A Novel Vasoactive Peptide Endothelin Stimulates Mitogenesis through Inositol Lipid Turnover in Swiss 3T3 Fibroblasts*

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Endothelin, a novel vasoactive peptide derived from endothelial cells (Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yasaki, Y., Goto, K., and Masaki, T. (1988) Nature 332, 411–415), acts as a potent mitogen in Swiss 3T3 fibroblasts. This effect is dose-dependent with a half-maximal effect obtained at approximately 3 \times 10^{-11} M and is synergistically enhanced by a low concentration of insulin-like growth factor-I. Endothelin specifically binds to a single class of high affinity receptors in intact Swiss 3T3 cells and stimulates phospholipase C with the production of second messengers inositol trisphosphate and 1,2-diacylglycerol, leading to biphasic increases in the intracellular free Ca²⁺ concentration, as measured with a fluorescent indicator fura-2, phosphorylation of a putative cellular substrate of 80 kDa for protein kinase C, and transient expression of cellular protooncogenes, c-fos and c-myc. Mitogenic effect of endothelin is markedly attenuated in phorbol ester-pretreated, protein kinase C-depleted cells. Endothelin-induced inositol phosphates production is not affected by removal of extracellular Ca²⁺, suggesting that endothelin-induced phospholipase C activation is not the result of stimulation of Ca²⁺ influx across the plasma membrane. These composite results indicate that the inositol lipid signaling pathway plays an important role in endothelin-induced mitogenesis in Swiss 3T3 fibroblasts.

The mitogenic effect of endothelin is considerably smaller than that of bombesin, another well characterized mitogen acting through the inositol lipid pathway, despite comparable potencies in eliciting initial second messenger signals. In endothelin-treated cells, an increase in cellular 1,2-diacylglycerol content is transient, and cellular cyclic AMP content is reduced. By contrast, bombesin induces a more prolonged increase in cellular 1,2-diacylglycerol content and a slight increase in cellular cyclic AMP content. Because both 1,2-diacylglycerol and cyclic AMP are thought to serve as signals for promoting DNA synthesis in Swiss 3T3 fibroblasts, these differences in the signal generation may contribute to the differences in potencies between the two mitogens.

Endothelin is a newly identified 21-residue peptide produced by vascular endothelial cells, which exhibits a potent vasoconstrictive activity (1). Endothelin has a quite unique structure and does not belong to any other mammalian peptide family so far known, but shows regional homologies to a group of peptide neurotoxins such as α-scorpion toxins and ω-conotoxin that act on voltage-dependent ion channels (1). In vascular smooth muscle, endothelin-induced contraction was shown to be greatly inhibited by low concentrations of nardipine, a dihydropyridine Ca²⁺ channel antagonist (1). Moreover, we have recently found that endothelin actually increases the voltage-dependent Ca²⁺ channel antagonist (2). These observations have led to the proposal that endothelin may act as an endogenous modulator of a dihydropyridine-sensitive, voltage-dependent Ca²⁺ channel (1). Therefore, it is of particular interest whether endothelin acts on other types of cells which possess a dihydropyridine-sensitive, voltage-dependent Ca²⁺ channel. In Swiss 3T3 fibroblasts the existence of this type of Ca²⁺ channel has recently been demonstrated (3). This report prompted us to examine the effect of endothelin on Swiss 3T3 fibroblasts.

The present study reports that endothelin acts on Swiss 3T3 cells as a potent mitogen. Unexpectedly, endothelin is found to induce phospholipase C activation leading to production of two messengers, inositol trisphosphate and diacylglycerol, and appears to exert a mitogenic effect mainly through activation of protein kinase C (Ca²⁺/phospholipid-dependent enzyme) in Swiss 3T3 fibroblasts.

