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Involvement of Phospholipase D Activation in Endothelin-1–Induced Release of Arachidonic Acid in Osteoblast-Like Cells

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Abstract In a previous study, we have shown that endothelin-1 (ET-1) activates phospholipase D independently from protein kinase C in osteoblast-like MC3T3-E1 cells. It is well recognized that phosphatidylycholine hydrolysis by phospholipase D generates phosphatidic acid, which can be further degraded by phosphatidic acid phosphohydrolase to diacylglycerol. In the present study, we investigated the role of phospholipase D activation in ET-1–induced arachidonic acid release and prostaglandin E_2 (PGE_2) synthesis in osteoblast-like MC3T3-E1 cells. ET-1 stimulated arachidonic acid release dose-dependently in the range between 0.1 nM and 0.1 μM. Propranolol, an inhibitor of phosphatidic acid phosphohydrolase, significantly inhibited the ET-1–induced arachidonic acid release in a dose-dependent manner as well as the ET-1–induced diacylglycerol formation. 1,6-bis-(cyclohexyloxyminocarbonylamino)-hexane (RHC-80267), an inhibitor of diacylglycerol lipase, significantly suppressed the ET-1–induced arachidonic acid release. The pretreatment with propranolol and RHC-80267 also inhibited the ET-1–induced PGE_2 synthesis. These results strongly suggest that phosphatidylcholine hydrolysis by phospholipase D is involved in the arachidonic acid release induced by ET-1 in osteoblast-like cells. J. Cell. Biochem. 64:376–381.

Key words: endothelin-1; phospholipase D; arachidonic acid; osteoblasts

Endothelin (ET) is a potent vasoconstrictive peptide consisting of three isotypes, ET-1, ET-2, and ET-3 [Yanagisawa et al., 1988; Simonson and Dunn, 1990; Masaki, 1993]. It is nowadays recognized that ET has a wide variety of effects on both vascular and nonvascular tissues through its binding to specific receptors [Simonson and Dunn, 1990; Masaki, 1993]. In bone tissue, it has been shown that ET receptors exist in osteoblasts [Takuwa et al., 1990]. ET-1 has been reported to induce bone resorption and stimulate collagen and noncollagen protein synthesis and DNA synthesis in cultured neonatal mouse calvaria [Takuwa et al., 1989, 1990; Sakurai et al., 1992; Tatrai et al., 1992].

As for intracellular signaling system of ET, it has been reported that ET induces phosphoinositide hydrolysis by phospholipase C and mobilizes Ca^{2+} from extra- and intracellular pools in osteoblast-like MC3T3-E1 cells [Takuwa et al., 1989, 1990], which have been derived from newborn mouse calvaria [Kodama et al., 1981; Sudo et al., 1983]. Two second messengers, inositol 1,4,5-trisphosphate and diacylglycerol, are produced from phosphoinositide hydrolysis [Berridge, 1993]. It is well known that diacylglycerol is a physiological activator of protein kinase C [Nishizuka, 1986]. However, phosphoinositide hydrolysis is not the only pathway of diacylglycerol formation [Exton, 1990; Zeisel, 1993]. It is recognized that phospholipase D catalyzes the hydrolysis of phosphatidylcholine, resulting in the formation of phosphatidic acid [Billah and Anthes, 1990; Exton, 1990; Zeisel, 1993]. Phosphatidic acid, which itself could be a potential intracellular mediator, can be further degraded by phosphatidic acid phosphohydrolase to diacylglycerol [Billah and Anthes, 1990; Exton, 1990; Zeisel, 1993]. It is nowadays recognized that phospholipase D

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plays an important role in modulating cellular functions through the activation of protein kinase C, since phosphatidylcholine is the principal phospholipid in cell membranes [Billah and Anthes, 1990; Exton, 1990; Zeisel, 1993]. We have recently shown that ET-1 stimulates phosphatidylcholine-hydrolyzing phospholipase D independently of protein kinase C in osteoblast-like MC3T3-E1 cells [Suzuki et al., 1994].

