proximity of endothelial cells to bone marrow (12), and the effects of ETs on osteoblasts and osteoclasts (13–16) strongly suggest that ETs might play an important role in bone remodeling.

Regulation of ET receptors and their functional role have attracted considerable attention (17). For example, ET receptors are down-regulated during myocardial ischemia (18) and by agents such as thrombin (19) and glucocorticoids (20) and are up-regulated during cyclosporin A-induced nephrotoxicity (21). Vit D_3 and its analogs play a very important role in regulating the functions of bone-derived cells (22). Clinically, vitamin D administration has been shown to stimulate bone formation *in vivo* (23). It was therefore of interest to test the effects of this class of agents on ET receptor regulation in an osteoblast population. This investigation was undertaken to specifically examine (a) the subtypes of ET receptors and their involvement in osteocalcin production and (b) receptor cross-

ABBREVIATIONS: ET, endothelin; Vit D_3, 1,25-dihydroxy-vitamin D_3; S6c, sarafotoxin 6c; RIA, radioimmunoassay; KRH, Krebs-Ringer-HEPES; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AM, acetoxymethyl ester; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; SDS, sodium dodecyl sulfate; SSC, standard saline citrate.

talk, if any, between two hormones that may be involved in similar functions such as bone remodeling.

Using the rat osteoblast-like osteosarcoma cell line ROS 17/2.8 and subtype-selective ligands such as S6c, which is a selective agonist for ET_B receptors (24), and BQ123, which is a selective antagonist for ET_A receptors (25), we have demonstrated the presence of ET_A and ET_B receptors on ROS osteoblasts. Both receptors are down-regulated by Vit D_3 treatment. In addition, it appears that ET_A receptors mediate the induction of osteocalcin in these cells.

Materials and Methods

Reagents. The osteocalcin RIA kit was purchased from Biomedical Technologies (Stoughton, MA). Vit D_3 was a gift from Dr. M. Uskokovic (Hoffman-La Roche, Nutley, NJ). The Fast Track mRNA isolation kit was purchased from Invitrogen (San Diego, CA). *myo*-[^{3}H]Inositol (80–120 mCi/mmol) was obtained from Amersham (Arlington Heights, IL).

Cell culture. ROS 17/2.8 cells were maintained in Ham's F-12 medium supplemented with 10% FCS (Hyclone, Logan, UT), 0.1 mM penicillin-streptomycin, and 0.2 mM L-glutamine, at 37° in 5% CO_2 with humidification.

Radioligand binding. ^{125}I-ET-1 binding to ROS 17/2.8 cells was performed as described previously (20). Saturation binding experiments were performed using increasing concentrations of ET-1 (50–700 pM) in the absence (total binding) and presence (nonspecific binding) of 1 μM unlabeled ET-1. For competition binding studies, the binding of 0.3 nM ^{125}I-ET-1 was examined in the presence of increasing concentrations of the indicated compounds. Binding of ^{125}I-ET-3 and ^{125}I-IRL-1620 were done the same way as with ^{125}I-ET-1.

Measurement of intracellular calcium. Intracellular calcium release in ROS 17/2.8 cells was determined following the procedure of Nambi *et al.* (20). Briefly, cells grown in T-150 flasks were trypsinized for 3 min at 37°, washed once with KRH buffer (118 mM NaCl, 4.6 mM KCl, 24.9 mM $NaHCO_3$, 1.0 mM KH_2PO_4, 11.1 mM glucose, 1.1 mM $MgSO_4$, 1.0 mM $CaCl_2$, 5 mM HEPES buffer, pH 7.4, 0.1% bovine serum albumin), and then incubated with fura 2/AM (2 μM) for 30 min at 37°. At the end of the 30-min incubation, the cells were washed and incubated for another 10 min in the absence of fura 2/AM. At the end of this incubation, the cells were washed with KRH buffer, resuspended at 5×10^6 cells/ml, and kept on ice until use. Calcium transients were measured with a spectrofluorometer (designed by Biomedical Instrumentation Group, University of Pennsylvania, Philadelphia, PA), at 5×10^5 cells/ml of KRH buffer. Interexperimental variability was <10%.