**MATERIALS AND METHODS**

**Cell Culture and Materials**—Swiss 3T3 fibroblasts (3T3) are a generous gift from Dr. E. R. Rozengurt (Imperial Cancer Research Fund, London, United Kingdom). Cells were routinely maintained in a subconfluent state as described (4). For experimental purposes, cells were grown to confluence and made quiescent as described previously (5). Porcine endothelin was obtained from Peptide Institute, Inc. (Osaka, Japan). Endothelin was dissolved at 10⁻⁴ M in Dulbecco's phosphate-buffered saline containing 0.5% bovine serum albumin and stored in aliquots at −20 °C as stock solutions. Dilutions of the stock solution were prepared immediately before use. Insulin-like growth factor-I was generously provided by Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan). Bombesin, phorbol-12,13-dibutyrate, bovine serum albumin (RIA grade) were obtained from Sigma. Fura-2/AM and nicardipine were purchased from Wako (Tokyo, Japan). Myo-[2-³H]inositol (14.3 Ci/mmol), [methyl-³H]thymidine (85.6 Ci/mmol), adenosine 5'-β,γ-³H]triphosphate (3000 Ci/mmol), [³P]orthophosphate (carrier-free) were purchased from Du Pont-New England Nuclear Research Products. ³²P-Labeled endothelin (∼2000 Ci/mmol) was a gift from Amersham Corp.

**Labeled Endothelin Binding Experiments**—Confluent, quiescent 3T3 fibroblasts grown in 24-well plates were washed once with 1 ml of medium A (140 mM NaCl, 4 mM KCl, 1 mM NaHPO₄, 1 mM MgCl₂, 1.25 mM CaCl₂, 11 mM glucose, 5 mM Hepes (pH 7.4), and 0.2% bovine serum albumin) and incubated in 1 ml of the same...
Effect of Endothelin on DNA Synthesis in Swiss 3T3 Fibroblasts—Shown in Fig. 1 are the effects of endothelin on [3H]thymidine incorporation into DNA in Swiss 3T3 fibroblasts. When quiescent cells are incubated with endothelin alone for 24 h, DNA synthesis of 3T3 cells is stimulated in a dose-dependent manner. The half-maximal and maximal effects are obtained at approximately $3 \times 10^{-11}$ and $10^{-9}$ M of endothelin, respectively. This effect of endothelin is synergistically enhanced by a low concentration of insulin-like growth factor-I (IGF-I) (1 ng/ml), which by itself shows only a minimal mitogenic activity.

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The abbreviations used are: IGF-I, insulin-like growth factor-I; 1,2-DG, 1,2-diacylglycerol; [Ca$^{2+}$]$^{99}{\text{I}}$ intraocular free Ca$^{2+}$ concentration; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

cells show specific binding of [125I]-labeled endothelin which is displaced by unlabeled endothelin in a concentration-dependent manner (Fig. 2A). Specific binding of [125I]-labeled endothelin is a saturable process, and Scatchard analysis of the data reveals a single class of high affinity sites with the $k_d$ value of $1.8 \times 10^{-10}$ M and the number of the binding sites ($B_{max}$) of 380,000 sites/cell (Fig. 2B). The half-maximal concentration needed for DNA synthesis is nearly one order of magnitude lower than the $k_d$ value for receptor binding. These data may suggest the existence of an amplification step between receptor binding and the following cascade of signaling pathway, or alternatively, large numbers of spare receptors.

Effect of Endothelin on Inositol Phosphates Production in Swiss 3T3 Fibroblasts—In Swiss 3T3 fibroblasts, a group of growth factors has been known to exhibit enhanced mitogenic activities in the presence of IGF-I or high concentrations of insulin. Bombesin, vasopressin, platelet-derived growth factor, tumor-promoting phorbol ester, and a diacylglycerol an-
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alog, oleoylacetoylglycerol belong to this group of mitogens (13). One of the outstanding characteristics common to this group of mitogens is that they activate protein kinase C directly or indirectly via phosphoinositide hydrolysis. Therefore, we examined the effects of endothelin on phosphoinositide hydrolysis, 1,2-diacylglycerol (1,2-DG) metabolism, protein kinase C activation, and cellular Ca2+ metabolism.

First we studied the effect of endothelin on inositol phosphates production. As shown in Fig. 3, endothelin (10^-7 M) induces marked increases in all four inositol phosphates in [3H]inositol-prelabeled Swiss 3T3 cells. Inositol bis-, tris-, and tetrakisphosphates increase promptly, peak within 20 s, and then gradually fall down to new steady-state levels at 5 min. In contrast to these three inositol phosphates, inositol monophosphate rises more slowly and reaches a plateau at 1 min. These results indicate that endothelin activates phospholipase C which catalyzes the breakdown of polyphosphoinositides in Swiss 3T3 cells.

