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INHIBITION OF ENDOTHELIAL CELL CLEARANCE OF ATRIAL NATRIURETIC PEPTIDE BY CYCLIC GMP TREATMENT

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Summary: In a previous study, we reported that cyclic GMP (cGMP) selectively down-regulates the clearance receptor (C-receptor) for atrial natriuretic peptide (ANP) in the cultured bovine pulmonary artery endothelial (CPAE) cell line. The present study was undertaken in order to examine the effect of cGMP on the internalization of the ANP-receptor complex in CPAE cells. Maximum binding of [^{125}I]APIII to the cells significantly decreased following the treatment with 1 mM 8-bromo-cGMP for 48 or 72 h. Scatchard analysis of the binding assay data from the treated cells showed a decrease in Bmax (616 to 411 fmol/mg protein) without a significant change in Kd. Removal of cell surface-bound APIII by acetic acid revealed that not only the surface binding, but also the internalization of APIII significantly decreased in 8-bromo-cGMP-treated cells, indicating a decrease in receptor-mediated uptake of ANP into the cells. These results suggest that cGMP regulates the clearance of ANP by vascular endothelial cells.

Atrial natriuretic peptide (ANP) is a circulating hormone which regulates blood pressure and extracellular fluid volume (1). ANP elicits vasodilator and natriuretic effects by specific binding to cell surface receptors (1). At least two subtypes of ANP receptors have so far been identified in target organs or cultured cells including vascular endothelial

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Abbreviations: ANP, atrial natriuretic peptide; APIII, rat ANP (103-126); CPAE cells, cultured bovine pulmonary artery endothelial cells; cGMP, cyclic GMP; DMEM, Dulbecco's modification of Eagle's media; FBS, fetal bovine serum.

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cells (2, 3). One is a biologically active receptor coupled with the membrane type guanylate cyclase (B-receptor) and the other is a "clearance" receptor (C-receptor) which lacks guanylate cyclase activity (2-4). ANP binding to the B-receptor results in an accumulation of intracellular cyclic GMP (cGMP), a major second messenger for ANP (1). The precise function of the C-receptor is still unclear; however, a "clearance" function of ANP from the circulation is thought to be a major role (4, 5). ANP receptors have been reported to be regulated by glucocorticoids or angiotensin II in cultured cells (6, 7). Recently, we reported that cGMP selectively down-regulates the C-receptor in the cultured bovine pulmonary artery endothelial (CPAE) cells (8). The present study was carried out in order to determine if cGMP regulation of the ANP C-receptor affects uptake of ANP by CPAE cells.

Materials and Methods

Cell culture and chemicals: A cultured bovine pulmonary artery endothelial (CPAE) cell line (CCL 209, American Type Culture Collection) was obtained at passage 17. The cells were plated in 24- or 48-well culture plates (Costar, Cambridge, MA) and grown at 37° C in 5% CO_2 humidified incubator in Dulbecco's modification Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. After reaching confluence, the cells were subjected to experiments at passages 18-20. APIII, rat ANP (103-126), was provided by the Searle Co., and 8-bromo-cGMP was purchased from Sigma, St. Louis, MC [^{125}I]APIII was prepared by the Iodogen method (9) with a specific activity of 300-600 mCi/mg.

Cell treatment and binding assay: Confluent monolayers of CPAE cells were incubated with 1 mM 8-bromo-cGMP in DMEM with 10% FBS at 37° C for various time periods. Incubated cells were washed twice with 1.0 ml/well of binding assay buffer (Earle's media, 0.05% BSA, 25 mM HEPES, pH 7.2) and subjected to the binding assay. Binding assay was performed as reported previously (6). All binding data were corrected with total cellular protein concentrations determined by the Lowry method (10). Equilibrium binding parameters were calculated based on the LIGAND program (11).

Measurement of cell surface-bound and internalized APIII: Confluent CPAE cells treated with 1 mM 8-bromo-cGMP for 48 h were washed twice with 0.5 ml/well DMEM at 37° C. The cells were then incubated with 100 fmol of unlabeled APIII and 25 fmol of [^{125}I]APIII in 0.5 ml/well binding assay buffer at 37° C for various time periods. After the given time incubation, binding reaction was terminated by aspirating media and quickly washing four times with 1.0 ml/well ice cold binding assay buffer. Cell surface-bound and internalized APIII were determined according to the methods described by Haigler et al. (12) Briefly, the washed cells were treated with 0.5 ml/well of 0.2 M acetic acid (pH 2.5) containing 0.5 M NaCl for 4 min to remove cell surface-bound radioactivity. After the removal of acetic acid, the cells were solubilized in 0.5 ml 1 N NaOH for the measurement of internalized [^{125}I]APIII.

