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RAPID COMMUNICATION

EFFECT OF CELL DENSITY ON ENDOTHELIN RELEASE FROM ENDOTHELIAL CELLS AND PHOSPHORAMIDON DEPENDENT INHIBITION

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ABSTRACT——The modulation of endothelin (ET) release from endothelial cells was investigated as a function of cell density. The present study examined the release of ET from bovine pulmonary artery endothelial cells (BPAEC) and bovine aortic endothelial cells (BAEC) at various cell densities, as well as the effects of phosphoramidon, thiorphan and pepstatin on ET release at different densities. ET release from BPAEC and BAEC decreased as cell density increased. This cell density effect was not observed with prostacyclin release from either BPAEC or BAEC. Phosphoramidon (1 mM) inhibited ET release at every density examined for both BPAEC and BAEC. Thiorphan (1 mM) inhibited ET release from BPAEC weakly at low density and had no effect on ET release from BAEC. Pepstatin (1 mM) slightly inhibited ET release in BPAEC at the lowest density and had no effect at any other cell density for either cell type. These protease inhibitors had no effect on cell viability as determined by trypan blue exclusion and a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide conversion assay. This study supports the concept that ET release is modulated by endothelial cell density. In addition, these data demonstrate that phosphoramidon, which presumably inhibits the endothelin converting enzyme, can inhibit ET release over a range of cell densities without affecting cell viability.

INTRODUCTION

The vascular endothelium plays an active role in maintaining the integrity of the vasculature. This continuous monolayer of endothelial cells (EC) not only serves as a barrier between the blood and tissue, but also regulates the exchange of molecules between plasma components and underlying tissue. EC have been shown to modulate vascular resistance by producing vasodilators such as endothelium-derived relaxing factor (EDRF) and prostacyclin, as well as vasoconstrictors such as endothelium-derived constricting factors (EDCF) and endothelin (ET) [1-3].

The isolation and identification of ET from cultured porcine EC by Yanagisawa et al. [3] led to much research focusing on ET release from EC in an effort to define the possible physiological and pathophysiological roles of this peptide. It has been proposed that cell to cell contact may modulate ET release from EC [4]. It is suggested [5] that damaged EC may release higher levels of ET. It is possible that ET may be released to stimulate the growth of new EC. In this connection, ET has been reported to be a mitogen for vascular smooth muscle [6-8], fibroblast [9] and glomerular [10] cells. Thus, looking at ET release from non-confluent EC may provide a model of regenerating EC after physical damage. The present study examined this concept and demonstrated that as cell density increased, ET release from both bovine pulmonary artery endothelial cells (BPAEC) and bovine aortic endothelial cells (BAEC) decreased. This increase in EC density had no effect on the release of the potent vasodilator, prostacyclin. In addition, phosphoramidon was found to inhibit ET release independent of cell density as has been reported for confluent EC [11]. Although phosphoramidon was used at high concentration, the ET release inhibition was not due to effects on cell viability. Furthermore, thiorphan and pepstatin had little if any inhibitory effect at the cell densities tested.

MATERIALS AND METHODS

BPAEC were obtained from American Type Cell Culture (Catalog No. CCL209). BAEC were a gift from Dr. Jane Chin (VA Medical Center, Palo Alto, CA). BPAEC were grown in modified Eagle's medium (MEM, Irvine Scientific, Santa Ana, CA), and BAEC were

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grown in Dulbecco's modified Eagle's medium (DMEM), both containing 10% fetal bovine serum in 150 cm^2 flasks at 37° in a 95% air and 5% CO_2 incubator. Once confluent (10.6 x 10^6 cells/150 cm^2 flask) the cells were subcultured onto 24 well plates at various densities (11.6 x 10^3 to 70 x 10^3 cells/cm^2) and used within 3 days after subculturing.

The endothelin release assay was initiated by removing growth medium from the cells and adding 200 µL of serum-free medium containing 0.1% bovine serum albumin (BSA) in the absence or presence of phosphoramidon (1 mM), thiorphan (1 mM) or pepstatin (100 µM) (Sigma Chemical Co., St. Louis, MO). After 24 hr, the media were collected, spun at 12,000 g for 5 min to remove cell debris, transferred to a separate microfuge tube and stored at -20° until assayed for immunoreactive ET or the stable inactive metabolite of prostacyclin, 6-keto-PGF_{1} alpha.

