Extracellular Adenosine 5'-Triphosphate Decreases the Permeability of an Endothelial Cell Monolayer through Protein Kinase C

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Adenosine 5'-triphosphate (ATP) is known to have various effects on cells as an extracellular nucleotide. It decreases the degree of edema and the requirement for intravenous hydration in critically ill patients. We examined the direct effects of ATP on the permeability of a vascular endothelial cell monolayer using an *in vitro* co-culture chamber system.

Human umbilical vein endothelial cells were cultured on filter membranes of co-culture chambers to form a confluent monolayer. FITC-labeled 70 k dalton dextran and ATP at various concentrations were added to the upper chamber. The concentrations of FITC-dextran, which permeated to the lower chamber through the endothelial cell monolayer, were determined in 4 hr. A significant decrease in the permeability of

FITC-dextran was observed with 10^{-6} to 10^{-3} M of ATP. One, 10, 100, and 1000 μ M ATP decreased the permeability from 1.00 ± 0.05 to 0.75 ± 0.05 , 0.66 ± 0.04 , 0.61 ± 0.07 , and 0.58 ± 0.08 respectively (mean $\pm$ SD, $n=5$, $p<0.05$). Adenosine, an ATP metabolite, did not change the permeability. Phorbol 12-myristate 13-acetate (PMA), a protein kinase C stimulator, decreased the permeability. Treatment with staurosporine, a protein kinase inhibitor, and myristoylated protein kinase C (19-27), a protein kinase C specific inhibitor, suppressed the decrease of the permeability by ATP.

These results indicated that ATP decreases the permeability of endothelial cell monolayers through protein kinase C (PKC) in a signal transduction system.

Key words: Adenosine 5'-triphosphate, Endothelial cell, Protein kinase C

I. Introduction

Third spacing of body fluid is a critical problem in seriously ill patients. Previous reports showed that ATP decreases leakage from the intravascular lumen in shock models [8, 10]. Clinically, many investigators reported beneficial effects of magnesium-adenosine 5'-triphosphate in shock, ischemia and sepsis [4]. Purine nucleotide and nucleosides not only are involved in cellular metabolism but also act as extracellular mediators and initiate or modulate cellular responses via cell surface receptors. However, the direct effect of ATP on the permeability of an endothelial cell monolayer has not been examined in an *in vitro* system. An *in vitro* assay system, developed by the authors to evaluate the permeability of cell monolayer [11], was used in this study to examine the direct effect of

ATP on an endothelial cell monolayer and to elucidate how the contribution of protein kinase C (PKC) modifies its effect.

II. Materials and Methods

Cell culture

Human endothelial cells from an umbilical vein (Kurabo, Ltd., Osaka, Japan) were grown in plastic culture flasks. The cell culture medium was made of: MCDB 131 (Sigma Chemical Co., St. Louis, MO, U.S.A.) with 2% heat inactivated newborn calf serum (Life Technologies, Inc., Grand Island, NY, U.S.A.), 10 mg/ml bovine pituitary extract (Upstate Biotechnology Inc., Lake Placid, NY, U.S.A.), 5 μ g/ml heparin, 100 mg/ml Streptomycin, 100 U/ml penicillin-G and additional 10 mM glutamine (Sigma Chemical Co.). Cells were grown in an incubator at 37°C in a water-saturated atmosphere of 5% CO₂ in air. The cells were used at 6–12 population doubling level.

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Permeability assay

Permeability of the endothelial cell monolayer was assayed using the *in vitro* system [11]. Co-culture chambers (Becton Dickinson and Company, Franklin Lakes, NJ, U.S.A.) were placed in each well of 24 well culture plates (Becton Dickinson and Company). Six hundred μ l of culture medium consist of MCDB B131, 2% heat inactivated newborn calf serum, 100 mg/ml Streptomycin, 100 U/ml penicillin-G and additional 10 mM glutamine was added in the each well. Each chamber was also filled with 200 μ l of the culture medium. Forty thousand endothelial cells per square centimeters were grown to confluence on the membrane for 44 hr. Six hundred μ l of assay media made from MCDB 131, 1 μ M fluorescein isothiocyanate-labeled 70,000 dalton dextran (FITC-dextran, Sigma Chemical Co.) and several concentrations of substance were added gently to each chamber. After incubation for 4 hr, the FITC-dextran concentrations in the well were measured by fluorimetry with an excitation wavelength of 485 nm and an emission of 520 nm. The final concentrations were calculated from relative percentage values ranging between zero to 100%. 0% permeability was represented as background measured in the medium without FITC-dextran, and 100% permeability was represented as the FITC-dextran concentration that results in the well when no endothelial cells are present on the chamber membrane. The degree of permeability was expressed as ratio to the control; permeability (%) at each well divided by permeability in the control (%).