Statistical analysis: Multiple comparisons were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's t-test. Student's t-test was used for comparisons between two experimental groups. All data were expressed as means ± S.E.M. and P value less than 0.05 was considered to be significant.

Results and Discussion

In order to examine the effect of cGMP on APIII binding, cultured bovine pulmonary artery endothelial (CPAE) cells were treated with 1 mM 8-bromo-cGMP, a membrane permeable cGMP analog, for various time periods (Figure 1). The treatment with 8-bromo-cGMP for 48 or 72 h significantly decreased maximum [^{125}I]APIII binding to 90.1 ± 2.4% or to 85.5 ± 2.1% of control, respectively. Figure 2 shows Scatchard analysis of the binding assay data from control CPAE cells and those treated with 1 mM 8-bromo-cGMP for 48 h. As we reported previously (6), control CPAE cells contained a single class of high affinity binding sites with an equilibrium dissociation constant (Kd) of 114 pM and a maximum binding (Bmax) of 616 fmol/mg cellular protein. The treatment with 8-bromo-cGMP decreased Bmax (616 to 411 fmol/mg protein) without a significant change in Kd. As previously reported, cGMP, a major second messenger of ANP, selectively down-regulates the ANP clearance receptor (C-receptor) in CPAE cells (8). The data presented above are consistent with our previous observations.

Two subtypes of ANP receptors have been reported to be widely distributed in target organs or cultured cells including vascular endothelial cells (1-3). The biologically active receptor (B-receptor) contains the membrane type guanylate cyclase in its molecule, and produces cGMP in response to the binding of ANP (1-3). Meanwhile, the function of the C-receptor is still controversial. Almeida et al. (5) reported that [^{125}I]ANP infused intravenously was rapidly degraded into small fragments in rats. They showed an inhibition of the ANP degradation by the simultaneous infusion of C-ANF, a specific ligand for

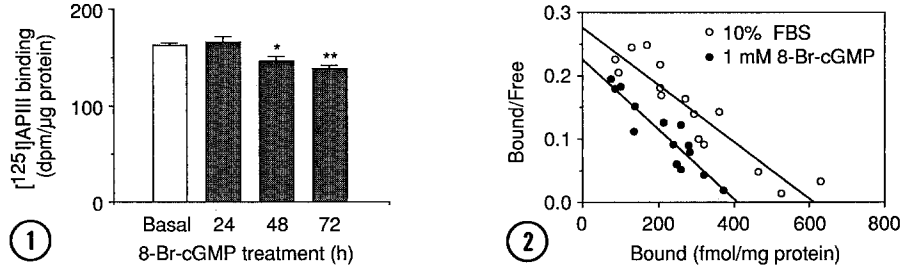

Figure 1. The effect of 1 mM 8-bromo-cGMP on maximum [^{125}I]APIII binding to CPAE cells. Confluent CPAE cells were incubated with 1 mM 8-bromo-cGMP in DMEM with 10% FBS for indicated time periods. Incubated cells were washed and subjected to the binding assay. Values are means ± S.E.M. of twelve wells examined. *P<0.05, **P<0.01, compared to control.

Figure 2. Scatchard analysis of binding assay data from control (O) and 8-bromo-cGMP-treated cells (●). Confluent CPAE cells were incubated with 1 mM 8-bromo-cGMP in DMEM with 10% FBS for 48 h. Incubated cells were washed and subjected to the binding assay.

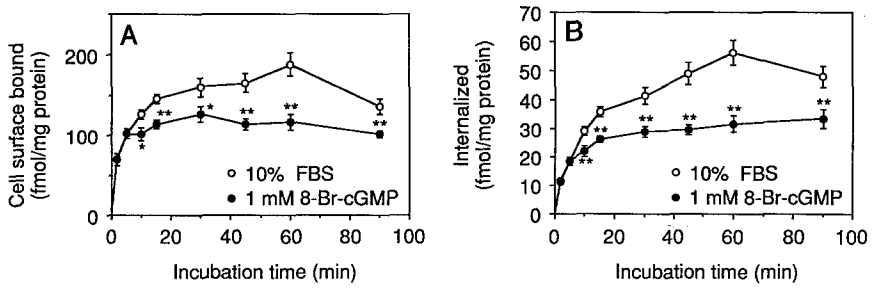

Figure 3. Surface-bound (**A**) and internalized APIII (**B**) in CPAE cells without (O) or with (●) 1 mM 8-bromo-cGMP pretreatment for 48 h. After the pretreatment, the cells were washed and subjected to the binding experiments. Cell surface-bound and internalized APIII were determined as described under "Materials and Methods". Values are means ± S.E.M. of eight to twenty wells examined. *P<0.05, **P<0.01, compared to untreated cells.