ET present in media samples was determined by radioimmunoassay (RIA) using standard ET-1 (human, porcine; American Peptide, Santa Clara, CA), anti-ET antibody (prepared as described below) and 3-[^{125}I]iodotyrosyl-13-endothelin (porcine; Amersham, Arlington Heights, IL).

Antiserum specific for ET-1 was prepared using a conjugate of the ET-1 C-terminal pentapeptide (Leu-Asp-Ile-Ile-Trp) with thyroglobulin as immunogen. Pentapeptide (1 mg) and 10 mg bovine thyroglobulin (Sigma) were coupled in 1.5 mL of phosphate-buffered saline (PBS) with 7.5 µL of 25% glutaraldehyde (Sigma) for 18 hr at room temperature. Conjugate (0.2 mL) was emulsified with 1.8 mL PBS and 2 mL Complete Freund's Adjuvant and used to immunize two New Zealand white rabbits at approximately ten intradermal sites. The animals were boosted with an emulsion of 0.1 mL conjugate, 1.9 mL PBS and 2.0 mL Incomplete Freund's Adjuvent after 5 weeks; antisera were prepared 2 weeks subsequently. Additional boosts were performed at 2- to 4-month intervals.

The RIA was as follows: briefly, ET-1 standard in PBS containing 0.1% BSA, 0.1% Triton X-100, 1 mM EDTA, and 0.01% NaN_3 (PBS RIA buffer) or sample (25 µL) was incubated with antiserum (50 µL at 1:10,000 dilution) at 4° for 24 hr. (^{125}I)ET-1 (10,000 cpm/50 µL) was added and incubated at 4° for an additional 24 hr. Antibody bound and free [^{125}I]ET-1 were separated by adding 50 µL goat antirabbit immunoglobulin and normal rabbit serum (Peninsula Laboratories, Belmont, CA) for 2 hr at room temperature. The reaction was terminated by adding 250 µL of cold PBS RIA buffer and centrifuging samples at 3000 rpm for 20 min. The supernatant buffer was aspirated and the activity in the pellet analyzed using a Gamma counter. The ET-1 specific RIA had an EC_{50} of 100 fmol/tube and less than 0.01% cross-reactivity with big ET-1.

Prostacyclin present in the media samples was determined by measuring 6-keto-PGF_{1alpha} using an RIA system from Amersham. The media samples (150 µL) were assayed undiluted.

Cell viability was determined morphologically by trypan blue exclusion and cell counting. A second functional assay was employed in which the dehydrogenase activity in viable cells converts 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to an insoluble formazan which can be solubilized with dimethyl sulfoxide (DMSO) yielding a purple color [12]. The optical density of this color reaction was read at 570 nm. The data are expressed as MTT conversion (% of control) and were analyzed using the One Group Student's *t*-test (Statview II, Abacus Concepts, Berkeley, CA).

RESULTS

ET release from BPAEC decreased with increasing cell number as shown in Fig. 1. Immunoreactive ET in the medium decreased from 69 fmol/10^4 cells at a subconfluent density of 52,500 cells/cm^2 to 7.5 fmol/10^4 cells at a confluent density of 177,813 cells/cm^2. As EC density increased, prostacyclin release did not change and remained between 18 and 21 pg/10^4 cells.

The effect of protease inhibitors on ET release from BPAEC at different cell densities was also examined (Fig. 1). ET release was inhibited by 1 mM phosphoramidon (between 61 and 100%) at every cell density examined. Thiorphan (1 mM) was found to inhibit ET release weakly by 43 and 46% but only at subconfluent cell densities of 52,500 and 75,625 cells/cm^2, respectively. Thiorphan had no apparent effect on ET release as cell densities approached confluence (155,000 cells/cm^2). Pepstatin (100 µM) appeared to slightly inhibit ET release at the lowest cell density but had no effect at any of the higher cell densities examined.

The effect of cell density on ET release from BAEC was also investigated and data are summarized in Fig. 2. As with BPAEC, ET release from BAEC decreased as cell density increased (i.e. from 61.3 fmol/10^4 cells at a subconfluent density of 52,422 cells/cm^2 to 4.2 fmol/10^4 cells at a confluent density of 520,938 cells/cm^2). In BAEC, prostacyclin release remained at 1.1 pg/10^4 cells as EC density increased. In these cells, phosphoramidon inhibited ET release (71-94%) at every cell density examined. Thiorphan and pepstatin had no effect on ET release at any cell density.