Effect of Endothelin on Celluar 1,2-Diacylglycerol Content and Phosphorylation of an 80-kDa Protein—Next, the effect of endothelin on 1,2-DG production was studied. Table I shows the time course of endothelin-induced changes in cellular 1,2-DG content. Within 20 s of the addition of 10^-8 M endothelin, cellular 1,2-DG increases significantly and continues to rise for 5 min, when the maximal value of 1.7-2-fold over the basal level is observed. It then gradually declines but remains higher than the basal level for at least 1 h.

An acidic 80-kDa protein in Swiss 3T3 fibroblasts is a well-known specific substrate for protein kinase C (14). Previous studies have shown that tumor-promoting phorbol esters and other protein kinase C activators induce stimulation of phosphorylation of this protein in Swiss 3T3 cells (5, 9, 13-15). In order to see whether endothelin induces activation of protein kinase C in vivo in Swiss 3T3 cells, phosphorylation changes of the 80-kDa protein were examined in intact quiescent cells prelabeled with [32P]orthophosphate, by use of two-dimensional polyacrylamide gel electrophoresis. Stimulation of cells with endothelin (10^-8 M) induces a rapid increase in the incorporation of 32P into the 80-kDa protein, which was evident within 5 min and is sustained for at least 1 h (Fig. 4). These results indicate that endothelin activates protein kinase C in intact Swiss 3T3 fibroblasts and suggest a role of this kinase in endothelin-induced mitogenesis. In fact, in protein kinase C-depleted cells where the kinase is down-regulated by pretreatment with 1 µM phorbol 12,13-dibutyrate for 42 h (5, 16), the mitogenic effect of endothelin is markedly attenuated (Table II).

Effect of Endothelin on Intracellular Free Ca2+ Concentration—The endothelin-induced Ca2+ mobilization was studied by measurement of changes in intracellular free Ca2+ concentration ([Ca2+]i), employing fura-2 as a Ca2+ indicator (Fig. 5). Addition of endothelin (10^-8 M) induces a prompt rise in [Ca2+]i, which peaks within 15 s of endothelin addition (Fig. 5A). The [Ca2+]i then falls down close to the base-line value.

![Fig. 3. The effect of endothelin on inositol phosphates generations. Confluent quiescent monolayer cells in 60-mm dishes were labeled with [3H]inositol (10 µCi/ml) for 24 h and made quiescent at the same time. The media were replaced by fresh medium, and the cells were incubated at 37 °C for 10 min and then stimulated with endothelin (10^-7 M) for indicated time periods in the absence of LiCl. The reaction was stopped by aspirating media and adding ice-cold 10% perchloric acid solution. Each inositol phosphate was then separated as described (4). The data represent the mean ± S.E. of three determinations. IP1, inositol monophosphate; IP2, inositol bisphosphate; IP3, inositol trisphosphate; IP4, inositol tetrakisphosphate.

| Table I |
| Effect of endothelin on cellular 1,2-diacylglycerol content in Swiss 3T3 fibroblasts |

Confluent quiescent cells were incubated with 10^-8 M endothelin for indicated times and cellular 1,2-DG content was measured. The data are the mean ± S.E. of three determinations.

<table>
<thead>
<tr>
<th>1,2-Diacylglycerol</th>
<th>nmol/dish</th>
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<tbody>
<tr>
<td>0</td>
<td>0.204 ± 0.004</td>
</tr>
<tr>
<td>20 s</td>
<td>0.250 ± 0.015</td>
</tr>
<tr>
<td>1 min</td>
<td>0.295 ± 0.004</td>
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<tr>
<td>5 min</td>
<td>0.353 ± 0.006</td>
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<tr>
<td>30 min</td>
<td>0.309 ± 0.008</td>
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<tr>
<td>60 min</td>
<td>0.233 ± 0.006</td>
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| Table II |
| Effect of pretreatment of cells with phorbol dibutyrate on the mitogenic effect of endothelin |

