Vol. 172, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1990 Pages 178-183

ENDOGENOUS DIGITALIS-LIKE FACTOR AS A STIMULATOR OF ENDOTHELIN SECRETION FROM ENDOTHELIAL CELLS

Kaoru Yamada, Atsuo Goto, Chen Hui and Tsuneaki Sugimoto

The Second Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo 113, Japan

Received August 20, 1990

Summary: Effects of human urine-derived endogenous digitalis-like factor (EDLF) and ouabain on endothelin (ET) secretion were examined in cultured endothelial cells. ET was secreted in a linear fashion over 5 hours from bovine pulmonary artery endothelium into serum-free medium. EDLF stimulated ET secretion in a dose-dependent manner. In contrast, ouabain did not affect ET secretion at the concentration of 10^{-9}-10^{-5}M. These results indicate that human urine-derived EDLF is distinct from plant-derived ouabain and act as a stimulator of ET secretion by endothelial cells.

We have recently isolated two endogenous digitalis-like factors (EDLFs) from human urine with use of reverse-phase HPLC based on the inhibitory effects on [^{3}H]-ouabain binding to intact human erythrocytes (1). Ouabain-displacing compound 1 (ODC-1) and ODC-2 were reproducibly eluted with 18% and 31% acetonitrile in water from reverse-phase C_{18} column, respectively. Urinary excretion of polar ODC-1 significantly increased on a high salt diet (2). ODC-1 raised intracellular free calcium concentration of cultured vascular smooth muscle cells (1). Thus, ODC-1 apparently fullfils the criteria for the putative natriuretic and vasoactive digitalis-like factor. Endothelin (ET) is a potent vasoconstrictive peptide generated by endothelial cells and ET secretion has been shown to be controlled by various stimuli (3-6). In this study, we examind the effects of ODC-1 on endothelin secretion to investigate the possible interaction between these two vasoactive substances.

0006-291X/90 $1.50

Materials and Methods

Purification of ouabain-displacing compounds: Different batches of 60 l each of human urine collected from normal male volunteers were processed as described previously (1). Human urine after filtration through 0.45 μm filter paper was applied to reverse-phase HPLC (D-ODS-5 column) and was fractionated with a gradient of acetonitrile (0-40%) in water over 80 minutes. One minutes fractions were collected, freeze-dried and assayed for digitalis-like activity using the inhibitory effects on [^{3}H]-ouabain binding to intact human erythrocytes under the assay condition described elsewhere (7). Polar ODC-1 and less polar ODC-2 emerged at retention times of 38-41 minutes and 70-75 minutes, respectively. The fractions containing ODC-1 were pooled and rechromatographed on a D-ODS-5 columu under the same condition mentioned above. The active fractions were again pooled, freeze-dried and reconstituted in 100 mM Tris-HCl (pH 7.4) at the concentration of 20 units/100 μl. Buffer with high ionic strength was used to keep the pH of the sample neutral, but this buffer alone had no effect on ET secretion. 20 units of ODC-1 inhibited [^{3}H]-ouabain binding by 95%. One unit of ODC was defined as that amount required to inhibit [^{3}H]-ouabain binding by 50% under our assay condition.

Cell culture: CPAE (ATCC;CCL 209, bovine pulmonary artery endothelium) cell lines were cultured in Dulbecco's modified Eagles' medium (DMEM) containing 10% fetal bovine serum and antibiotics at 37 ℃ in an atmosphere of 5% CO_2 / 95% air in 12-well-culture plates. After reaching confluency, the culture medium was aspirated and serum-free DMEM was added to the cells. To examine the effects of ODC-1, 0.9 ml serum-free DMEM and 0.1 ml of 100 mM Tris-HCl with or without ODC-1 were added. Ouabain was dissolved in serum-free DMEM to the concentration of 10^{-9}-10^{-5}M and 1 ml of DMEM or DMEM containing ouabain was added. Experiments on ODC-1 were carried out twice with different batches of ODC-1. ET released during 3 hours' incubation was measured by a radioimmunoasay (RIA).

Radioimmunoassay for ET: One ml of culture medium was acidified with 0.25 ml of 2 M HCl and was loaded onto Sep-pak C18 cartridge which was preequilibrated by washing with 5 ml of methanol followed by 10 ml of water. The cartridge was washed with 15 ml of water containing 0.1% trofluoacetic acid (TFA) and ET was eluted with 3 ml of 80% acetonitrile containing 0.1% TFA. The eluent was evaporated, freeze-dried and reconstituted in 0.25 ml of 0.02 M borate buffer (pH 7.4). RIA for ET was performed using Endothelin-1,2 [^{125}I]-assay system (Amersham; Amersham, U.K.)