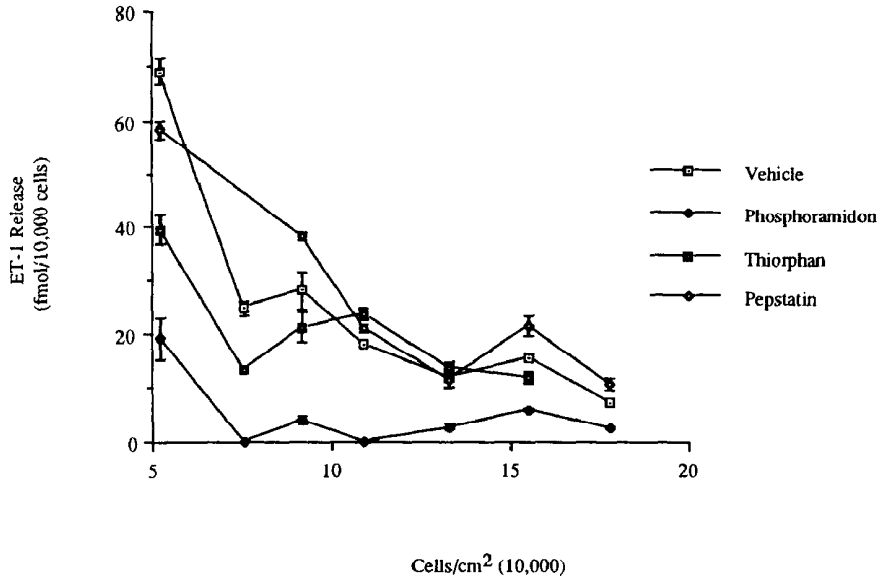

Fig. 1. Endothelin release from BPAEC. Cells were incubated with serum-free medium containing 0.1% BSA (vehicle) alone, or in the presence of 1 mM phosphoramidon, 1 mM thiorphan or 100 μM pepstatin, and ET released after 24 hr was determined by RIA. Data are presented as fmol/10^4 cells (mean +/- SE, N=6) as a function of cells/cm^2.

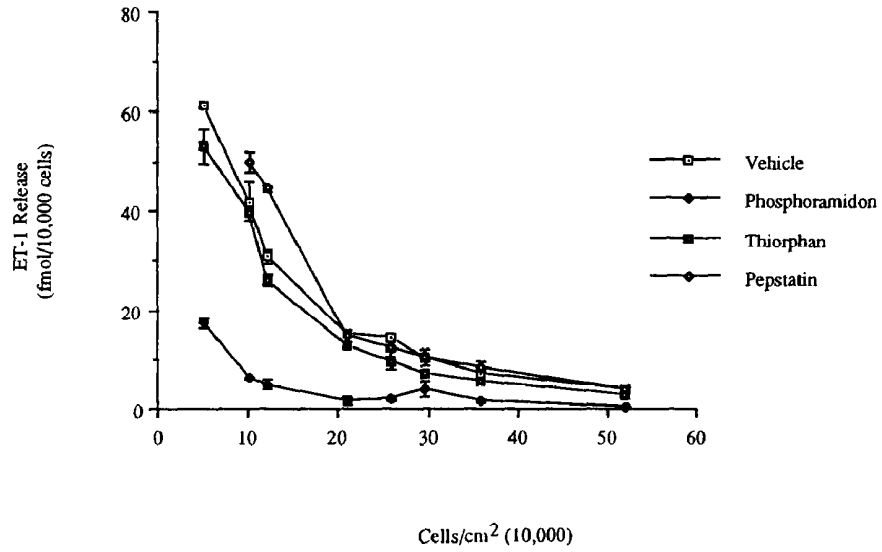

Fig. 2. Endothelin release from BAEC. Cells were incubated with serum-free medium containing 0.1% BSA (vehicle), 1 mM phosphoramidon, 1 mM thiorphan or 100 μM pepstatin, and ET released after 24 hr was determined by RIA. Data are presented as fmol/10^4 cells (mean +/- SE, N=6) as a function of cells/cm^2.

Inhibition of ET release with phosphoramidon was found to be concentration-dependent for both BPAEC and BAEC as shown in Fig. 3. At 1 μM phosphoramidon, no effect on ET release was observed. As the concentration of phosphoramidon was increased from 10 μM to 1 mM, ET release was inhibited from 7.4 to 83.9% and from 8.7 to 85.4% in BPAEC and BAEC, respectively.

