Inhibitory Effect of Atrial Natriuretic Peptide on Accelerated Endothelin Secretion from Cultured Human Endothelial Cells

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This study investigated the effect of atrial natriuretic peptide (ANP) on endothelin (ET) secretion from cultured human endothelial cells. Confluent umbilical venous endothelial cells were incubated with experimental agents in multi-well plates, and the level of immuno-reactive ET in the medium was measured by radioimmunoassay. There was no significant effect of ANP (10^{-8} , 10^{-7} and 10^{-6} M) on ET secretion after a 3- or 6-hour incubation. However, with 24-hour incubation, ANP significantly inhibited ET secretion from cultured human endothelial cells (control, 139.0 ± 7.2 fmol/well; 10^{-8} M, 89.4 ± 4.7 fmol/well; 10^{-7} M, 79.4 ± 8.2 fmol/well; 10^{-6} M, 71.0 ± 10.1 fmol/well, $P < 0.01$). Furthermore, the addition of 8-bromo-cyclic GMP to the medium inhibited ET secretion (control, 147.2 ± 2.9 fmol/well; 10^{-5} M, 140.9 ± 2.3 fmol/well; 10^{-4} M, 143.0 ± 1.0 fmol/well; 10^{-3} M, 96.6 ± 6.3 fmol/well, $P < 0.01$). These findings demonstrate that ANP inhibits accelerated ET secretion from cultured human endothelial cells, probably due to augmentation of intracellular cyclic GMP levels by ANP-activated guanylate cyclase. *J Atheroscler Thromb*, 1994; 1: 76-79.

Key words : Cyclic GMP, Cell culture, Endothelin, Atrial natriuretic peptide

Atrial natriuretic peptide (ANP) is a polypeptide which regulates body fluid volume and sodium balance through its potent natriuretic properties (1). Furthermore, ANP is known to relax vascular smooth muscle cells in the absence of endothelium (2). It has therefore been speculated that endothelial cells do not have an important role to play in the effect of ANP on vasodilation. However, recent observations have demonstrated that ANP receptors are present on endothelial cells (3), and that ANP increases cyclic GMP levels in these cells (4). These observations suggest that ANP influences on endothelial function. However, this effect has received very little scrutiny, especially concerning the release or synthesis of endothelium-derived vasoactive substances.

Endothelin (ET) is a recently discovered 21-amino-acid sequenced polypeptide which is secreted from vascular endothelial cells (5). This peptide has been reported to have potent vasoconstrictive and proliferative properties

on vascular smooth muscle cells (6, 7). Systemic administration of synthetic ET has induced significant biological effects such as an increase of blood pressure, a reduction in cardiac output, and antinatriuresis in humans and animals (8, 9). Furthermore, elevated plasma ET levels have been reported in patients with essential hypertension (10), atherosclerosis (11) and congestive heart failure (12). These findings suggest that ET may contribute to the pathogenesis of these conditions (13). However, the mechanism of ET secretion from vascular endothelial cells is unclear. This study was carried out to clarify the effects of ANP on ET secretion from cultured human vascular endothelial cells.

Materials and Methods

Materials

ANP was purchased from the Peptide Institute (Osaka, Japan), and 8-bromo-cyclic GMP was obtained from Sigma Chemical Co. (St Louis, MO). Modified MCDB 131 (EBM or EGM-UV, Kurabou, Osaka, Japan) was used as the culture medium.

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Cell culture and experiments

Endothelial cells were obtained from human umbilical cord veins according to the modified method previously described by Jaffe *et al.* (14). These were subcultured with calf-serum containing medium (EGM-UV) at 37°C under 5%CO₂/95% air. The cultured cells were identified as endothelial cells by the typical cobble-stone morphology and immunoenzymatic staining of factor VIII, which are characteristic features of vascular endothelial cells. The cultured cells were detached from the culture flasks by trypsin, and were seeded at a concentration of 2×10^4 cells/ml onto 24 multi-well plates. Confluent cells were obtained 5–7 days after subseeding, at which point the incubation medium was replaced with 0.5 ml of fresh serum-free medium (EBM) and preincubated for 1 hour. After preincubation, the medium was exchanged for 1.0 ml of serum-free medium (EBM) with or without the experimental agents added, and this was incubated at 37°C. At the end of incubation, the medium was removed from the wells and stored immediately at –70°C until assayed.

Assay of ET

The concentration of ET in the medium was measured by direct radioimmunoassay using a specific ¹²⁵I-ET assay system (RPA-555, Amersham, Buckinghamshire, UK). This antibody has a cross-reactivity of 100% with ET-1, 144% with ET-2, 52% with ET-3 and 0.4% with big ET-1. The sensitivity of ET concentration was 0.5 fmol/tube.

Statistics

The data were expressed as mean $\pm$ SEM, and were evaluated by one-way analysis of variance. A value of $p < 0.05$ was considered statistically significant.

Results

Basal ET accumulation in control wells

The accumulation of ET in the medium increased in a time-dependent manner from 3 to 24 hours after the commencement of incubation. The concentration of ET in the control wells was 3.1 ± 3.1 fmol/well for 3 hrs, 16.3 ± 5.1 fmol/well for 6 hrs, 58.7 ± 3.2 fmol/well for 12 hrs, and 139.0 ± 7.2 fmol/well for 24 hrs (Fig. 1).