Measurement of inositol phosphates. ROS 17/2.8 osteoblasts were grown to confluency in 24-well multidishes in DMEM containing 1% FCS. The day before the experiment, the medium was changed to DMEM without inositol. The cell monolayers were incubated for 14 hr in this medium in the presence of 1.0 μCi/ml *myo*-[^{3}H]inositol. On the day of the experiment, cells were washed and resuspended in Dulbecco's phosphate-buffered saline containing 0.2% bovine serum albumin, 5 mM glucose, and 10 mM $MgCl_2$ and were then challenged with agonists or antagonists in the presence of 10 mM LiCl as indicated. After a 15-min incubation at room temperature, the reaction was stopped and inositol phosphates were measured as described (26). Interexperimental variability was <10%.

Measurement of osteocalcin release. ROS osteoblasts were plated in 24-well multidishes at a density of 3×10^4 cells/ml, in Ham's F-12 medium containing 1% FCS, glutamine, penicillin, and streptomycin, as described above under Cell culture. The cultures were pretreated with or without the indicated concentration of BQ123 for 15 min and then with ET-1 or S6c for 48 hr at 37°, in a 5% CO_2 humidified incubator. At the end of the incubation, osteocalcin in culture supernatants was determined with the RIA kit, according to the manufacturer's instructions. The lower limit of this assay was typically 30 pg/ml. The treatment time of 48 hr was selected based on a time-course study of ET-1-induced osteocalcin release. Measurable levels of osteocalcin were detected after 48-hr treatment, and levels increased slightly after 72-hr and 96-hr treatment.

Isolation and Northern blot analysis of poly(A)$^+$ RNA. Poly(A)$^+$ RNA was isolated from ROS osteoblasts after treatment with or without 10 nM Vit D_3 (24-hr treatment), using the Fast Track mRNA isolation kit (27). RNA was fractionated by electrophoresis on 1% agarose-formaldehyde gels, transferred to a UV-Duralon nylon membrane, and cross-linked to the membrane using a UV Stratalinker 180 (Stratagene, La Jolla, CA). Nylon blots were prehybridized at 37° in 10 ml of 35% formamide, 50 mM $NaH_2PO_4 \cdot H_2O$, 0.75 M NaCl, 5 mM EDTA, pH 7.4, 0.2% sodium pyrophosphate, 0.5% SDS, 8% dextran sulfate, 250 μg/ml salmon sperm DNA. Next, [^{32}P]dCTP-labeled ET_A or ET_B receptor cDNA (9) and β-actin cDNA (specific activity, 1×10^8 cpm/μg of DNA), labeled by the random primer method, were added as probes and hybridized at 42° for 18 hr. The blots were washed twice with 6× SSC (0.9 M NaCl, 90 mM sodium citrate, pH 7)/0.1% SDS, followed by two washes with 2× SSC/0.1% SDS and finally two washes with 0.2× SSC/0.1% SDS at 65°. Blots were exposed to Kodak X-Omat AR film, with intensifying screens, at −70°.

Results and Discussion

Identification and Characterization of ET Receptors on ROS Osteosarcoma Cells

Addition of increasing concentrations of either ^{125}I-ET-1, ^{125}I-ET-3, or ^{125}I-IRL-1620 (50–700 pM) to ROS cells resulted in saturable binding of all three ligands. The Scatchard transformation of the specific binding from saturation binding experiments yielded one-site binding for all three ligands, with apparent dissociation constants (K_d) of 133, 166, and 266 pM for ^{125}I-ET-1, ^{125}I-ET-3, and ^{125}I-IRL-1620, respectively (Fig. 1). The maximum binding (B_{max}) values were 55,000, 19,500, and 20,500 sites/cell for ^{125}I-ET-1, ^{125}I-ET-3, and ^{125}I-IRL-1620, respectively (Fig. 1), indicating the presence of both ET_A and ET_B receptor subtypes. ^{125}I-ET-3 and ^{125}I-IRL-1620 (ET_B receptor-selective agonists) labeled the same number of binding sites,