Adenosine 5'-triphosphate disodium salt (ATP, Wako Pure Chemical Industries, LTD, Tokyo, Japan) was applied to the assay media in each well at 10^{-7} – 10^{-3} M. Drug diluent was used as a control. Because ATP is metabolized to adenosine quickly by cells, adenosine (Wako Pure Chemical Industries, LTD) was also tested to evaluate the effect. To examine the contribution of PKC on the permeability of a monolayer stimulated by ATP, 10^{-8} – 10^{-6} staurosporine (Wako Pure Chemical Industries, LTD), a protein kinase inhibitor, or 10^{-8} – 10^{-6} M myristoylated protein kinase C (19-27) (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA, U.S.A.), a cell membrane permeable PKC specific inhibitor, was added in combinations with or without 10^{-5} M ATP. A PKC stimulator, 10^{-10} – 10^{-7} M phorbol 12-myristate 13-acetate (PMA; Wako Pure Chemical Industries, LTD), was also applied to examine the effect of PKC activation. To confirm the inhibitory effect of the PKC inhibitors in this assay model, 10^{-8} – 10^{-6} staurosporine or 10^{-8} – 10^{-6} M myristoylated protein kinase C (19-27), with or without 10^{-7} M PMA were also added.

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) with Bonferroni's modification of the *t*-test. Results were deemed significant when $P < 0.05$.

III. Results

The endothelial cells formed a confluent monolayer on the membrane of a co-culture chamber as verified by phase-contrast microscopy. The permeability of the endothelial cell monolayer was decreased by 10^{-6} – 10^{-3} M of ATP in a dose-dependent manner (Fig. 1). One, 10, 100, and 1000 μ M ATP decreased the permeability from 1.00 ± 0.05 to 0.75 ± 0.05 , 0.66 ± 0.04 , 0.61 ± 0.07 , and 0.58 ± 0.08 respectively (mean $\pm$ SD, $n=5$, $p < 0.05$). Adenosine, an ATP metabolite, did not change the permeability, suggesting the effect is directly due to ATP (Fig. 2).

As a positive control to examine the effect of PKC activation on the permeability, effect of PMA, a PKC stimulator, was assessed. Significant decrease in the permeability of the endothelial cell monolayer was observed with 10^{-10} – 10^{-7} M of PMA, indicating PKC activation of the endothelial cells leads to the decrease of the permeability (Fig. 3).

To examine the contribution of PKC on the permea-

Fig. 1. Permeability of the endothelial cell monolayers to FITC-dextran at different concentrations of ATP. ATP between 10^{-6} – 10^{-3} M significantly decreased permeability of the endothelial cell monolayer ($p < 0.05$). Data are expressed as the mean $\pm$ SD ($n=5$).

Fig. 2. Permeability of the endothelial cell monolayers to FITC-dextran at different concentrations of adenosine. Adenosine between 10^{-7} – 10^{-3} M did not decrease permeability of the endothelial cell monolayer. Data are expressed as the mean $\pm$ SD ($n=5$).

Fig. 3. Permeability of the endothelial cell monolayers to FITC-dextran at different concentrations of PMA. PMA between 10^{-10} – 10^{-7} M significantly decreased permeability of the endothelial cell monolayer ($p < 0.05$). Data are expressed as the mean $\pm$ SD ($n = 5$).

bility of the monolayer stimulated by ATP, staurosporine, a protein kinase inhibitor, or myristoylated PKC (19-27), a PKC inhibitor, was added with or without ATP. In the presence of the PKC inhibitors, the decrease of the per-

meability by 10^{-7} M PMA was suppressed (Fig. 4), indicating both of drugs inhibited the decrease of permeability by modifying PKC activity. Both PKC inhibitors suppressed the decrease of the permeability by 10^{-5} M ATP (Fig. 5).