Although phosphoramidon is thought to affect release by inhibiting the putative endothelin converting enzyme, it is possible that phosphoramidon may affect ET release by an alternative mechanism such as altering cell viability. To examine this possibility we evaluated the viability of BPAEC and BAEC following incubation with phosphoramidon as well as with thiorphan and pepstatin. Cell viability was assessed morphologically using trypan blue exclusion, and using an MTT conversion assay [12]. Figure 4 shows the amount

of MTT conversion (expressed as percent of control) following treatment with each of the above protease inhibitors. We found that phosphoramidon and thiorphan had no effect on BPAEC viability since the conversion of MTT to the formazan was the same as in the controls. Pepstatin on the other hand appears to slightly inhibit MTT conversion in BPAEC ($P<0.01$). However, neither phosphoramidon, thiorphan nor pepstatin had any effect on BPAEC viability when assessed by trypan blue exclusion (data not shown). In BAEC, none of the protease inhibitors had any effect on cell viability as determined by both MTT conversion and trypan blue exclusion.

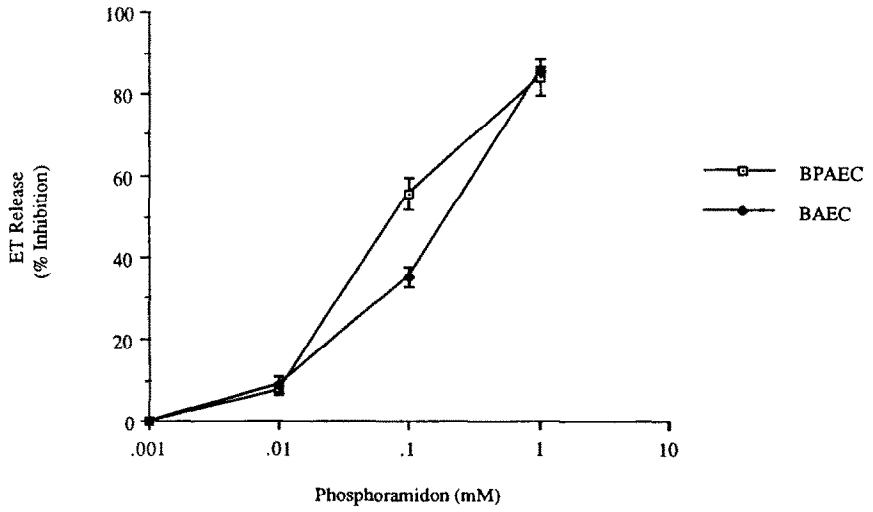

Fig. 3. Phosphoramidon inhibition of ET release in BPAEC and BAEC. Cells were incubated with serum-free medium containing 0.1% BSA in the absence or presence of 1 μM, 10 μM, 100 μM or 1 mM phosphoramidon, and ET release was measured at 24 hr. Values are means+/-SE, N=8.

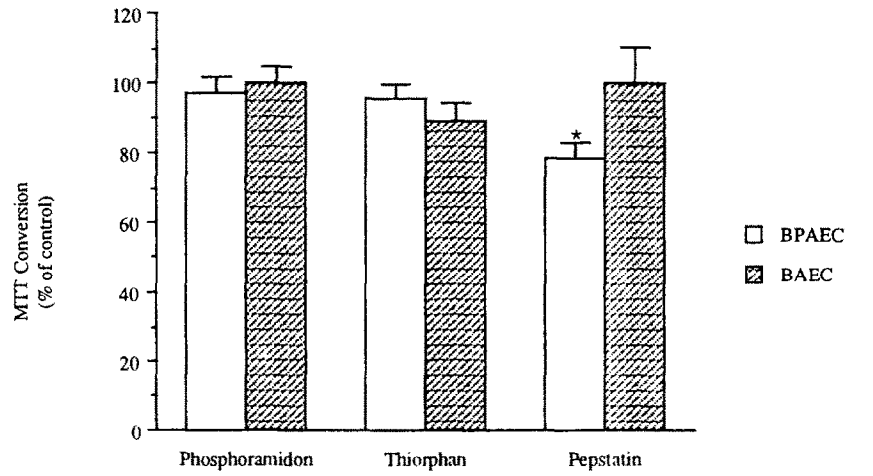

Fig. 4. MTT conversion in BPAEC and BAEC. BPAEC and BAEC were incubated with serum-free medium containing 0.1% BSA in the absence and presence of 1 mM phosphoramidon, 1 mM thiorphan or 100 μM pepstatin. After 24 hr media were collected for RIA. Cells were washed with fresh medium and MTT (1 mg/mL) in DMEM was added to the cells and incubated at 37° for 4 hr. After removal of MTT and incubation with DMSO for 15 min, optical density was read at 570 nm. The optical density for MTT conversion in control BPAEC and BAEC was different in each experiment but averaged 0.6 and 0.5, respectively. The data are expressed as MTT conversion (% of control), and was analyzed using One Group Student's *t*-test (*$P < 0.01$). Values are means+/-SE, N=5.

