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Atriopeptin inhibition of thrombin-mediated changes in the morphology and permeability of endothelial monolayers

(atrial natriuretic peptide/cytoskeleton/receptors/actin/rhodamine-conjugated phalloidin)

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ABSTRACT **To determine the role of endothelial atriopeptin (AP) receptors, we examined the effect of AP-III on the morphology and macromolecular permeability of monolayer cultures of bovine aortic endothelial cells. AP-III alone (10^{-9}–10^{-6} M) had no observable effect on the morphology of the monolayers or their permeability to ^{125}I-labeled albumin. However, incubation of the endothelial monolayers with AP-III (10^{-8}–10^{-6} M) antagonized thrombin-induced (1 unit/ml) cell-shape change and the formation of intercellular gaps. AP-III also opposed the effect of thrombin on the distribution of actin filaments in the endothelial cytoskeleton. Further, thrombin caused a 2-fold increase in monolayer permeability to ^{125}I-labeled albumin, which was abolished by 10^{-8}–10^{-6} M AP-III pretreatment. Taken together with the findings that AP-III exhibited specific and saturable binding in these cells, these data suggest that AP regulates endothelial permeability through a receptor-mediated process.**

Atriopeptin (AP), a peptide hormone synthesized and secreted by atrial myocytes, is thought to play a role in circulatory homeostasis (1). The actions of AP relevant to the regulation of extracellular fluid volume include natriuresis, diuresis, and inhibition of aldosterone synthesis, vasopressin secretion, and thirst (1). Prominent among the tissues that possess AP receptors are vascular smooth muscle and the endothelia of heart, lung, and kidney (2). In vascular smooth muscle, AP elicits relaxation (3); however, other than evoking a pronounced increase in cGMP synthesis (4), the role of AP in the regulation of endothelial cell function is unknown (5). Because one primary function of the endothelium is regulation of the movement of water, solutes, and macromolecules between vascular and extravascular spaces, a function of AP may be the regulation of vascular permeability through specific receptors on endothelial cells.

One of the first observations relating AP to vascular permeability was an increase in hematocrit in nephrectomized rats following bolus injection of AP (6). These data compelled consideration as to whether AP directly affected endothelial permeability. However, attempts to elicit increases in capillary permeability by AP infusion in isolated microvascular preparations were unsuccessful (7). More recently, AP has been proposed to increase hematocrit by altering the dynamics of peripheral vascular pressure despite a decreased loss of plasma albumin (8). The discrepancy between the effect of atrial natriuretic peptide on hematocrit and capillary macromolecular permeability may be a result of the several different pathways by which solutes and water cross the endothelial barrier. The flux of solutes and water across nonfenestrated capillary endothelium is thought to occur via channels (pores), vesicles, and at the intercellular junctions (9). The intercellular junctions are thought to be a major site of the conductance of water and hydrophilic solutes, but not of macromolecules *under basal conditions* (9). However, the intercellular junctions may become permeable to macromolecules as a result of endothelial cell-shape change (retraction) in response to humoral mediators of inflammation (10) and oxidant-induced injury (11). This opening of the intercellular junction to macromolecules is thought to result in edema formation (12).

Many agents, including thrombin, oxidants, and endotoxins, have been shown to increase endothelial permeability in association with a change in cell shape, a rearrangement of cytoskeletal elements, and the development of intercellular gaps (13). We developed the hypothesis that AP modulates macromolecular permeability and morphology of vascular endothelium in varying physiological states. Therefore, we examined the ability of AP to antagonize thrombin-enhanced macromolecular permeability across isolated endothelial cell monolayers.

MATERIALS AND METHODS

Isolation of Bovine Aortic Endothelial Cells (BAEC). BAEC were obtained by an established method (14). Briefly, cells were harvested by collagenase digestion from fresh bovine aortas and grown in a T-25 flask with Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum, penicillin at 100 international units/ml, and streptomycin at 100 μg/ml. Cells were passaged at near confluency with 0.25% trypsin and cloned initially at a theoretical concentration of 10–100 cells per well in 24-well dishes. Experiments were performed with a line of cloned cells in their 4th–10th passage at 1–7 days past confluency. The cells were characterized phenotypically by their morphology, immunocytochemical localization of factor VIII antigen (15), and by uptake of fluorescent acetylated low density lipoprotein (16).