Prostaglandins (PGs), which are synthesized from arachidonic acid by cellular enzymes, are important bioactive substances and modulate diverse cellular functions in ubiquitous cells [Samuelsson et al., 1978; Smith, 1989]. In bone metabolism, it has been reported that PGE_2 is a major eicosanoid product in osteoblasts including osteoblast-like MC3T3-E1 cells and that it is a potent bone resorptive agent [Nijweide et al., 1986; Raisz and Martin, 1984; Yokota et al., 1986]. It is generally accepted that arachidonic acid is released from the esterified stores of membrane phospholipids by phospholipase A_2 [Irvine, 1982]. However, arachidonic acid could be also released via membrane phospholipids by other phospholipases [Smith, 1989; Dennis et al., 1991]. In the present study, we investigated the role of phospholipase D activation in ET-1–induced arachidonic acid release in osteoblast-like MC3T3-E1 cells. Herein we show that phosphatidylcholine hydrolysis by phospholipase D is involved in the arachidonic acid release induced by ET-1 in osteoblast-like cells.

METHODS

Materials

[5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (208 Ci/mmol) and the *sn*-1,2-diacylglycerol assay system and PGE_2[^{125}I]assay system were purchased from Amersham Japan (Tokyo, Japan). ET-1 was purchased from Peptide Institute Inc. (Minoh, Japan). *dl*-propranolol hydrochloride (propranolol) was purchased from Wako Pure Chemical Industries (Osaka, Japan). 1,6-bis-(cyclohexyloximinocarbonylamino)-hexane (RHC-80267) was purchased from Funakoshi Pharmaceutical Co. (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. Propranolol and RHC-80267 were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide in the culture medium was 0.1%, and this did not affect the measurement of arachidonic acid release, diacylglycerol formation, and assay for PGE_2.

Cell Culture

Cloned osteoblast-like MC3T3-E1 cells were maintained as previously described [Kozawa et al., 1994]. In brief, 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 (5×10^4) were seeded into 35 mm diameter dishes in 2 ml of α-MEM containing 10% FCS. After 5 days, the medium was exchanged for 2 ml of α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h. When indicated, the cells were pretreated with propranolol or RHC-80267 for 20 min.

Measurement of Arachidonic Acid Release

The measurement of arachidonic acid release was performed as previously described [Suzuki et al., 1993]. In brief, the cultured cells were labeled with [^{3}H]arachidonic acid (0.5 μCi/dish) for 24 h. The medium was removed, and the cells were then washed four times with 1 ml of the assay buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, and 1 mM $CaCl_2$). The cells were preincubated subsequently with 1 ml of the assay buffer containing 0.1% essentially fatty acid–free bovine serum albumin (BSA) at 37°C for 20 min, and the cells were then stimulated by various doses of ET-1. After the indicated periods, the medium was collected, and the radioactivity of the medium was determined.

Measurement of Diacylglycerol Formation

The cultured cells were incubated in the assay buffer containing 0.01% BSA at 37°C for 20 min and then stimulated by ET-1 for 20 min. The reaction was terminated by adding 0.75 ml of ice-cold methanol, and the lipids were extracted as previously described [Bligh and Dyer, 1959]. Diacylglycerol was quantitated using the *sn*-1,2-diacylglycerol assay reagent system. The radioactive spot corresponding to phosphatidic acid was analyzed by a BAS2000 (Tokyo, Japan) equipped with imaging plates used as previously described [Amemiya and Miyahara, 1988].

Assay for PGE_2

Procedures were done as described under Measurement of Arachidonic Acid Release except for using unlabeled cells. The cultured

cells were pretreated with propranolol or RHC-80267 for 20 min and then stimulated by ET-1. After 2 h, the medium was collected, and PGE_2 in the medium was measured with a radioimmunoassay kit.