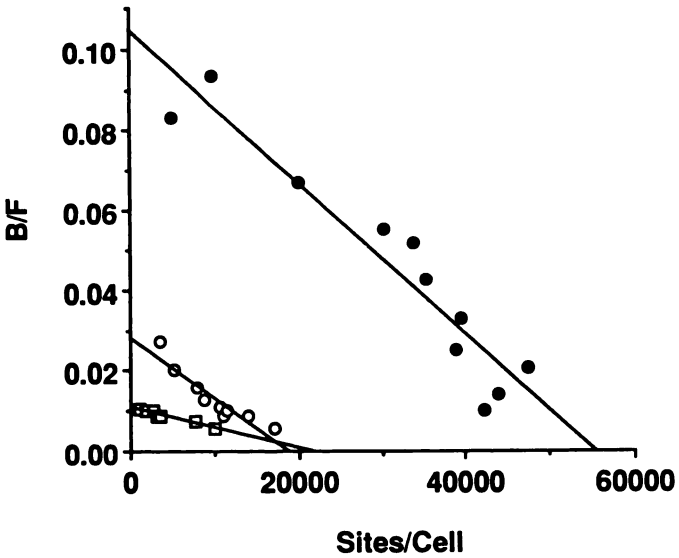

Fig. 1. Scatchard analysis of ^{125}I-ET-1, ^{125}I-ET-3, and ^{125}I-IRL-1620 binding to ROS 17/2.8 osteosarcoma cells. ROS osteoblasts in 24-well plates were assayed for ^{125}I-ET-1 (●), ^{125}I-ET-3 (○), or ^{125}I-IRL1620 (□) binding with increasing concentrations of the respective ligands (50–700 pM), in the presence (nonspecific binding) or absence (total binding) of 1 μM of the appropriate unlabeled ligand. Scatchard plots from the saturation binding data were analyzed and plotted using the Lundon I program. The data presented are from one experiment, which is representative of two similar experiments.

which was ~35% of the number labeled with ^{125}I-ET-1 (which binds to ET_A and ET_B receptors with similar affinities), suggesting that the ratio of ET_A to ET_B receptors in these cells is 65:35.

The presence of ET_A and ET_B receptor subtypes was further confirmed by performing competition binding experiments using ^{125}I-ET-1, unlabeled ET-1, and the subtype-selective ligands S6c (an ET_B receptor-selective agonist) and BQ123 (an ET_A receptor-selective antagonist). The data presented in Fig. 2 indicate that, whereas unlabeled ET-1 displayed a monophasic competition curve, S6c and BQ123 inhibited ^{125}I-ET-1 binding by only ~35 and 65%, respectively. These data agree well with the saturation binding results and confirm the presence as well as the proportion of ET_A and ET_B receptors present in these cells. Data from previous studies (8, 10, 14, 28) on the distribution of ET receptor subtypes have been confusing. Sakurai *et al.* (8) demonstrated the presence of ET_B receptor mRNA in osteoblast-like osteosarcoma ROS 17/2 cells, whereas Takuwa *et al.* (10) showed that cultured osteoblastic cells from rat calvaria displayed much higher sensitivity to ET-1, compared with ET-3, indicating the presence of ET_A receptors. Our data clearly demonstrate the presence of both ET_A and ET_B receptors in the ROS 17/2.8 clone. The reasons for these different observations may be due to the cells being in different stages of differentiation, the type of osteoblast-like cells used, or clonal variation.