DISCUSSION

ET-1 was first identified as a potent vasoconstrictor produced by EC [3]. It is also produced by tracheal epithelial cells [13], renal epithelial cells [14], and neural epithelial cells [15] as well as vascular smooth muscle cells [16]. Subsequent *in situ* hybridization studies have demonstrated that ET mRNA is distributed in organs where ET receptors have also been identified (i.e. brain, lung, kidney and intestine) [17, 18]. The observations that ET specific receptors have been identified in a variety of tissues [17, 19] and cultured cells [20-22] suggest that ET may have diverse physiological functions. In this connection, ET has not only been found to be a potent vasoconstrictor in a number of vessels [3, 23] but has also been reported to stimulate the proliferation of rat vascular smooth muscle cells [6-8], fibroblasts [9], and glomerular mesangial cells [10], and to stimulate the expression of c-*fos* and c-*myc* [6]. Thus, ET may act as a paracrine factor.

In endothelial cells, ET may also act in an autocrine fashion to stimulate growth [24]. Our data support this concept since we observed that when EC density is subconfluent, ET levels are higher, possibly to stimulate their growth. As EC density approaches a confluent monolayer, ET levels are lower, possibly in an effort to slow proliferation. Its role as an autocrine factor has also been suggested in rat cultured mesangial cells [25] and parathyroid cells [26]. It has been shown that damaged EC release ET [5], and that ET can act synergistically with growth factors such as epidermal growth factor and transforming growth factor alpha to stimulate vascular smooth muscle proliferation [16] and c-*fos* and c-*myc* mRNA [6]. It is possible that regenerating EC may release ET to stimulate their own growth as well as vascular smooth muscle proliferation in an effort to repair the site of vascular injury. However, such stimulation may also be deleterious, since continued cell proliferation may lead to vessel wall thickening and atherosclerotic plaque formation which could contribute to the development of vascular disorders.

Our study agrees with previous observations by Sugimoto et al. [4] that ET release is inversely proportional to EC density. We also observed that a completely different vasoactive substance, prostacyclin, release was independent of cell density. In addition, we found that only phosphoramidon, which presumably inhibits the endothelin converting enzyme, blocked ET release at every cell density examined for both BPAEC and BAEC. This study supports the idea that ET may act in an autocrine fashion to regulate its own release from EC and possibly to regulate EC proliferation. Thus, endothelin converting enzyme inhibitors may have a clinical utility in managing vasoproliferative disorders.

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REFERENCES

1. Griffith TM, Lewis MJ, Newby AC and Henderson AH, Endothelium-derived relaxing factor. *Am J Cardiol* **12**: 797-806, 1988.
2. Vanhoutte PM and Katusic ZS, Endothelium-derived contracting factor: endothelin and/or superoxide anion. *Trends Pharmacol Sci* **9**: 229-230, 1988.
3. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K and Masaki T, A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* **332**: 411-415, 1988.
4. Sugimoto T, Hirata Y, Matsuoka H and Yagi S, Cell density related changes in endothelin (ET) secretion from cultured vascular endothelial cells. *Clin Res* **38**(N2): A404A, 1990.
5. Sugiura M, Inagami T and Kon V, Endotoxin stimulates endothelin release *in vivo* and *in vitro* as determined by radioimmunoassay. *Biochem Biophys Res Commun* **161**: 1220-1227, 1989.
6. Komuro I, Kurihara H, Sugiyama T, Takaku F and Yazaki Y, Endothelin stimulates c-fos and c-myc expression and proliferation of vascular smooth muscle cells. *FEBS Lett* **238**: 249-252, 1988.
7. Hirata Y, Takagi Y, Fukuda Y and Marumo F, Endothelin is a potent mitogen for rat vascular smooth muscle cells. *Atherosclerosis* **78**: 225-228, 1989.
8. Bobik A, Grooms A, Millar JA, Mitchell A and Grinpukel S, Growth factor activity of endothelin on vascular smooth muscle. *Am J Physiol* 258: C408-C415, 1990.