Determination

The radioactivity of ^3H-labeled samples was determined with a Beckman LS-6000IC liquid scintillation spectrometer (Fullerton, CA). The radioactivity of ^{125}I samples was determined with an Aloka ARC-600 autowell gamma system (Tokyo, Japan).

Statistical Analysis

The data were analyzed by Student's *t*-test, and $P < 0.05$ was considered significant. All data are presented as the mean ± S.E. of triplicate determinations.

RESULTS

Effect of ET-1 on Arachidonic Acid Release in MC3T3-E1 Cells

ET-1 (0.1 μM) significantly stimulated arachidonic acid release, compared to the control, in a time-dependent manner up to 20 min in osteoblast-like MC3T3-E1 cells (Fig. 1). The effect of ET-1 on arachidonic acid release was dose-dependent in the range between 0.1 nM and 0.1 μM (Fig. 2). The maximum effect of ET-1 was observed at 0.1 μM.

Effect of Propranolol on ET-1–Induced Arachidonic Acid Release and Diacylglycerol Formation in MC3T3-E1 Cells

We examined the effect of propranolol, an inhibitor of phosphatidic acid phosphohydrolase [Pappu and Hauser, 1983], on ET-1–induced arachidonic acid release in MC3T3-E1 cells. The pretreatment with propranolol, which by itself had little effect on arachidonic acid release, significantly inhibited the ET-1–induced arachidonic acid release in these cells (Fig. 3). The effect of propranolol was dose-dependent in the range between 100 and 300 μM. The inhibitory effect of propranolol (300 μM) on the arachidonic acid release was 51%. In addition, we examined the effect of propranolol on the diacylglycerol formation induced by ET-1 in MC3T3-E1 cells. ET-1 (0.1 μM)–

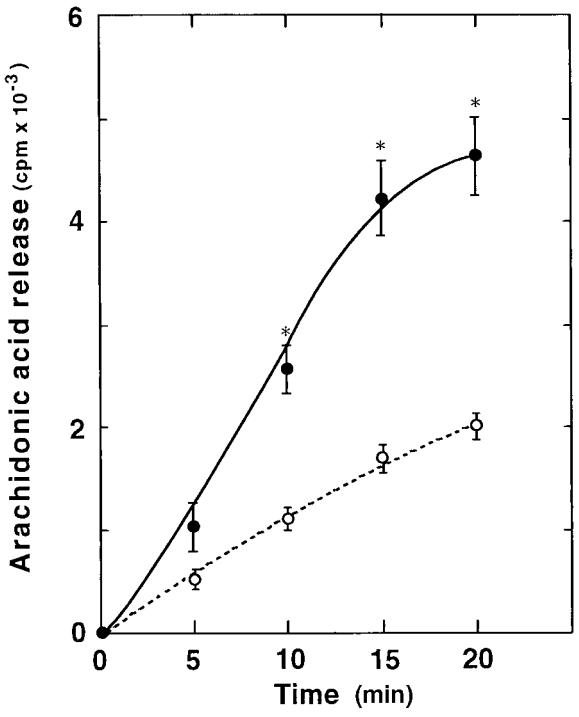

Fig. 1. Time-dependent effect of ET-1 on arachidonic acid release in MC3T3-E1 cells. The [^3H]arachidonic acid–labeled cells were stimulated by 0.1 μM ET-1 (●) or vehicle (○) for the indicated periods. Values represent the means ± S.E. of triplicate determinations of a representative experiment carried out three times. *$P < 0.05$ vs. control values.