Functional Studies

Inositol phosphate accumulation. Next, experiments were performed to test whether these ET receptors were functional. Because most of the studies performed on the signal transduction pathways of ET have shown the activation of phospholipase C by ET-1, we measured ET-1-mediated inositol phosphate accumulation in ROS cells. Exposure of *myo*-[^{3}H] inositol-prelabeled ROS cells to ET-1 resulted in increased accumulation of inositol phosphates that was dependent on the concentration of ET-1 used, with an EC_{50} value of 0.3 nM (Fig. 3A). S6c also induced inositol phosphate accumulation, but the maximum accumulation was only 30% of that obtained with ET-1, further confirming the presence of ET_B receptors that are ~30% of the total ET receptors. These data also demonstrate that both ET_A and ET_B receptors are coupled to the activation of phospholipase C. Pretreatment of ROS cells with BQ123, an ET_A receptor-selective antagonist, resulted in dose-dependent inhibition of ET-1-mediated inositol phosphate accumulation (Fig. 3B). The maximum inhibition by BQ123 of ET-1-mediated inositol phosphate accumulation was 60–65%, suggesting that 65% of the ET-1-mediated response was through ET_A receptors and that the remainder was through the activation of ET_B receptors. In the same experiments, BQ123 did not have any effect on S6c-induced inositol phosphate accumulation (Fig. 3B).

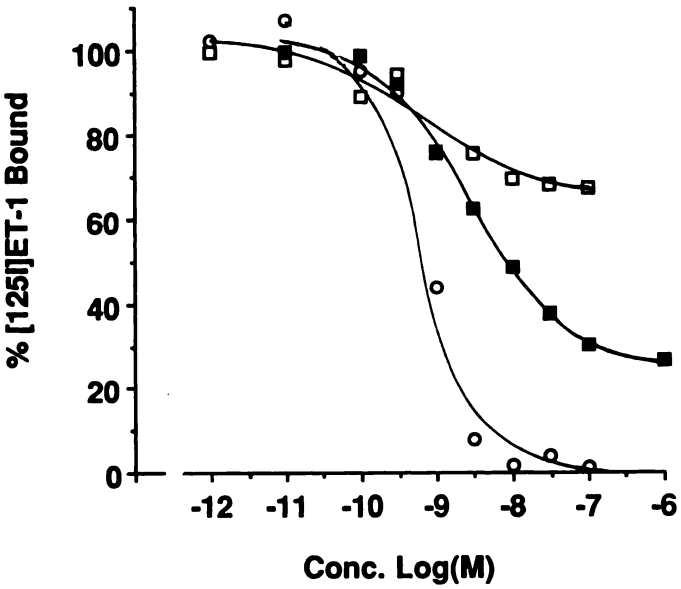

Fig. 2. Competition with ^{125}I-ET-1 binding to ROS 17/2.8 cells by various unlabeled ligands. Cells in 24-well plates were incubated with 0.3 nM ^{125}I-ET-1 in the absence or presence of increasing concentrations of unlabeled ET (○), S6c (□), or BQ123 (■). At the end of a 1-hr incubation at room temperature, the cells were washed four times with Dulbecco's phosphate-buffered saline, solubilized with 1% SDS, and counted. The data presented are from one of two experiments.

Intracellular calcium release. The increase in inositol phosphate accumulation was accompanied by a corresponding increase in intracellular calcium concentration, as shown in Fig. 3C. The extent of calcium release in response to S6c was ~30% of that obtained with ET-1 and compares well with inositol phosphate responses obtained with these two ligands (Fig. 3A). Addition of BQ123 blocked the ET-1-mediated calcium release and did not have any effect on S6c-mediated calcium release (data not shown).