Quiescent Swiss 3T3 fibroblasts were pretreated with either 1 µM phorbol 12,13-dibutyrate or 0.1% dimethyl sulfoxide (control) for 42 h, washed extensively, and then incubated with [3H]thymidine in the presence or absence of 10^-8 M endothelin for 24 h. The results are the mean ± S.E. of three determinations.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>[3H]Thymidine incorporation into DNA</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>dpm/well</td>
</tr>
<tr>
<td>Phorbol dibutyrate-pretreated</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>7,300 ± 100</td>
</tr>
<tr>
<td>Endothelin</td>
<td>8,200 ± 900</td>
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<td></td>
<td>11,800 ± 2,100</td>
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After this initial Ca\(^{2+}\) transient, there is a second, small but significant, increase in [Ca\(^{2+}\)], which persists for at least 10 min of the observation period. When extracellular Ca\(^{2+}\) is lowered by adding 2 mM EGTA (free Ca\(^{2+}\) 200 nM), the basal [Ca\(^{2+}\)] declines to a new steady-state level (Fig. 5B). Under such a condition, endothelin (10\(^{-8}\) M) still induces an initial Ca\(^{2+}\) transient, but not a second plateau of the [Ca\(^{2+}\)] response (Fig. 5B). In the presence of 1.25 mM CaCl\(_2\), addition of 1 mM Ni\(^{2+}\), an inorganic Ca\(^{2+}\) channel blocker, promptly abolishes the second plateau phase of the [Ca\(^{2+}\)] response to endothelin (Fig. 5C). These findings suggest that endothelin induces mobilization of Ca\(^{2+}\) from both intra- and extracellular pools; the initial Ca\(^{2+}\) transient is mainly due to mobilization of extracellular Ca\(^{2+}\), which is probably mediated by inositol 1,4,5-trisphosphate, and the second plateau phase is mainly due to Ca\(^{2+}\) influx across the plasma membrane.

Previous studies suggested that endothelin acts on vascular smooth muscle cells to activate a voltage-sensitive Ca\(^{2+}\) channel of "L-type" (1). However, in the present study a dihydropyridine Ca\(^{2+}\) channel antagonist nicardipine did not inhibit the second plateau of the [Ca\(^{2+}\)] response to endothelin at concentrations up to 3 \times 10^{-7} M (Fig. 5D).

**Effect of Extracellular Ca\(^{2+}\) Removal on Endothelin-induced Inositol Phosphates Production**—The above results also suggest that endothelin-induced phosphoinositide hydrolysis is not dependent on stimulation of Ca\(^{2+}\) influx from outside the cells. In order to examine this possibility more directly, endothelin-evoked inositol phosphates production was compared in the presence and absence of extracellular Ca\(^{2+}\). As shown in Table III, endothelin induces stimulation of inositol phosphate production even when cells are incubated in the Ca\(^{2+}\)-free media containing 1 mM EGTA prior to addition of endothelin. Thus, endothelin-induced stimulation of phosphoinositide hydrolysis is not a consequence of stimulation of Ca\(^{2+}\) influx across the plasma membrane.

**Effect of Endothelin on Protooncogenes c-fos and c-myc Expression**—It is well known that mitogens which stimulate phosphoinositide hydrolysis and activate protein kinase C induce expression of cellular protooncogenes including c-fos and c-myc (17-19). We examined whether endothelin also exerts this effect in Swiss 3T3 fibroblasts. Shown in Fig. 6 are Northern blot analysis of the expression of c-fos and c-myc mRNAs. Endothelin (10\(^{-8}\) M) induces the expression of both of the protooncogenes with distinct time courses: c-fos expression is rapid and complete by 15 min, whereas c-myc expression is not induced until 30 min.
mRNA appears within 15 min, reaches the maximal level at 15–30 min, and then rapidly disappears to a background level by 60 min; by contrast, c-myc mRNA appears more slowly, peaks at 60 min, and gradually decreases but is still detectable at 180 min.

