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Endothelin-1 Stimulates c-fos mRNA Expression and Acts as a Modulator on Cell Proliferation of Rat FRTL5 Thyroid Cells

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SUMMARY In FRTL5 thyroid cells, endothelin(ET)-1 alone had no effect on DNA synthesis but caused a transient increase in c-fos mRNA levels and stimulated IGF-I induced DNA synthesis and cell proliferation. By contrast, ET-1 inhibited the stimulatory effects of TSH actions on DNA synthesis, cell proliferation and c-AMP production. 8-Bromo-cAMP-induced DNA synthesis was also inhibited by ET-1, suggesting that ET-1 exerts its inhibitory effects at step(s) involving cAMP production and post cAMP pathway. ET-1-induced suppression of TSH actions were reversed by a C-kinase inhibitor,H-7. These results suggest that the effect of ET on functions of FRTL5 cells is, at least, in part mediated by C-kinase dependent pathway.

Endothelin(ET) was originally isolated from the culture medium of porcine aortic endothelial cells and shown to be one of the most potent vasoconstrictor peptides(1). In addition, it has been reported that ET is able to induce cell proliferation in a number of cultured cells such as vascular smooth muscle cells(2), glomerular mesangial cells(3), osteoblastic cells(4) or Swiss 3T3 fibroblasts(5). Furthermore, we have found that ET inhibits TSH-induced iodine metabolism in porcine thyroid cells in culture via interaction with ET receptors(6). In the present study, we investigated the effects of ET-1 on growth of a rat thyroid epithelial cell line(FRTL5 cells). We show here that ET-1 modifies growth of FRTL5 cells stimulated by TSH or insulin-like growth factor(IGF)-1.

MATERIALS AND METHODS

Materials ET-1 and ET-2 were purchased from Peptide Institute Inc.(Osaka, Japan) and other chemicals or hormones were from Sigma Chemical Co.(St.Louis, MO).

0006-291X/92 $1.50

Cell culture FRTL5 thyroid cells were kindly provided by Dr.L.D. Kohn, NIH, Bethesda, MD(7) and used between the third and 20th passage. Cells were grown in Coom's modified Ham's F-12 medium, supplemented with 5% calf serum and a six-hormone mixture (6H), which contained bovine TSH(1mU/ml), insulin (10μg/ml), cortisol(0.4ng/ml), transferrin(5μg/ml), glycyl-L-histidyl-L-1-lysine acetate(10ng/ml),and somatostatin(10ng/ml) (8). When cells reached confluence, medium was changed to 5H(without TSH) or 4H(without TSH and insulin) medium depending on the purpose of experiments. All assays were performed in triplicate and were repeated on at least three separate occasions with different batches of cells.

^{3}H-thymidine incorporation into DNA The assay was performed as described previously(9). After replaced in 5H medium for 2 days, test materials and ^{3}H-thymidine(0.1 μCi/well) were added to the culture medium and cultured for 3 days. ^{3}H-thymidine incorporated into TCA-insoluble cellular fractions was counted with a β-counter. Cell numbers were counted by a coulter counter(Coulter Electronics, Hialeah, FL).

cAMP levels in cells Assays were performed as previously described(10). The medium was shifed to 500μl of Hanks' balanced salt solution with 0.5 mM 3-isobutyl-1-methyl-xanthine(IBMX) and 20 mM HEPES(pH7.4) containing the indicated concentration of TSH with or without endothelin. After incubation at 37 C for 2 h, the reactions were terminated by adding 500μl of 20% TCA. The cAMP content in the extract was determined by RIA using commercial kits(New England Nuclear/ Dupon, Boston, MA)

c-fos mRNA levels Total cellular RNA was isolated essentially as described by Chirgwin et al.(11). For Northern blot analysis, RNA samples were electrophoresed in 1% agarose gels containing 0.66 M formaldehyde and blotted on nylon filters. The filters were hybridized to v-fos probe; Oncor(Gaithersburg,MD)(2-4 $\times 10^{5}$ cpm/ml each) and then subjected to autoradiography. Hyblidization and washing were performed as previously described(12); final washings were carried out at 65 C in 1 x SSPE(20 x SSPE is 2.9M NaCl, 0.2 M NaH_2PO_4, 0.01M NaOH, and 0.01M EDTA).