Osteocalcin production. In addition to these second messengers, ET-1 also mediated the release of osteocalcin when ROS cells were challenged with ET-1 for 48 hr. The overall increase in osteocalcin levels in ET-1-treated culture supernatants was rather modest (1.5-fold over control) but significant. Unlike the other responses described above, S6c did not have any effect on osteocalcin production, indicating that this is an ET_A receptor-mediated effect. This was further confirmed by the use of BQ123, which inhibited ET-1-mediated osteocalcin release completely (Fig. 4). It is interesting to note that Shiode and Noda (14) recently demonstrated a 2-fold increase in osteocalcin mRNA production in ROS 17/2.8 cells that were challenged with ET-1. In addition, there was no increase in osteocalcin mRNA production when the cells were challenged with ET-3, indicating the involvement of ET_A receptors. These observations agree very well with the data presented here, which show a 1.5-fold increase in osteocalcin protein production in response to ET-1 that is mediated by ET_A receptors. The levels of osteocalcin release induced by ET-1 treatment are rather modest; however, comparable increases in osteocalcin have been reported with other well characterized stimuli, such as Vit D_3 and parathyroid hormone treatment (29). Osteocalcin, also called bone γ-carboxyglutamic acid protein, is an abundant Ca^{2+}-binding protein indigenous to the organic matrix of bone, dentin, and possibly other mineralized tissues. It is regarded as a highly specific osteoblast marker produced during bone formation (30), and its expression has been shown to be positively influenced by calcitropic hormones and cytokines. The fact that ET is among the effectors that enhance the release of osteocalcin, as shown in the present study, strongly supports a role for this peptide in osteoblastic function.

Vit D_3 Effects

Because Vit D_3 has been shown to regulate the functions of bone-derived cells (31, 32), it was of interest to test whether