the C-receptor (4, 5). Hirata et al. (13) observed that internalized ANP-receptor complex was delivered to lysosomes in cultured vascular smooth muscle cells. These findings suggest that circulating ANP is taken into cells by internalization of C-receptor-ANP complex, and subsequently degraded by lysosomal enzymes. Vascular endothelial cells have been reported to possess both subtypes of receptors and to produce cGMP following the ANP binding to the B-receptor (2, 8). In comparison with other tissues, endothelial cells have abundant C-receptors with more than 95% of ANP receptors being of this type (2, 8). Taken together with the fact that vascular endothelium is always exposed to the blood stream, it seems likely that endothelial cells have an important role in the metabolism of circulating ANP by C-receptor-mediated uptake of ANP. Figure 3 demonstrates the time course of the cell surface-bound (**A**) and the internalized APIII (**B**) following incubation with 200 pM unlabeled APIII and 50 pM ^{125}I-APIII, a physiological range of ANP concentration for plasma (14). Interestingly, not only cell surface-bound but also the internalized APIII significantly decreased by about 40% in cells treated with 8-bromo-cGMP compared to untreated cells. The decreased internalization may result in a concomitant decrease in degradation of ANP. Hence, cGMP is likely to influence circulating ANP levels by regulating the C-receptor density in vascular endothelial cells.

In summary, the chronic treatment of cultured endothelial cells with cGMP induced the down-regulation of the ANP C-receptor. This decrease in the density of the C-receptor was associated with a decrease in the uptake of ANP by the endothelial cells. The clearance of ANP from the circulation, in part, is thought to be mediated by the C-receptor. Thus, these results suggest that cGMP may regulate plasma ANP levels by regulating the C-receptor density in vascular endothelium.

References

1. Needleman, P., Blaine, E.H., Greenwald, J.E., Michener, M.L., Saper, C.B., Stockmann, P.T., and Tolunay, H.E. (1989) Annu. Rev. Pharmacol. Toxicol. 29, 23-54.
2. Leitman, D.C., Andresen, J.W., Kuno, T., Kamisaki, Y., Chang, J.K., and Murad, F. (1986) J. Biol. Chem. 261, 11650-11655.
3. Takayanagi, R., Inagami, T., Snajdar, R.M., Imada, T., Tamura, M., and Misono, K.S. (1987) J. Biol. Chem. 262, 12104-12113.
4. Maack, T., Suzuki, M., Almeida, F.A., Nussenzveig, D., Scarborough, R.M., McEnroe, G.A., and Lewicki, J.A. (1987) Science 238, 675-678.
5. Almeida, F.A., Suzuki, M., Scarborough, R.M., Lewicki, J.A., and Maack, T. (1989) Am. J. Physiol. 256, R469-R475.
6. Lanier-Smith, K.L., and Currie, M.G. (1990) Eur. J. Pharmacol. 178, 105-109.
7. Chabrier, P.E., Roubert, P., Lonchampt, M.O., Plas, P., and Braquet, P. (1988) J. Biol. Chem. 263, 13199-13202.
8. Kato, J., Lanier-Smith, K.L., and Currie, M.G. (1991) J. Biol. Chem. 266, 14681-14685.
9. Fraker, P.J., and Speck, J.C. (1978) Biochem. Biophys. Res. Commun. 80, 849-857.
10. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
11. Munson, P.J., and Rodbard, D. (1980) Anal. Biochem. 107, 220-239.
12. Haigler, H.T., Maxfield, F.R., Willingham, M.C., and Pastan, I. (1980) J. Biol. Chem. 255, 1239-1241.
13. Hirata, Y., Takata, S., Tomita, M., and Takaichi, S. (1985) Biochem. Biophys. Res. Commun. 132, 976-984.
14. Kato, J., Kida, O., Kita, T., Nakamura, S., Sasaki, A., Kodama, K., and Tanaka, K. (1988) Biochem. Biophys. Res. Commun. 153, 1084-1089.