Statistical analysis Statistical evaluation was performed by Student's t-test when variation of the data was uniform or by Cochran-Cox's test when variation was not uniform. P values less than 5% were considered significant. Unless otherwise indicated, all values presented represent the mean±SD.

RESULTS

ET-1 alone had no effects on DNA synthesis and cell replication at concentrations between 10^{-10} and 10^{-7}M in FRTL5 cells cultured in basal medium (without TSH, insulin and with 0.5% calf serum). Intracellular cAMP levels were also not changed by ET-1. However, 10^{-8}M ET-1 or its isopeptide, ET-2, increased c-fos mRNA expression as shown in Fig. 1. The c-fos mRNA levels increased significantly at 30 min after the addition of ET and disappeared in 120 min. The time course is similar to that previously reported for c-fos mRNA expression by TSH(12).

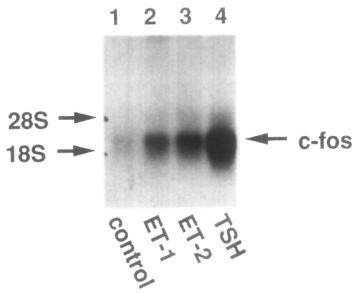

Fig.1. Effect of endothelin(ET) on c-fos mRNA levels. The cells grown to near confluency were shifted to the medium without TSH and were incubated for 3 days. Then cells were exposed to TSH (10^{-10}M) or ET(10^{-8}M) for 30 min. Total RNA was isolated and subjected to Northern blot analysis(10μg/lane) as described in Materials and Methods.

Although ET-1 alone did not affect growth of FRTL5 cells, expression of c-fos, a competent gene, suggested a possible role of ET-1 on growth of FRTL5 cells. Earlier studies have shown that IGF-I is mitogenic to FRTL5 cells(13) or porcine thyroid cells(14). Fig.2A illustrates that ET-1 is able to potentiate DNA synthesis of FRTL5 cells stimulated by IGF-I in a dose-dependent manner. To demonstrate that ^{3}H-thymidine incorporation really reflect mitogenic activity, FRTL5 cells were cultured with IGF-I in the absence or presence of increasing

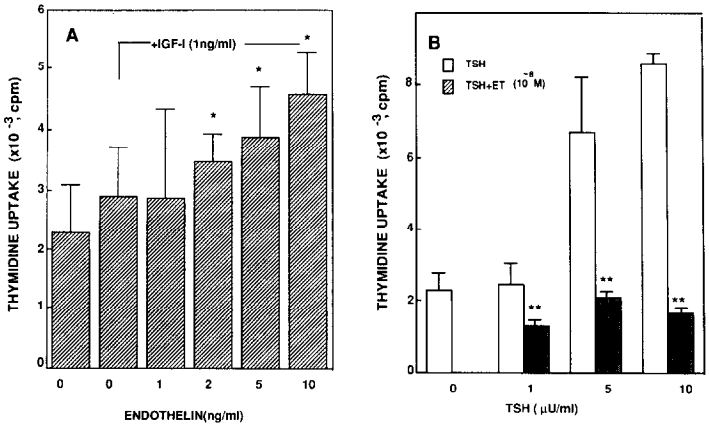

Fig.2. Effect of ET-1 on IGF-I(A) or TSH(B) stimulated ^{3}H-thymidine incorporation into DNA. Cells were preincubated for 3 days in 4H or 5H medium plus 0.5% calf serum and then exposed to fresh medium containing the indicated concentrations of ET-1 with IGF-I or TSH. ^{3}H-thymidine incorporation into DNA was measured 72 hr later, as described in Materials and Methods. The bars represent the mean±SD of triplicated determines of one of three experiments.
* Significantly different from IGF-I alone(P<0.05).
**Significantly different from TSH alone(P<0.01).