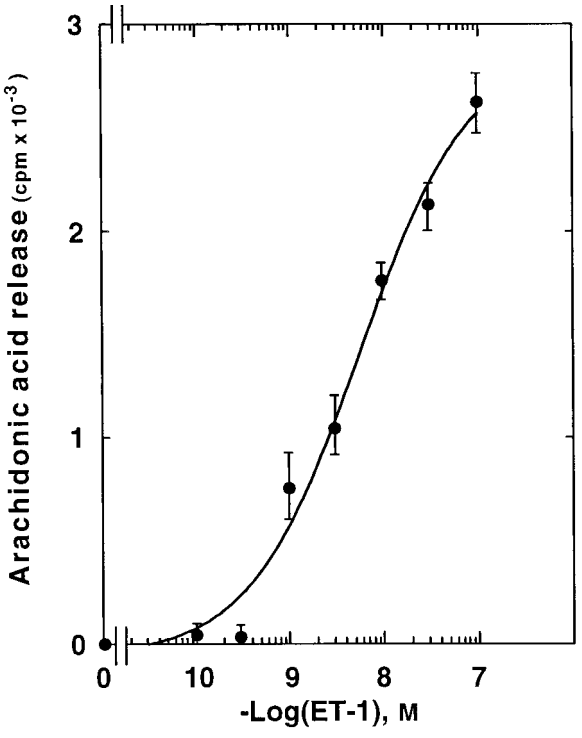

Fig. 2. Dose-dependent effect of ET-1 on arachidonic acid release in MC3T3-E1 cells. The [^3H]arachidonic acid–labeled cells were stimulated by various doses of ET-1 for 20 min. Values for control cells have been subtracted from each data point. Values represent the means ± S.E. of triplicate determinations of a representative experiment carried out three times.

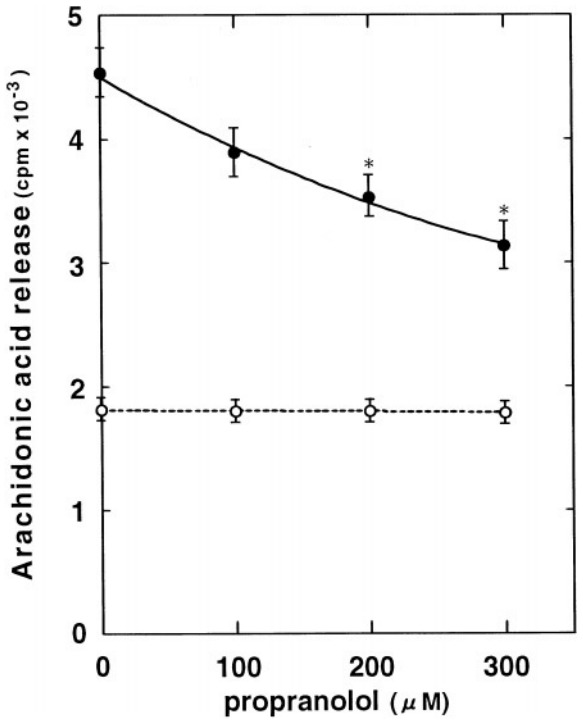

Fig. 3. Effect of propranolol on ET-1–induced arachidonic acid release in MC3T3-E1 cells. The [^{3}H]arachidonic acid–labeled cells were pretreated with various doses of propranolol for 20 min and then stimulated by 0.1 μM ET-1 (●) or vehicle (○) for 20 min. Values represent the means ± S.E. of triplicate determinations of a representative experiment carried out three times. *$P < 0.05$ vs. value of ET-1 without propranolol pretreatment.