Equilibrium Binding Assay. Cultured endothelial cells were grown to confluency in 24-well plates. After removal of growth medium (DMEM plus 20% fetal calf serum), the cells were washed two times with 1.0 ml of DMEM containing 0.05% bovine serum albumin (BSA) and 25 mM Hepes/Earle's salts buffer (25 mM Hepes/116 mM NaCl/5.4 mM KCl/0.89 mM NaH_2PO_4/0.81 mM $MgSO_4$/1.8 mM $CaCl_2$/5.8 mM glucose, pH 7.2; binding buffer) at 37°C. The cells were then covered with 0.5 ml of binding buffer and incubated at 37°C with a set concentration of ^{125}I-labeled AP-III and various concentrations of AP-III (rat atrial natriuretic peptide 103–126, a gift from Searle). Nonspecific binding was determined in the presence of 1 μM AP-III. After incubation for 30 min, cells were washed four times with 0.5 ml of binding buffer (0°C) and solubilized in 0.6 ml of 1 M NaOH. Aliquots of 0.5 ml were assayed for radioactivity in a LKB γ counter

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Abbreviations: AP, atriopeptin; BSA, bovine serum albumin; BAEC, bovine aortic endothelial cells.

with 80% efficiency. K_d and B_{max} were subsequently determined by Scatchard analysis.

cGMP Determination. Confluent endothelial cells, in the presence of 0.5 mM isobutylmethylxanthine, a phosphodiesterase inhibitor, were incubated with various concentrations of AP-III for 5 min at 37°C. The reaction was terminated by the addition of 6% (vol/vol) trichloroacetic acid. Trichloroacetic acid was extracted using four changes of ether, and the ether was removed by boiling the samples. The samples were lyophilized and resuspended in 50 mM sodium acetate buffer, pH 6.4, and assayed for cGMP with antiserum raised by our laboratory (cross reactivity: <0.001% for guanosine, 5'-GMP, GDP, GTP, inosine, 5'-AMP, and cAMP) by RIA as described by Steiner *et al.* (17). cAMP was measured similarly (17).

^{125}I Labeling of Albumin. BSA was labeled by the Iodo-Gen method (18). Briefly, 1 mg of Iodo-Gen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril) (Pierce) was dissolved in 25 ml of methylene chloride. Fifty microliters of this solution was dried onto the sides of a plastic Eppendorf tube using N_2 gas. The Iodo-Gen was redissolved in 80 μl of 250 mM sodium phosphate buffer, pH 7.0, to which 3 μl of BSA at 1 mg/ml was added. NaI (0.5 mCi; 1 Ci = 37 GBq) was added to this solution, and after 10 min, ^{125}I-labeled BSA was separated chromatographically from unbound NaI using a Sep-Pak C_{18} cartridge (Millipore–Waters). Prepared label was tested for bound versus unbound NaI by trichloroacetic acid precipitation of bound protein before each experiment. Preparations <92% bound were discarded.

Permeability Assay. Endothelial cells were seeded into 12-mm culture plate inserts (Millicell-HA; Millipore) containing a 0.45-μm-pore cellulose filter and grown to confluency (6–8 days) in 24-well plates before use. With medium placed in both the inner and outer wells, two chambers were created that were separated by the endothelial monolayer grown on the microporous filter. For experimentation, the cells were washed with Hepes/Earle's salts buffer and placed in a 37°C shaking water bath. To ensure equal mixing throughout the experiment, the permeability chambers were shaken at a rate of 60 oscillations per min. To the inner chamber (which contained cells), 265 μl of Hepes/Earle's salts buffer containing 2% BSA was added. To the outer chamber, 435 μl of Hepes/Earle's salts buffer without albumin was added. These specific volumes were added to equalize fluid heights in the two chambers, so that only diffusive forces would be involved in permeability to albumin. The cells were preincubated for 10 min at 37°C, and AP-III was added 5 min into the preincubation period. At t_0 other reagents including ^{125}I-labeled BSA (10^6 cpm/well) were added. Fifty-microliter samples were removed from the outer well at 10-min intervals, up to 60 min. To keep hydrostatic forces constant, an equal amount of Hepes/Earle's salts buffer was added back to the outer well. Fifty microliters of Hepes/Earle's salts buffer plus 2% BSA was added to each sample followed by 100 μl of 20% trichloroacetic acid. Each sample was then mixed, centrifuged, and the precipitate containing ^{125}I-labeled BSA was counted. Data from filters with subconfluent monolayers, as evidenced by the free passage of ^{125}I-labeled BSA, were discarded.