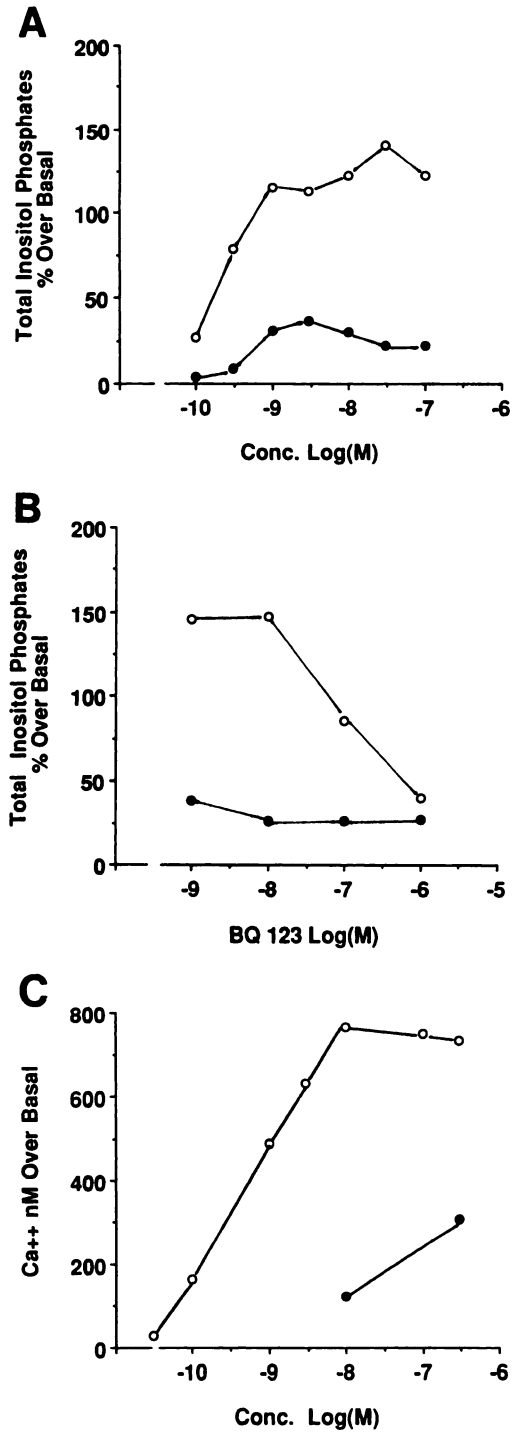

Fig. 3. A, Inositol phosphate accumulation in ROS 17/2.8 cells in response to ET-1 and S6c. Cells in 24-well plates were labeled with 1.0 μCi/ml *myo*-[^{3}H]inositol for 16 hr, in DMEM without inositol. At the end of labeling, the cells were washed and challenged with increasing concentrations of ET-1 (○) or S6c (●) for 15 min, and the accumulated inositol phosphates were measured as described. The data presented are from one of three similar experiments. B, Effect of BQ123 on ET-1- and S6c-mediated inositol phosphate accumulation in ROS 17/2.8 cells. *myo*-[^{3}H]Inositol-prelabeled cells were exposed to 10 nM ET-1 (○) or S6c (●) in the presence of increasing concentrations of BQ123 for 15 min, and the accumulated inositol phosphates were quantitated as described in Materials and Methods. The data are from one experiment, which is representative of two similar experiments. C, Intracellular calcium release in response to ET-1 and S6c in ROS 17/2.8 cells. Increasing concentrations of ET-1 (○) or S6c (●) were added to fura-2/AM-preloaded ROS 17/2.8 cells, and calcium release was measured as described in Materials and Methods. The basal intracellular calcium concentration was 120 nM. The data are from one of two similar experiments.

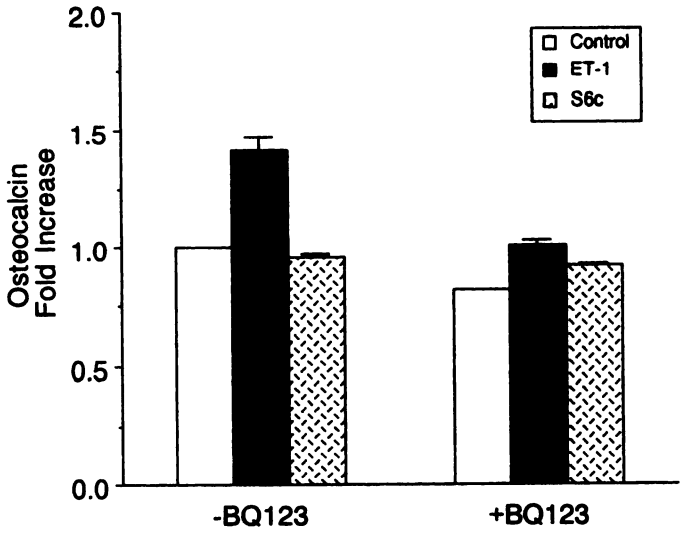

Fig. 4. Osteocalcin induction in response to ET-1 treatment. Cells in 24-well plates were treated with 10 nM ET-1 or S6c in the presence or absence of 1 μM BQ123. After a 48-hr incubation at 37°, culture supernatants were assayed for osteocalcin levels by RIA. The data presented here are representative of three individual experiments. *Error bars*, standard errors from triplicate cultures.

pretreatment of ROS cells with Vit D_3 had any effect on ET binding and/or function. Exposure of ROS cells to 10 nM Vit D_3 for 24 hr resulted in a 40–60% decrease in ^{125}I-ET-1 binding (Fig. 5, *top* and *middle*). Based on a dose-response study (data not shown) using increasing concentrations of Vit D_3 (0.01–100 nM), 10 nM Vit D_3 was found to elicit a maximal response for the induction of osteocalcin production. Hence, it was chosen as the optimal concentration for all of the subsequent experiments. The Vit D_3-induced decrease in ^{125}I-ET-1 binding was due to a decrease in maximum binding with no change in affinity, as shown in Fig. 5, *bottom*. Also, the decrease was due not to an increase in the nonspecific binding but to a decrease in total binding (Fig. 5, *top* and *middle*). A similar decrease in ^{125}I-IRL-1620 binding was also observed in Vit D_3-treated osteoblast cells, indicating down-regulation of ET_B receptors also by Vit D_3 (data not shown). A kinetic analysis to determine the time course of Vit D_3 exposure from 30 min to 24 hr suggested that the maximal down-regulation of ET receptors occurred between 7 and 8 hr after treatment, with no subsequent change up to 24 hr (data not shown). Because there was no difference in the extent of ET receptor down-regulation between 7 and 24 hr of treatment, the latter time point was selected for the sake of convenience in subsequent studies. The Vit D_3-induced decrease in ET receptors was reflected in ET-1-mediated inositol phosphate accumulation as well (Fig. 6). There was a ~50% decrease in ET-1-mediated inositol phosphate accumulation in Vit D_3-treated ROS cells (Fig. 6), but there was no difference between the EC_{50} values obtained in control and Vit D_3-treated ROS cells for the ET-1-mediated response. This agrees well with the binding data, where the change observed in Vit D_3-treated cells was only in the maximum binding, with no change in affinity.