Effects of ANP on ET secretion

ANP (10^{-8} , 10^{-7} and 10^{-6} M) significantly inhibited ET secretion from cultured human endothelial cells over a 24-hour incubation (control, 139.0 ± 7.2 fmol/well; 10^{-8} M, 89.4 ± 4.7 fmol/well; 10^{-7} M, 79.4 ± 8.2 fmol/well; 10^{-6} M, 71.0 ± 10.1 fmol/well, $P < 0.01$; $n = 6$, Fig. 1). However, no significant effect of ANP on ET secretion was noted in the experiments employing a shorter incubation period of 3 or 6 hours.

Effects of 8-bromo-cyclic GMP on ET secretion

The addition of 8-bromo-cyclic GMP (10^{-5} , 10^{-4} and 10^{-3}

Fig. 1. Time course of the effects of various concentrations of atrial natriuretic peptide (ANP) on endothelin (ET) secretion from cultured human endothelial cells. The accumulation of ET in the control well increased in a time-dependent manner. The ANP-induced inhibitory action of ET secretion was apparent in the 12- and 24-hour experiments (at 12 hrs, $p < 0.05$; at 24 hrs, $p < 0.01$), while any effect after 3 or 6 hours was obscure. open bar, control; hatched bar, ANP 10^{-8} M; cross-hatched bar, ANP 10^{-7} M; black bar, ANP 10^{-6} M. Data was expressed as mean $\pm$ SEM.

M) to the medium used in the 24-hour incubation resulted in inhibition of ET secretion only at the 10^{-3} M concentration (control, 147.2 ± 2.9 fmol/well; 10^{-5} M, 140.9 ± 2.3 fmol/well; 10^{-4} M, 143.0 ± 1.0 fmol/well; 10^{-3} M, 96.6 ± 6.3 fmol/well, $P < 0.01$; $n = 4$, Fig. 2).

Discussion

This study demonstrates that ANP inhibits ET secretion in a concentration-dependent like manner from cultured human endothelial cells. This effect was clearly evident after 24-hours incubation, but was obscure after 3- or 6-hours incubation.

There is no previous report investigating the time course of ANP's effects on ET secretion in the manner of the present study. Kohno *et al.* (15) examined the effect of ANP on ET secretion over a 4-hour incubation period using cultured human endothelial cells, and reported that although ANP had no effect on ET secretion under basal conditions, secretion was inhibited when ET secretion was stimulated by thrombin.

In the present experiment, the mean value of ET secretion in the control wells during the first 3 hours was 3.1 fmol/well. If it is assumed that ET was secreted at a constant rate during the entire 24-hour period, the total

Fig. 2. Effects of 8-bromo-cyclic GMP (10^{-5} , 10^{-4} and 10^{-3} M) on endothelin (ET) secretion during 24 hour incubation with cultured human endothelial cells. CT=control. 8-bromo-cyclic GMP inhibited ET secretion at 10^{-3} M ($p < 0.01$ vs. control). Data was expressed as mean $\pm$ SEM.

amount of ET in the control wells would be calculated at 24.8 fmol/well. However, the actual accumulated amount of ET in the culture medium after 24 hours of incubation (139.0 ± 7.2 fmol/well) was 5 times greater than this calculated value. In view of this fact, we speculated that ET secretion accelerates during the incubation period. We therefore hypothesize that ANP does not consistently inhibit ET secretion from cultured human endothelial cells, but that its inhibitory effect is evident in this acceleration of ET secretion.

Although this study did not determine which factors caused this acceleration of ET secretion from cultured endothelial cells, endothelial cells have been known to produce several vasoactive factors (5, 16–18) (e.g. ET, transforming growth factor β). Recently, ET receptors were identified in endothelial cells (19), and it was demonstrated that ET stimulates its own synthesis (20) and mitogenesis (21) of endothelial cells in cultured human endothelial cells. In addition, transforming growth factor β was also reported to stimulate ET secretion from cultured endothelial cells (22, 23). We speculated that these endogenous vasoactive factors produced by cultured endothelial cells accumulate gradually in the medium during the incubation periods, and thus stimulate ET secretion in an autocrine fashion.

It was reported that ANP increases cyclic GMP concentration in endothelial cells (4). Therefore, we investigated whether 8-bromo-cyclic GMP would inhibit ET secretion from cultured human endothelial cells, and observed an inhibitory effect at the highest incubation concentration. In addition, we reported previously that nitroprusside, which is known to increase cyclic GMP concentration in endothelial cells, inhibited the stimulation of ET secretion induced by calf-serum rich conditions (24). Taken

together, these findings suggest that the increase in cyclic GMP in the cells may be important for the natriuretic peptide-induced inhibitory action of ET secretion from cultured human endothelial cells.

The concentration of ANP added to the medium of the present study was more than 100 times higher than that observed in plasma under various disease conditions (25–27). However, under culture conditions, the peptide in the medium may be degraded during incubation, whereas *in vivo*, intact ANP is supplied constantly from the heart to the local vascular tissue via blood circulation. If this ANP-induced inhibitory action of ET secretion occurs *in vivo*, the pathophysiological implications of this effect in the cardiovascular system must be addressed. Since increased circulating ANP levels have been reported in patients with congestive heart failure (25), hypertension (26) and chronic renal failure (27), ANP may antagonize abnormal vasoconstriction, impaired renal function and exaggerated proliferation of vascular smooth muscle cells possibly caused by ET under these pathological conditions (10, 28, 29).

In conclusion, ANP inhibits accelerated ET secretion from cultured human endothelial cells, probably due to augmentation of intracellular cyclic GMP levels by ANP-stimulated guanylate cyclase.

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