Table 1. Effects of endothelin on IGF-I or TSH-induced cell replication in rat FRTL5 cells

	cell number/well
control	3949± 597
IGF-I 10ng/ml	5541±1240
+ET10^{-10}M	6785±1348
+ET10^{-9}M	10321± 861[a]
+ET10^{-8}M	14894±4621[a]
+ET10^{-7}M	16518±1931[a]
TSH 10μU/ml	16483±1032
+ET10^{-10}M	16776±1017
+ET10^{-9}M	13164±1190[b]
+ET10^{-8}M	13824±1443[b]
+ET10^{-7}M	9667± 608[b]

FRTL5 cells were cultured with 10ng/ml IGF-I or 10 μU/ml TSH in the presence or absence of the indicated concentrations of endothelin for 3 days. Results are the mean±SD of triplicate wells from one representative experiment.
[a] P<0.01 compared to IGF-I alone
[b] P<0.01 compared to TSH alone

concentrations of ET-1, and the number of cells was counted (Table1). It can be seen that cell proliferation induced by IGF-I was actually enhanced by ET-1. The effect of ET-1 was dose-dependent and detected at 10^{-9}M.

It is well established that TSH is a potent growth promoting hormone in FRTL5 cells. Actually, as shown in Fig.2B, TSH dose-dependently increased ^{3}H-thymidine incorporation at 72 hr culture. In contrast to the case of IGF-I, however, ET-1(10^{-8}M) significantly(P<0.01) reduced DNA synthesis stimulated by TSH. The response of 100μU/ml TSH was reduced to 19.3% of initial by ET-1. The inhibitory effect of ET-1 was also confirmed by cell proliferation study(Table1). Inhibition was dependent on ET-1 concentration and detected at 10^{-8}M. Since growth promoting effect of TSH is largely mediated by cAMP(15), we compared TSH-stimulated cAMP production in the cells incubated with and without ET-1. Fig.3 illustrates that pretreatment with ET-1 (10^{-8}M) significantly reduced cAMP production by TSH, suggesting that the inhibitory effect of ET-1 on cell growth is due to inhibition of cAMP generation. However, DNA synthesis stimulated by 8-Br-cAMP was also attenuated by ET-1, although the concentration of ET-1 required for half maximal inhibition was approximately 10-fold higher compared to that for inhibition of TSH-stimulated DNA synthesis(data not shown).

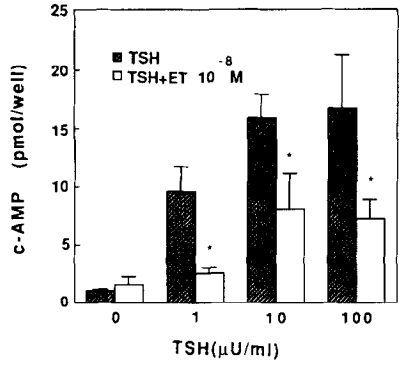

Fig.3. Effect of ET-1 on TSH-stimulated cAMP production. FRTL5 cells were preincubated in 5H medium containing 0.5% calf serum for 3 days. Cells were washed with buffered HBSS and incubated for 2 h with 1 to 100 μU/ml TSH in the presence or absence of 10^{-8} M ET-1. cAMP levels were measured by RIA as described in Materials and Methods. Assays were performed in triplicate on three separate occasions with different batches of cells. *Significantly different from TSH alone($P<0.01$).

It has been suggested that C-kinase dependent pathway is involved in growth regulation by ET-1 in some types of cells (2,5). We examined, therefore, the effect of a putative C-kinase inhibitor, 1-(5-isoquinolynyl sulfonyl)-2-methylpiperazine(H-7), on the effect of ET-1. As can be seen in Table 2, the inhibitory effect of ET-1 on TSH-induced DNA synthesis was completely reversed by 10^{-6}M of H-7. Similarly, the inhibition of TSH-stimulated cAMP production was also reversed by H-7(data not shown).