Analysis of culture medium: The extract of culture medium was applied to reverse-phase HPLC and was eluted with a linear gradient of acetonitrile in water containing 0.1% TFA. Each fraction was subjected to RIA and immunoreactive ET peak was compared with the elution position of synthetic ET-1.

Results

Serial dilution curve of the extract of the culture medium from CPAE cells was parallel to that of standard ET-1 (Fig.1). Immunoreactive ET was secreted in a linear fashion over 5 hours at a level of 20 to 50 fmol/well (Fig. 2). Analysis of extract with reverse-phase HPLC revealed the presence of one major immunoreactive ET peak corresponding to synthetic ET-1. In the experiment with first batch of ODC-1 sample, ODC-1 stimulated ET secretion in a dose-dependent manner. Immunoreactive ET secreted under control condition was 32.7±2.1[SE] fmol/well/3 hours. 2 units of ODC-1 slightly increased ET secretion (37.4±1.0 fmol/well/3 hours), but this increase was not statistically significant. 6 and 20 units of ODC-1 significantly increased ET secretion (44.6±1.7 [$p<0.05$] and 57.5±2.5 fmol/well/3 hours [$p<0.01$], respectively, n=3 for each, Fig.3). In the experiment with second batch of ODC-1 sample, similar results were obtained. 3.3 units of ODC-1 significantly increased ET secretion three-fold (52.1±0.75 and 171.7±16.1 fmol/well/3 hours at 0 and 3.3 units of ODC-1, respectively, $p<0.01$). 10 units of ODC-1 provoked prominent ET secretion (more than ten-fold). In contrast, ouabain even at the concentration of 10^{-7}-10^{-5} M,

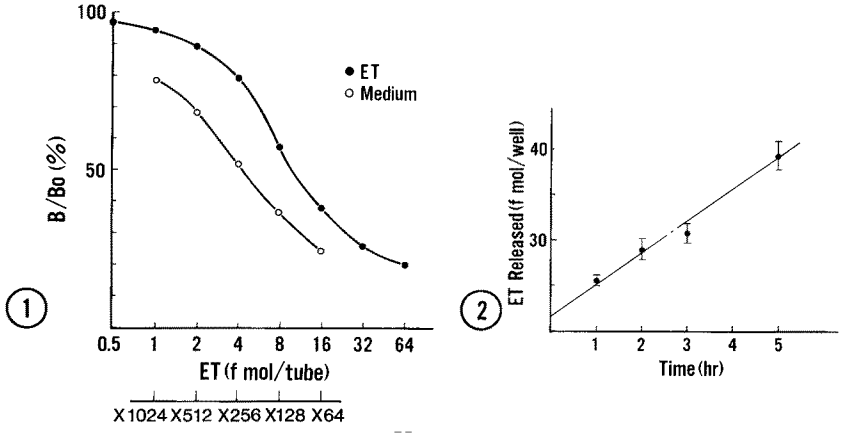

Fig. 1. Typical standard curve of the radioimmunoassay for endothelin (ET). Serial dilution curve of the culture medium from bovine pulumonary artery endothelial (CPAE) cells is also depicted.

Fig. 2. Release of immunoreactive endothelin (ET) from CPAE cells as a function of time. Each point is the mean of 3 measurements; bars indicate SE.

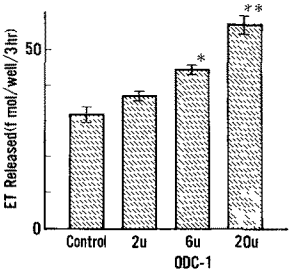

Fig. 3. Effect of ouabain displacing compound-1 (ODC-1) on the release of immunoreactive endothelin from CPAE cells. Confluent CPAE cells were incubated with various doses of ODC-1 in serum-free medium for 3 hours. Secreted endothelin was determined by radioimmunoassay. Each column represents the mean of triplicate experiments; bars indicate SE. Asterisks show statistically significant differences from control (* $p<0.05$, ** $p<0.01$).

which completely inhibits [^{3}H]-ouabain binding, did not affect ET secretion.