IV. Discussion

In this study, we demonstrated that ATP could decrease the permeability of an endothelial cell monolayer within 4 hr. The 4 hr of the assay duration was chosen because changes of the permeability were most conspicuous at that point in time in preliminary studies, which demonstrated that an alteration of the permeability began a few minutes after the extracellular stimulation, accelerated within several hours, and reached an equivalent after 6–8 hr in our assay system. ATP is supposed to be hydrolyzed by ecto-ATPase in 4 hr. It remains uncertain whether the effect observed with application of ATP is due to ATP itself or due to its metabolites. Our results suggested the metabolites, such as adenosine, do not affect the permeability as much as ATP. It is possible that other

Fig. 4. Permeability of the endothelial cell monolayers to FITC-dextran at different concentrations of staurosporine (A) or myristoylated protein kinase C (19-27) (B), with or without 10^{-7} M of PMA. Both protein kinase inhibitors significantly suppressed the decrease of the permeability by PMA ($p < 0.05$). Data are expressed as the mean $\pm$ SD ($n = 5$).

Fig. 5. Permeability of the endothelial cell monolayers to FITC-dextran at different concentrations of staurosporine (A) or myristoylated protein kinase C (19-27) (B), with or without 10^{-5} M of ATP. Both protein kinase inhibitors significantly suppressed the decrease of the permeability by ATP ($p < 0.05$). Data are expressed as the mean $\pm$ SD ($n = 5$).

ATP metabolites or signal messengers could contribute to the change in permeability in different ways, however, ATP could be considered as an important stimulator to initiate the signaling cascade to decrease the permeability.

Since our assay did not contain inflammatory cells, it was suggested that ATP modulates the endothelial cells and decreases the permeability directly. Since dextran passes only intercellularly in cultured endothelial cells [5, 9], ATP might alter cellular conformation by narrowing the intercellular junctional space in this model. The mechanism hypothesized for the narrowing of this space is thought to be a morphological change of the cell shape through its cytoskeleton, the tightening of cell-to-cell, cell-to-matrix adhesion or an increase of intercellular matrices.

ATP is reported to activate PKC in endothelial cells [2]. Activation of PKC by basic fibroblast growth factor is known to modify the cytoskeleton and induce proliferation and migration of endothelial cells [6]. These facts strongly support our hypothesis that ATP changes endothelial cell morphology through PKC in an intracellular signal transduction system. Although, the PKC activation by extracellular ATP or bradykinin has been reported to increase the permeability in the endothelium [2], the changes in the endothelium permeability have not been confirmed *in vitro*. Our *in vitro* system was designed to represent a simple model of capillary vessels. Whether this model is comparable or relevant to intact vessels *in vivo* remains to be seen. We have yet to account for the influence of the basement membrane, interactions between endothelial cells, other cells or other humoral factors *in vivo*. These factors may explain why there is a difference. However, we believe an *in vitro* model still has many advantages. It is a simple model with a limited cell type and it offers direct access to luminal and abluminal fluid for analysis. Our results suggest that the PKC modifier directly changes the permeability of the endothelial cell monolayer. This result might be supported by other studies reporting activation of PKC by PMA enhanced adenylate cyclase activity in bovine arterial endothelial cells [7] and a decrease in permeability by elevation of adenosine 3,5-cyclic phosphate level stimulated by histamine [11]. Moreover, in cultured intestinal cells, alteration of the intracellular level of adenosine 3,5-cyclic phosphate is known to modify cell-to-cell contact molecules [13]. In rat microvascular endothelial lining of the brain, Ca^{2+} -activated adenosine triphosphatase contributes to the capillary permeability [3]. ATP is known to elevate cytosolic Ca^{2+} level, which is a co-factor of PKC, through P_2 -receptor, while histamine also increases Ca^{2+} level through H_1 -receptor. However, decrease of the permeability by histamine is not mediated by Ca^{2+} as mentioned above. Taken together, these observations suggest that the signaling pathway seems different between ATP and histamine, and the precise mechanism remains to be elucidated.