DISCUSSION

The data presented here demonstrated for the first time that thyroid cell growth is modulated by ET-1. Since recent studies have shown that ET-1 immunoreactivity was observed in rat and

Table 2. Effect of H-7 to the inhibitory effect of endothelin on ^{3}H-thymidine incorporation into DNA

control	6417.5±1252.0
TSH	11195.6±1326.0[a]
TSH+ET	8463.5± 325.3[b]
TSH+ET+H-7	13030.5±2192.8[c]

Cells were incubated with 10μU/mlTSH and 10^{-8}M endothelin in the presence or absence of 10^{-6}M H-7 for three days. The data (cpm/well) are the mean±SD of triplicate determinations.
[a]Statistically significant($P<0.01$) difference from control.
[b]Statistically significant($P<0.05$) difference from TSH alone.
[c]Statistically significant($P<0.01$) difference from TSH+ET.

porcine thyroid follicular cells(16), ET-1 may act in an autocrine fassion in proliferation of thyroid cells. Although ET-1 alone did not affect on DNA synthesis of FRTL5 cells, it caused a rapid increase in c-fos mRNA expression and potentiated IGF-1 stimulated DNA synthesis. The result is similar to that reported for vascular smooth muscle cells; ET-1 elevated intracellular calcium levels and stimulated c-fos and c-myc mRNA expression, and caused stimulation of DNA synthesis in the presence of insulin but it failed to do so in the absence of insulin(17). Enhancement of IGF-I stimulated DNA synthesis by ET-1 has also been reported for Swiss 3T3 fibroblasts(5). Thus, it appears that ET-1 acts as a competent factor which primes the cells to respond to progression factors such as insulin and IGF-I.

In contrast to the effect on IGF-I-stimulated DNA synthesis, ET-1 was inhibitory to DNA synthesis as well as cAMP production stimulated by TSH. Furthermore, DNA synthesis stimulated by 8-Br-cAMP was also attenuated by ET-1. Thus, it appears that ET-1 exerts its inhibitory effect by reducing cAMP generation and by acting on the step(s) distal to cAMP. The mechanism by which ET-1 inhibits cAMP production is unknown. It has been shown that ET-1 induces calcium influx via voltage-dependent channel in smooth muscle cells(18,19). It is possible that ET-1 decreases cAMP content through Ca^{2+} dependent activation of phosphodiesterase. However, this is not likely because the inhibitory effect on TSH-induced cAMP production was not altered in the presence and absence of 1mM IBMX, a phosphodiesterase inhibitor(data not shown).

It is interesting to note that the effect of ET-1 is very similar to that of tetradecanoyl phorbol acetate(TPA), an activator of C-kinase. Lombardi et al.(20) reported that TPA stimulated DNA synthesis and cell replication in the presence of IGF-I, but, inversely, inhibited TSH-stimulated DNA synthesis, cAMP generation, and iodine uptake. These results are compatible with the notion that C-kinase is involved in the action of ET-1. Stimulation of C-kinase by ET-1 has already been reported for growth of Swiss 3T3 cells(5). In addition, Sugiura et al.(21) showed that ET-induced aortic vasoconstriction was reversed by

H-7, a C-kinase inhibitor. Consistent with these recent studies, the inhibitory effect of ET-1 on TSH-induced DNA synthesis and cAMP generation was reversed by treatment with H-7 in FRTL5 cells. Furthermore, our preliminary study have shown that in FRTL5 cells ET-1 stimulated a rapid and transient increase of inositol triphosphate(IP_3) which stimulates the release of intracellular Ca^{2+} stores. The levels increased by 1.6-fold within 1 min and returned to a baseline after 3 min. The results strongly suggests that ET-1 modulates TSH-induced DNA synthesis and cell replication through stimulation of phospholipid/Ca^{2+} and protein kinase C activation.

The present data support the hypothesis that ET-1 produced locally from capillary endothelial cells or from thyrocytes acts as a modulator in the regulation of thyroid follicular cell growth and function.

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