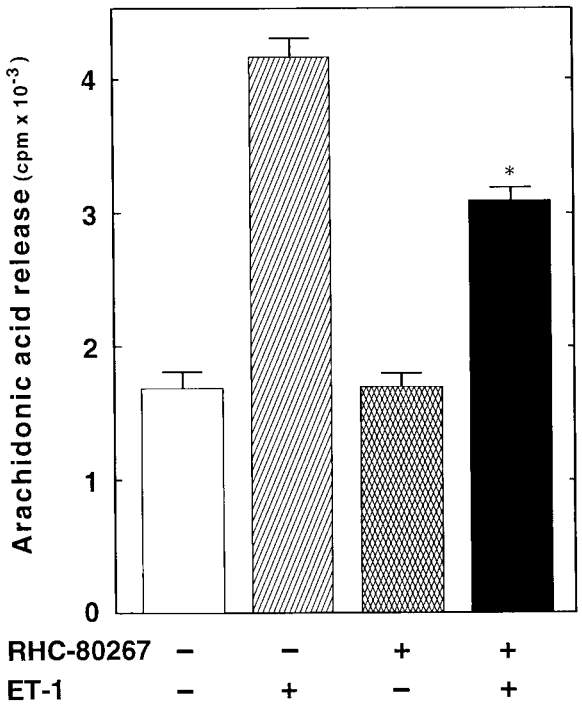

Fig. 4. Effect of RHC-80267 on ET-1–induced arachidonic acid release in MC3T3-E1 cells. The [^{3}H]arachidonic acid–labeled cells were pretreated with 30 μM RHC-80267 or vehicle for 20 min and then stimulated by 0.1 μM ET-1 or vehicle for 20 min. Values represent the means ± S.E. of triplicate determinations of a representative experiment carried out three times. *$P < 0.05$ vs. value of ET-1 without RHC-80267 pretreatment.

TABLE I. Effect of Propranolol on ET-1–Induced Diacylglycerol Formation in MC3T3-E1 Cells*

	Diacylglycerol formation (pmol/dish)
ET-1	1,069 ± 128
Propranolol + ET-1	566 ± 74

*The cultured cells were pretreated with 300 μM propranolol or vehicle for 20 min and then stimulated by 0.1 μM ET-1 for 20 min. Diacylglycerol formation was quantitated as described in Methods. Each value represents the mean ± S.E. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

induced diacylglycerol formation was also reduced by propranolol (300 μM) as well as arachidonic acid release in these cells (Table I). The inhibitory effect of propranolol on the diacylglycerol formation was 53%.

Effect of RHC-80267 on ET-1–Induced Arachidonic Acid Release in MC3T3-E1 Cells

Diacylglycerol is recognized to be an important cellular source of arachidonate which may be generated subsequently by diacylglycerol lipase [Bell et al., 1979]. RHC-80267 has been reported to inhibit selectively diacylglycerol lipase activity [Sutherland and Amin, 1982]. Pretreatment with 30 μM RHC-80267, which by itself had little effect on arachidonic acid release, significantly suppressed the ET-1–induced arachidonic acid release in MC3T3-E1 cells (Fig. 4).

The inhibitory effect of RHC-80267 (30 μM) on the arachidonic acid release was about 44%.

Effects of Propranolol or RHC-80267 on ET-1–Induced PGE_2 Synthesis in MC3T3-E1 Cells

We next examined the effect of propranolol or RHC-80267 on PGE_2 synthesis induced by ET-1 in these cells. The pretreatment with propranolol, which by itself had no effect on PGE_2 synthesis, significantly inhibited PGE_2 synthesis induced by ET-1 in MC3T3-E1 cells (Table II). The inhibitory effect of propranolol (300 μM) was about 85%. Pretreatment with RHC-80267, which by itself had no effect on PGE_2 synthesis, also suppressed PGE_2 synthesis induced by ET-1

TABLE II. Effect of Propranolol or RHC-80267 on ET-1–Induced PGE_2 Synthesis in MC3T3-E1 Cells†

	PGE_2 synthesis (pg/ml)
Control	27 ± 4
Propranolol	28 ± 5
RHC-80267	30 ± 3
ET-1	443 ± 39
Propranolol + ET-1	88 ± 10*
RHC-80267 + ET-1	120 ± 13*

†The cultured cells were pretreated with 300 μM propranolol, 30 μM RHC-80267, or vehicle for 20 min and then stimulated with 0.1 μM ET-1 or vehicle for 2 h. Each value represents the mean ± S.E. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.
*$P < 0.05$ compared to the value of ET-1 alone.

in MC3T3-E1 cells (Table II). The inhibitory effect of RHC-80267 (30 μM) was about 79%.