Discussion

In the present study, we have demonstrated that semipurified human urine-derived polar EDLF (ODC-1) increased ET secretion by cultured endothelial cells. Many studies have suggested the existence of EDLF and its involvement in the mechanisms of hypertension (8-12). However, the nature, source and precise mechanism of action have not been elucidated. We have been able to purify two ODCs from human urine and demonstrated that the polar ODC-1 is reversible, competitive and specific inhibitor of [^{3}H]-ouabain binding to intact human erythrocytes (7, 13, 14). Urinary excretion of ODC-1 increased in normotensive subjects on the high salt diet (2). Furthermore, the polar ODC-1 (10 units/ml) increased intracellular free calcium concentration ($[Ca]_i$) of cultured rat vascular smooth muscle cells (VSMC) (1). In contrast, ouabain (10^{-5}-10^{-4} M) had no effect on $[Ca]_i$ of cultured VSMC. In this study, ET secretion from endothelial cells has not been influenced by ouabain at the concentration of 10^{-9}-10^{-5} M.

In cultured endothelial cells, the gene expression of ET can be stimulated by thrombin, transforming growth factor beta, epinephrine and calcium ionophore A23187 (15). Furthermore, it has been reported that thrombin, arginine-vasopressin and

angiotensin Ⅱ dose-dependently stimulated ET secretion into serum-free medium of cultured bovine endothelial cells (3). However, the precise mechanism of ET release from endothelial cells has not been clarified yet. Recent reports indicate that intracellular calcium mobilization and activation of protein kinase-C may play important roles in ET release (3,16). In view of these findings, it is likely that ODC-1 increased ET secretion from endothelial cells through its action on $[Ca]_i$.

The tone of vasculature could be modulated by the intricate balance between vasodilatory substances and vasoconstrictive substances released from endothelial cells. Although the effects of ODC-1 on the release of endothelium-derived relaxing factors were not determined in this study, ET release was dose-dependently stimulated by ODC-1. These results suggest that ODC-1 may increase the vascular tone not only by its direct effect on $[Ca]_i$ of VSMC but also by stimulatory effect on ET release from endothelial cells. Whether these actions of ODC-1 may contribute to pathophysiological mechanisms of hypertension remains to be determined.

References

1. Goto, A., Yamada, K., Ishii, M., Yoshioka, M., Ishiguro, T., Eguchi, C. and Sugimoto, T. (1989) Hypertension 13, 916-921.
2. Goto, A., Yamada, K., Ishii, M. and Sugimoto, T. (1990) Nephron 54, 99-100.
3. Emori, T., Hirata, Y., Ohta, K., Shichiri, M. and Marumo, F. (1989) Biochem. Biophys. Res. Commun. 160, 93-100.
4. Shini, V.B., Hendrickson, H., Heublein, D.M., Burnett, J.C.Jr. and Vanhoutte, P.M. (1989) Eur. J. Pharmacol. 165, 333-334.
5. Yoshizumi, M., Kurihara, H., Morita, T., Yamashita, T., Ohhashi, Y., Sugiyama, T., Takaku, F., Yanagisawa, M., Masaki, T. and Yazaki, Y. (1990) Biochem. Biophys. Res. Commun. 166, 324-329.
6. Boulanger, C. and Lüscher, T.F. (1990) J. Clin. Invest. 85, 587-590.
7. Goto, A., Yamada, K., Ishii, M., Yoshioka, M., Ishiguro, T. and Sugimoto, T. (1988) Hypertension 11, 645-650.
8. Tamura, M., Lam, T-T. and Inagami, T. (1988) Biochemistry 27, 4244-4253.
9. Hamlyn, J.M., Harris, D.W., Clark, M.A., Rogowski, A.C., White, R.J. and Ludens, J.H. (1989) Hypertension 13, 681-689.
10. Cloix, J-F. (1987) Hypertension 10 (suppl 1), I 67-I 70.

11. Haddy, F.J., Pamnani, M.B. and Clough, D.L. (1987) Hypertension 10 (suppl 1), I 101-I 107.
12. Blaustein, M.P., Ashida, T. and Hamlyn, J.M. (1987) Klin. Wochenschr. 65 (suppl Ⅶ), 21-32.
13. Goto, A., Yamada, K., Ishii, M., Yoshioka, M., Ishiguro, T., Eguchi, C. and Sugimoto, T. (1988) Biochem. Biophys. Res. Commun. 152, 322-327.
14. Goto, A., Yamada, K., Ishii, M., Yoshioka, M., Ishiguro, T., Eguchi, C. and Sugimoto, T. (1988) Biochem. Biophys. Res. Commun. 154, 847-853.
15. Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, T., Goto, K. and Masaki, T. (1988) Nature 332, 411-415.
16. Yanagisawa, M., Inoue, A., Takuwa, Y., Mitsui, Y., Kobayashi, M. and Masaki, T. (1989) J. Cardiovasc.Pharmacol. 13 (suppl 5), S 13-S 17.