Comparisons of the Actions of Endothelin and Bombesin—The above findings demonstrate that endothelin activates phospholipase C with the production of two second messengers, inositol 1,4,5-trisphosphate and 1,2-diaclyglycerol, leading to intracellular Ca\(^{2+}\) mobilization and activation of protein kinase C. We demonstrated previously that this sequence of events also occurs when Swiss 3T3 fibroblasts are stimulated with bombesin (4). However, the comparison of the mitogenic potencies of endothelin and bombesin reveals that bombesin is evidently more potent than endothelin at any concentrations from 10\(^{-8}\) to 10\(^{-7}\) M either in the presence or absence of IGF-I (Table IV). We tried to explore the basis for the difference in the mitogenic potencies of these two growth factors. As shown in Table IV, 10\(^{-8}\) M endothelin causes even greater peak increases in [Ca\(^{2+}\)]\(_i\) than 10\(^{-9}\) M bombesin, although this concentration of endothelin is mitogenically less potent than 10\(^{-8}\) bombesin. However, when the time-dependent changes in the cellular 1,2-diaclyglycerol content induced by these two growth factors are compared, significant differences in the kinetics of this messenger is noted (Fig. 7). Endothelin (10\(^{-9}\) M) and bombesin (10\(^{-9}\) M) induce comparable 2-fold increases in the 1,2-DG content at 5 min. Then, in endothelin-stimulated cells the 1,2-DG content thereafter declines and completely returns to the basal level by 4 h. By contrast, in bombesin-stimulated cells, the 1,2-DG content decreases more gradually and stays at 130% over the basal level at 4 h.

Different effects of the two mitogens are also found on cellular cyclic AMP metabolism. Bombesin (10\(^{-9}\) M) induces a slight increase in the cellular cyclic AMP content (Fig. 8). By contrast, endothelin (10\(^{-8}\) M) causes a substantial decrease in cellular cyclic AMP content which is minimal after 5 min and slowly returns to the basal value by 60 min.

**DISCUSSION**

The discovery that vascular endothelial cells produce a novel potent vasoconstrictive peptide has developed a new field of investigation on the regulation of the vascular tone (1). Moreover, recent observations that a variety of tissues and cells are capable of binding endothelin with high affinities (20) raise an interesting possibility that endothelin may act on other cell types than vascular smooth muscle cells and induce diverse biological responses. The present study demonstrates that endothelin acts on Swiss 3T3 fibroblasts to stimulate mitogenesis.

As shown in Fig. 2, Swiss 3T3 cells have a single class of high affinity receptors for endothelin. The receptor activation by endothelin is found to be coupled to the signal transduction which is quite similar to that observed previously with bombesin in Swiss 3T3 cells (4). Thus, endothelin activates phospholipase C with the production of two second messengers, inositol trisphosphate and 1,2-diacylglycerol (Fig. 3 and Table I), leading to Ca\(^{2+}\) mobilization from both intra- and extracellular pools (Fig. 5) and activation of protein kinase C (Fig. 4). Endothelin induces increases in inositol phosphates production in the absence of extracellular Ca\(^{2+}\) nearly to the same extent as in its presence (Table III), indicating that the activation of phospholipase C by endothelin is not a consequence of endothelin-induced Ca\(^{2+}\) influx from outside the cells. The mitogenic effect of endothelin is markedly attenuated in phorbol ester-pretreated, protein kinase C-depleted cells. These results strongly suggest that the inositol lipid signaling pathway plays a crucial role in endothelin-induced stimulation of mitogenesis in Swiss 3T3 fibroblasts. This signaling pathway likely leads to the following biochemical cascade, including the expression of protooncogenes c-fos and c-myc (Fig. 6), which eventually results in the initiation of DNA synthesis.