DISCUSSION

In the present study, we showed that ET-1 stimulated arachidonic acid release time- and dose-dependently in osteoblast-like MC3T3-E1 cells, and propranolol, a phosphatidic acid phosphohydrolase inhibitor [Pappu and Hauser, 1983], significantly inhibited arachidonic acid release induced by ET-1. We previously reported that ET-1 stimulates phosphatidylcholine hydrolysis by phospholipase D independently of protein kinase C in these cells [Suzuki et al., 1994]. Phosphatidylcholine can be hydrolyzed by phospholipase D to yield phosphatidic acid, which is further degraded by phosphatidic acid phosphohydrolase to diacylglycerol [Billah and Anthes, 1990; Exton, 1990; Zeisel, 1993]. So, it seems that the conversion of phosphatidic acid to diacylglycerol is involved in ET-1–induced arachidonic acid release in MC3T3-E1 cells. In addition, we showed that ET-1 induced the formation of diacylglycerol and that propranolol significantly inhibited diacylglycerol formation induced by ET-1 as well as arachidonic acid release in these cells. The degrees of inhibition by propranolol were similar. Thus, these findings suggest that diacylglycerol formation induced by phosphatidylcholine hydrolysis by phospholipase D is involved in ET-1–induced arachidonic acid release in MC3T3-E1 cells. Next, we demonstrated that RHC-80267, which is known to inhibit selectively diacylglycerol lipase [Sutherland and Amin, 1982], significantly inhibited ET-1–induced arachidonic acid release in MC3T3-E1 cells. Thus, this finding suggests that the activation of diacylglycerol lipase is involved in ET-1–induced arachidonic acid release in these cells. Therefore, these results as a whole suggest that ET-1 stimulates arachidonic acid release via phosphatidylcholine hydrolysis by phospholipase D in osteoblast-like MC3T3-E1 cells.

PGE_2 is well known to be a major eicosanoid product in osteoblasts including MC3T3-E1 cells [Raisz and Martin, 1984; Yokota et al., 1986] and to be a potent bone resorbing agent [Nijweide et al., 1986; Zeisel, 1993]. In the present study, we demonstrated that ET-1 stimulated PGE_2 synthesis in MC3T3-E1 cells and that both propranolol and RHC-80267 suppressed ET-1– induced PGE_2 synthesis as well as arachidonic acid release in these cells. Our findings suggest that phosphatidylcholine hydrolysis by phospholipase D is involved in the mechanism of ET-1–induced arachidonic cascade in osteoblast-like MC3T3-E1 cells.

In conclusion, our findings strongly suggest that phosphatidylcholine hydrolysis by phospholipase D is involved in arachidonic acid release induced by ET-1 in osteoblast-like cells.

REFERENCES

Amemiya Y, Miyahara J (1988): Imaging plate illuminates many fields. Nature 336:89–90.

Bell RL, Kennerly DA, Stanford N, Majerus PW (1979): Diglyceride lipase: A pathway for arachidonic acid release from human platelets. Proc Natl Acad Sci U S A 76:3238–3241.

Berridge MJ (1993): Inositol trisphosphate and calcium signalling. Nature 361:315–325.

Billah MM, Anthes JC (1990): The regulation and cellular functions of phosphatidylcholine hydrolysis. Biochem J 269:281–291.

Bligh EG, Dyer WJ (1959): A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37: 911–917.

Dennis EA, Rhee SG, Billah MM, Hannun YA (1991): Role of phospholipases in generating lipid second messengers in signal transduction. FASEB J 5:2068–2077.

Exton JH (1990): Signaling through phosphatidylcholine breakdown. J Biol Chem 265:1–4.

Irvine RF (1982): How is the level of free arachidonic acid controlled in mammalian cells? Biochem J 204:3–16.