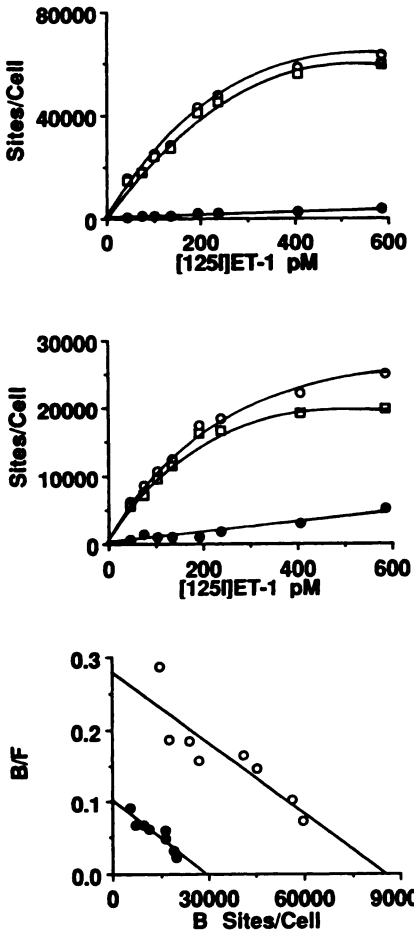

Fig. 5. Saturation binding of ^{125}I-ET-1 to control and Vit D_3-treated ROS 17/2.8 cells. *Top* and *middle*, Increasing concentrations of ^{125}I-ET-1 were added to control cells (*top*) or Vit D_3-pretreated cells (*middle*) in the absence or presence of 1 μM unlabeled ET-1, and cells were incubated for 60 min at room temperature. At the end of the incubation, the cells were washed, solubilized with 1% SDS, and counted. ○, Total binding; ●, nonspecific binding; □, specific binding. *Bottom*, Scatchard transformation of the specific binding data obtained from saturation binding experiments performed with control (○) or Vit D_3-treated (●) ROS 17/2.8 cells. The data are from one of three similar experiments.

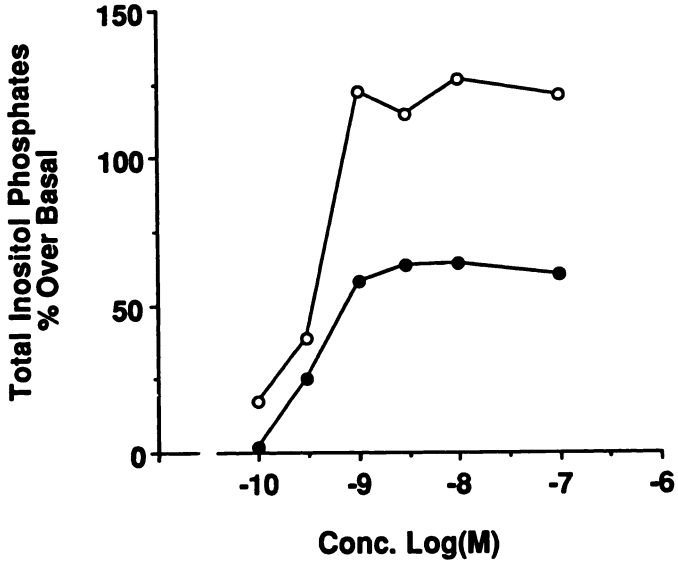

Fig. 6. Inositol phosphate accumulation in response to ET-1 in control and Vit D_3-treated ROS 17/2.8 cells. Control cells (○) and Vit D_3-treated cells (●) were prelabeled with *myo*-[^{3}H]inositol in 24-well plates and then challenged with increasing concentrations of ET-1. After a 15-min incubation at room temperature, the inositol phosphates were separated as described. The data are from one of three similar experiments.