After we finished this work, the paper by Klooq and his colleagues appeared (21), demonstrating that sarafotoxin, a snake venom that induces coronary vasoconstriction and has a high degree of structural similarity to endothelin, induces phosphoinositide hydrolysis in rat heart and brain (21). They also showed that this action of sarafotoxin was observed even in the absence of extracellular Ca\(^{2+}\). Another recent report by Komuro et al. (22) have shown that endothelin induces Ca\(^{2+}\) mobilization and stimulates DNA synthesis and expression of protooncogenes c-fos and c-myc in cultured rat aortic smooth muscle cells. However, they demonstrated that endothelin-induced Ca\(^{2+}\) mobilization is totally abolished in the absence of extracellular Ca\(^{2+}\), suggesting that endothelin causes an increase in [Ca\(^{2+}\)]\(_i\) solely by stimulating Ca\(^{2+}\) influx across the plasma membrane in cultured aortic smooth muscle cells. Further, Hirata et al. (23) have reported that endothelin does not stimulate phosphoinositide hydrolysis in cultured rat
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Aortic smooth muscle cells. Thus, the coupling mechanisms of endothelin receptor might be different among various type of cells. In addition, the property of the Ca\(^{2+}\) channel through which Ca\(^{2+}\) enters into cells in response to endothelin stimulation appears to be different between vascular smooth muscle cells and Swiss 3T3 fibroblasts. In Swiss 3T3 fibroblasts, the second phase of the [Ca\(^{2+}\)] response to endothelin, which is dependent on extracellular Ca\(^{2+}\), is abolished by an inorganic Ca\(^{2+}\) channel antagonist Ni\(^{2+}\) but not by a dihydropyridine Ca\(^{2+}\) channel antagonist nicardipine at concentrations up to \(3 \times 10^{-7}\) M (Fig. 5, C and D). By contrast, nicardipine effectively inhibits endothelin-induced increases in [Ca\(^{2+}\)]\(_i\) or contraction in cultured aortic smooth muscle (22) or coronary smooth muscle (2) over the similar range of concentration.

Another point which is noteworthy in the present study is the finding that the mitogenic effects induced by bombesin and endothelin are considerably different in terms of relative potency, despite the fact that the two mitogens induce comparable increases in the [Ca\(^{2+}\)]\(_i\), and the cellular 1,2-diacylglycerol content in the initial phase of signal generation (Table IV). In the present study, at least two major differences in the actions of the two mitogens have been demonstrated. First, a significant difference in the kinetics of changes in the second messenger 1,2-diacylglycerol is noted when the observation period is extended for up to 4 h: in endothelin-treated cells the second messenger level returns to the basal value by 4 h, whereas in bombesin-treated cells it stays significantly higher (130% over the basal) for at least 4 h (Fig. 7). Although the basis for the difference in 1,2-diacylglycerol metabolism between the two mitogens is not known at present, the results may suggest that the persistent action of the messenger 1,2-diacylglycerol for a certain period of time is required for full development of the mitogenic effect by this type of mitogens. This view is also supported by the recent observation by Matsuoka et al. (24) that the injection of a monoclonal antibody raised against phosphatidylinositol 4,5-bisphosphate into NIH 3T3 cells abolishes the mitogenic effect of platelet-derived growth factor, even when the antibody is injected 2 h after addition of the mitogen.

Another difference between the actions of bombesin and endothelin is observed in cellular metabolism of cyclic AMP. Previous studies have reported that certain mitogens which stimulate inositol lipid turnover, including platelet-derived growth factor and bombesin, increase this second messenger level (13, 25). By contrast to these mitogens, endothelin is shown to depress the cellular cyclic AMP content (Fig. 8). These findings indicate the existence of more than one mechanism by which this class of mitogens affects the cellular cyclic AMP metabolism. Rozengurt et al. (25) have shown that platelet-derived growth factor increases the production of prostaglandin E\(_2\), which in turn increases the cellular cyclic AMP content in Swiss 3T3 fibroblasts. Endothelin may not stimulate prostaglandin E\(_2\) synthesis, but rather depress cyclic AMP content through Ca\(^{2+}\)-dependent activation of phosphodiesterase. Alternatively, the receptor activation by endothelin may be coupled through G, to inhibition of adenyl cyclase. These possibilities remain to be evaluated. Since in Swiss 3T3 fibroblasts cyclic AMP is another mitogenic signal which acts in a synergistic manner with activators of protein kinase C (26), it is conceivable that a small increase or decline in cellular cyclic AMP content elicited by bombesin or endothelin may contribute to the observed difference in their mitogenic potencies.

Physiological significance of the present results remains to be clarified. However, the expression of preproendothelin mRNA in cultured endothelial cells was shown to be stimulated by a Ca\(^{2+}\) ionophore A23187 and bioactive substances such as thrombin and epinephrine (1), suggesting that production of endothelin by endothelial cells may indeed increase in vivo under such circumstances as endothelial damage, tissue injury, and hemorrhage. The results of the present study, together with those by Komuro et al. (22) raise the possibility that in these situations endothelin may play an important role as a growth factor in perivascular wound healing and development of atherosclerosis.

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REFERENCES