9. Takuwa N, Takuwa Y, Yanagisawa M, Yamashita K and Masaki T, A novel vasoactive peptide endothelin stimulates mitogenesis through inositol lipid turnover in Swiss 3T3 fibroblasts. *J Biol Chem* **264**: 7856-7861, 1989.

10. Simonson MS, Wann S, Mene P, Dubyak GR, Kester M, Nakazato Y, Sedor JR and Dunn MJ, Endothelin stimulates phospholipase C, Na^+/H^+ exchange, c-*fos* expression, and mitogenesis in rat mesangial cells. *J Clin Invest* **83**: 708-712, 1989.

11. Shields PP, Gonzalez TA, Charles D, Gilligan JP and Stern W, Accumulation of pepstatin in cultured endothelial cells and its effect on endothelin processing. *Biochem Biophys Res Commun* **177**: 1006-1012, 1991.

12. Mosmann T, Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**: 55-63, 1983.

13. Werber HI, Emancipator SN, Tykocinski ML and Seder JR, The interleukin 1 gene is expressed by rat glomerular mesangial cells and is augmented in immune complex glomerulonephritis. *J Immunol* **138**: 3207-3212, 1987.

14. Shichiri M, Hirata Y, Emori T, Ohta K, Nakajima T, Sato K, Sato A and Marumo F, Secretion of endothelin and related peptides from renal epithelial cell lines. *FEBS Lett* **253**: 203-206, 1989.

15. Giaid A, Gibson SJ, Ibrahim NBN, Legon S, Bloom SR, Yanagisawa M, Masaki T, Varndell IM and Polak JM, Endothelin 1, an endothelium-derived peptide, is expressed in neurons of the human spinal cord and dorsal root ganglia. *Proc Natl Acad Sci USA* **86**: 7634-7638, 1989.

16. Resink TJ, Hahn AWA, Scott-Burden T, Powell J, Weber E and Buhler FR, Inducible endothelin mRNA expression and peptide secretion in cultured human vascular smooth muscle cells. *Biochem Biophys Res Commun* **168**: 1303-1310, 1990.

17. Koseki C, Imai M, Hirata Y, Yanagisawa M and Masaki T, Autoradiographic distribution in rat tissues of binding sites for endothelin: a neuropeptide? *Am J Physiol* **256**: R858-R866, 1989.

18. MacCumber MW, Ross CA, Glaser BM and Snyder SH, Endothelin: Visualization of mRNAs by *in situ* hybridization provides evidence for local action. *Proc Natl Acad Sci USA* **86**: 7285-7289, 1989.

19. Takayanagi R, Ohnaka K, Takasaki C, Ohashi M and Nawata H, Multiple subtypes of endothelin receptors in porcine tissue: Characterization by ligand binding, affinity labeling and regional distribution. *Regul Pept* **32**: 23-37, 1991.

20. Sugiura M, Snajdar RM, Schwartzberg M, Badr KF and Inagami T, Identification of two types of specific endothelin receptors in rat mesangial cells. *Biochem Biophys Res Commun* 162: 1396-1401, 1989.

21. Martin ER, Brenner BM and Ballermann BJ, Heterogeneity of cell surface endothelin receptors. *J Biol Chem* **265**: 14044-14049, 1990.

22. Takuwa Y, Masaki T and Yamashita K, The effect of the endothelin family peptides on cultured osteoblastic cells from rat alvariae. *Biochem Biophys Res Commun* **170**: 998-1005, 1990.

23. Lovenberg W and Miller RC, Endothelin: A review of its effects and possible mechanisms of action. *Neurochem Res* **15**: 407-417, 1990.

24. Takagi Y, Fukase M, Takata S, Yoshimi H, Tokunaga O and Fujita T, Autocrine effect of endothelin on DNA synthesis in human vascular endothelial cells. *Biochem Biophys Res Commun* **168**: 537-543, 1990.

25. Sakamoto H, Sasaki S, Hirata Y, Imai T, Ando K, Ida T, Sakurari T, Yanagisawa M, Masaki T and Marumo F, Production of endothelin-1 by rat cultured mesangial cells. *Biochem Biophys Res Commun* **169**: 462-468, 1990.

26. Fujii Y, Moreira JE, Orlando C, Maggi M, Aurbach GD, Brandi ML and Sakaguchi K, Endothelin as an autocrine factor in the regulation of parathyroid cells. *Proc Natl Acad Sci USA* **88**: 4235-4239, 1991.