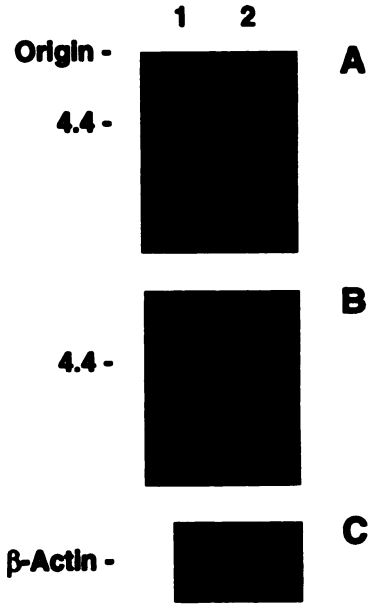

Fig. 7. Northern analysis of poly(A)$^+$ RNA from control and Vit D_3-treated ROS cells. Steady state levels of mRNA were determined by Northern blotting as described in Materials and Methods, using ET_A receptor (A), ET_B receptor (B), and β-actin (C) cDNAs from control (*lane 1*) and Vit D_3-treated (*lane 2*) ROS cells. The results shown here represent one of two separate experiments performed with cells from two different passages. Similar results were obtained in both experiments.

Northern Analysis

To test whether the Vit D_3-mediated decrease in ET receptors was at the transcriptional level, Northern blot analyses were performed using poly(A)$^+$ RNA isolated from control and Vit D_3-treated ROS cells. When the blots were probed for ET_A and ET_B receptors using appropriate cDNA probes, there was no detectable ET_A mRNA in Vit D_3-treated ROS cells but there was very little change in ET_B mRNA, indicating that there might be selective down-regulation of ET_A receptor message (Fig. 7A). Although there was a comparable loss (~50%) of ^{125}I-IRL-1620 (an ET_B receptor-selective agonist) binding in Vit D_3-treated cells, this may reflect a post-transcriptional event, because the ET_B receptor message level did not change (Fig. 7B). Differences observed between control and Vit D_3-treated cells in the levels of expression of ET_A receptor mRNA were not due to differences in the amounts of poly(A)$^+$ RNA loaded, because in the two preparations the levels of β-actin message were similar (Fig. 7C). It is interesting to note that only the subtype of ET receptors coupled to osteocalcin production (ET_A) was sensitive to Vit D_3 regulation at the mRNA level.

The physiological significance of ET receptors on osteoblasts and their subsequent down-regulation by Vit D_3 is not clear at this time. The demonstration of ET_A and ET_B receptors on ROS 17/2.8 osteosarcoma cells (shown in the present study), as well as on primary rat calvarial cells and human osteoblasts (data not shown), strongly suggests that ET receptors have a

major role in bone remodeling. One possibility is that, similarly to transforming growth factor-β (33), ET-1 could be instrumental in promoting communication between endothelial cells in the vasculature and bone cells in the local environment, to stimulate bone formation and inhibit bone resorption. Signals such as Vit D_3 could perhaps serve to regulate ET functions by down-regulating the responsive receptors, thereby helping to maintain homeostasis between the resorption and formation events. In conditions where there is an excess of ET-1, it may be necessary for the system to be down-regulated by agents such as Vit D_3 to maintain homeostasis. In this context, it is interesting to note that Vit D_3 regulates at the mRNA level only the ET receptor subtype that is involved in osteocalcin production. Clearly, additional experiments are necessary to delineate the mechanisms involved in the process of bone remodeling. In a very elegant study, Kurihara *et al.* (34) reported that one of the consequences of disruption of the ET-1 gene in mouse embryonic stem cells was that the ET-1 −/− homozygotes exhibited severely retarded mandibular bones, aberrant zygomatic temporal bones, and many other skeletal abnormalities and died of respiratory failure. These findings not only provide additional support for a role of ET-1 in bone remodeling but also provide evidence for the involvement of ET-1 in embryonic bone development.

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