ATP is known to have various effects as an extracellular nucleotide. Because concentration of intracellular

ATP is in the mM range, intracellular ATP would be a source for extracellular ATP in local inflammations with cell detachment and lytic injury [1]. Our results suggested the possibility that ATP leaked from injured cells affects the surrounding cells through a paracrine mechanism and tightens cell adhesion onto the matrix to maintain the monolayer structure [12].

Although further studies will be required to explain the exact mechanism in which ATP affects endothelial cell permeability, ATP may still be considered as a part of treatment to prevent third spacing in acute phases of critically ill patients.

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VI. References

1. Andreoli, S. P., Mallett, C., Williams, K., McAteer, J. A., Rothlein, R. and Doerschuk, C. M.: Mechanisms of polymorphonuclear leukocyte mediated peritoneal endothelial cell injury. *Kidney Int.* 46; 1100–1109, 1994.
2. Castro, A. F., Amorena, C., Müller, A., Ottaviano, G., Tellez-Iñon, M. T. and Taquini, A. C.: Extracellular ATP and bradykinin increase cGMP in vascular endothelial cells via activation of PKC. *Am. J. Physiol.* 275; C113–C119, 1998.
3. Fujimoto, K.: Alkaline-phosphatase and Ca^{2+} -ATPase cytochemistry of 'seamless' endothelial cells in the rat brain. *Acta Histochem. Cytochem.* 31; 17–23, 1998.
4. Harkema, J. M. and Chaudry, I. H.: Magnesium-adenosine triphosphate in the treatment of shock, ischemia, and sepsis. *Crit. Care Med.* 20; 263–275, 1992.
5. Hashida, R., Anamizu, C., Mizuno-Yagyu, Y., Ohkuma, S. and Takano, T.: Transcellular transport of fluorescein dextran through an arterial endothelial cell monolayer. *Cell Struct. Funct.* 11; 343–349, 1986.
6. Kent, K. C., Mill, S., Harrington, E. O., Chang, J. D., Mallette, S. and Ware, J. A.: Requirement for protein kinase C activation in basic fibroblast growth factor-induced human endothelial cell proliferation. *Circ. Res.* 77; 231–238, 1995.
7. Lefroy, D. C., Donnelly, L. E., McEwan, J. R. and MacDermot, J.: Phorbol ester enhances activation of adenylate cyclase in bovine aortic endothelial cells. *Life Sciences* 54; 87–94, 1994.
8. Martin, W., Cusack, N. J., Carleton, J. S. and Gordon, J. L.: Specificity of P_2 -purinoceptor that mediates endothelium-dependent relaxation of the pig aorta. *Eur. J. Pharmacol.* 108; 295–299, 1985.
9. Navab, M., Hough, G. P., Berliner, J. A., Frank, J. A., Fogelman, A. M., Haberland, M. E. and Edwards, P. A.: Rabbit beta-migrating very low density lipoprotein increases endothelial macromolecular transport without altering electrical resistance. *J. Clin. Invest.* 78; 389–397, 1986.

10. Needham, L., Cusack, N. J., Pearson, J. D. and Gordon, J. L.: Characteristics of the P_2 purinoceptor that mediates prostacyclin production by pig aortic endothelial cells. *Eur. J. Pharmacol.* 134; 199–209, 1987.
11. Takeda, T., Yamashita, Y., Shimazaki, S. and Mitsui, Y.: Histamine decreases the permeability of an endothelial cell monolayer by stimulating cyclic AMP production through the H_2 -receptor. *J. Cell Science* 101; 745–750, 1992.
12. Takeda, T., Yukioka, T., Matsuda, H. and Shimazaki, S.: Adenosine 5'-triphosphate decreases the permeability of a mesothelial cell monolayer. *Acta Histochem. Cytochem.* 30; 37–39, 1997.
13. Yuge, L., Yamamoto, M., Kawamata, S. and Kataoka, K.: Immunocytochemical localization of connexin 43 and E-cadherin in I-407 cells mediated by cAMP. *Acta Histochem. Cytochem.* 31; 427–433, 1998.