Kodama H, Amagai Y, Sudo H, Kasai S, Yamamoto S (1981): Establishment of a clonal osteoblastic cell line from newborn mouse calvaria. Jpn J Oral Biol 23:899–901.

Kozawa O, Suzuki A, Kotoyori J, Tokuda H, Watanabe Y, Ito Y, Oiso Y (1994): Prostaglandin $F_{2\alpha}$ activates phospholipase D independently from activation of protein kinase C in osteoblast-like cells. J Cell Biochem 55:375–379.

Masaki T (1993): Endothelins: Homeostatic and compensatory actions in the circulatory and endocrine systems. Endocrinol Rev 14:256–268.

Nijweide PJ, Burger EH, Feyen JHM (1986): Cells of bone: Proliferation, differentiation, and hormonal regulation. Physiol Rev 66:855–886.

Nishizuka Y (1986): Studies and perspectives of protein kinase C. Science 233:305–312.

Pappu AS, Hauser G (1983): Propranolol-induced inhibition of rat brain cytoplasmic phosphatidate phosphohydrolase. Neurochem Res 8:1565–1575.

Raisz LG, Martin TJ (1984): Prostaglandins in bone and mineral metabolism. In Peck WA (ed): "Bone and Mineral Research, Annual 2." Amsterdam: Elsevier, pp 286–310.

Sakurai T, Morimoto H, Kasuya Y, Takuwa Y, Nakauchi H, Masaki T, Goto K (1992): Level of ET_B receptor mRNA is down-regulated by endothelins through decreasing the intracellular stability of mRNA molecules. Biochem Biophys Res Commun 186:342–347.

Samuelsson B, Goldyne M, Grantstrom E, Hamberg M, Hammarstrom S, Malmsten C (1978): Prostaglandins and thromboxanes. Annu Rev Biochem 47:997–1029.

Simonson MS, Dunn MJ (1990): Cellular signaling by peptides of the endothelin gene family. FASEB J 4:2989–3000.

Smith WL (1989): The eicosanoids and their biochemical mechanisms of action. Biochem J 259:315–324.

Sudo H, Kodama H, Amagai Y, Yamamoto S, Kasai S (1983): In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol 96:191–198.

Sutherland CA, Amin D (1982): Relative activities of rat and dog platelet phospholipase A_2 and diglyceride lipase. J Biol Chem 257:14006–14010.

Suzuki A, Kotoyori J, Oiso Y, Kozawa O (1993): Prostaglandin E_2 is a potential mediator of extracellular ATP action in osteoblast-like cells. Cell Adh Commun 1:113–118.

Suzuki A, Oiso Y, Kozawa O (1994): Effect of endothelin-1 on phospholipase D activity in osteoblast-like cells. Mol Cell Endocrinol 105:193–196.

Takuwa Y, Ohue Y, Takuwa N, Yamashita K (1989): Endothelin-1 activates phospholipase C and mobilizes Ca^{2+} from extra and intracellular pools in osteoblastic cells. Am J Physiol 257:E797–E803.

Takuwa Y, Masaki T, Yamashita K (1990): The effects of the endothelin family peptides on cultured osteoblastic cells from rat calvariae. Biochem Biophys Res Commun 170: 998–1005.

Tatrai A, Foster S, Lakatos P, Shankar G, Stern PH (1992): Endothelin-1 actions on resorption, collagen and noncollagen protein synthesis, and phosphatidylinositol turnover in bone organ cultures. Endocrinology 131:603–607.

Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T (1988): A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332:411–415.

Yokota K, Kusaka M, Ohshima T, Yamamoto S, Kurihara N, Yoshino T, Kumegawa M (1986): Stimulation of prostaglandin E_2 synthesis in cloned osteoblastic cells of mouse (MC3T3-E1) by epidermal growth factor. J Biol Chem 261:15410–15415.

Zeisel SH (1993): Choline phospholipids: Signal transduction and carcinogenesis. FASEB J 7:551–557.