Morphological Studies. Endothelial cell monolayers grown in 24-well plates were photographed in real time during responses to thrombin and AP-III with a Nikon Diaphot equipped with Hoffman modulation contrast optics. A field was chosen in the center of each well at approximately the same location and no adjustments, other than focusing, were made between photographs. Experiments were terminated by fixation *in situ* with 2% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 24 hr. The monolayers were washed three times in the same buffer, postfixed for 60 min in 1% (vol/vol) OsO_4 in the same buffer, and rinsed briefly in distilled H_2O. Cells were then stained *en bloc* in 0.5% uranyl acetate in 0.1 M sodium hydrogen maleate buffer, pH 5.0, for 60 min, rinsed again in distilled water, dehydrated in graded alcohols, and embedded directly in epoxy resin. The epoxy discs were cracked out of the 24-well dishes, and fragments were oriented in flat embedding molds so as to obtain cross-sections.

To visualize actin, endothelial cell monolayers grown on 12-mm circular coverslips in 24-well plates were washed twice in phosphate-buffered saline (PBS) at 20°C, fixed in 3.7% formaldehyde in PBS at 20°C for 10 min, and washed twice in PBS before permeabilization for 3–5 min in −20°C acetone. The coverslips were air dried and incubated for 20 min at 20°C in 200 μl of 0.165 μM rhodamine-conjugated phalloidin (Molecular Probes), washed twice in PBS, and mounted on slides with PBS and glycerol (1:1). A Zeiss 1M35 microscope (40× objective) was used for epifluorescence microscopy. An arbitrary exposure time of 30 sec with Ektachrome 400 or Tri-X film (Kodak) facilitated qualitative comparisons.

Statistics. Results were analyzed for statistical significance by analysis of variance with repeated measures (19) and the Duncan multiple range test (20).

RESULTS

To investigate a functional role for AP receptors in endothelium, we studied the effect of AP on cultured BAEC. These cells stained positively for factor VIII antigen and incorporated fluorescently labeled low density lipoprotein, indicative of endothelial cells in culture. As reported by others (4, 5), we found ^{125}I-labeled AP-III displayed specific binding (>90%) to BAEC with a K_d of 5.05×10^{-10} M and a B_{max} of 289 fmol/mg of protein as determined by Scatchard analysis. By covalent cross-linking experiments with subsequent gel-electrophoresis under reducing conditions (4), these cells demonstrated a major binding site of M_r 60,000 and a lesser binding site of M_r 116,000 (data not shown). Furthermore, AP-III (at 10^{-9}–10^{-6} M) elicited a concentration-dependent increase in intracellular cGMP levels (Fig. 1) Corresponding experiments indicated that AP-III treatment (10^{-7} M) resulted in cAMP levels equivalent to basal levels, 16 ± 0.7 fmol per 10^5 cells and 13 ± 1.3 fmol per 10^5 cells, respectively, whereas forskolin (10 μM) increased cAMP levels to 135 ± 55 fmol per 10^5 cells.

To determine whether AP affects macromolecular monolayer permeability, we measured the effect of AP-III on the passage of ^{125}I-labeled BSA across endothelial monolayers grown on porous filters. Control monolayers showed a time-dependent increase in the amount of ^{125}I-labeled BSA that passed across the monolayer. AP-III alone had no effect on the passage of ^{125}I-labeled BSA across the monolayer,

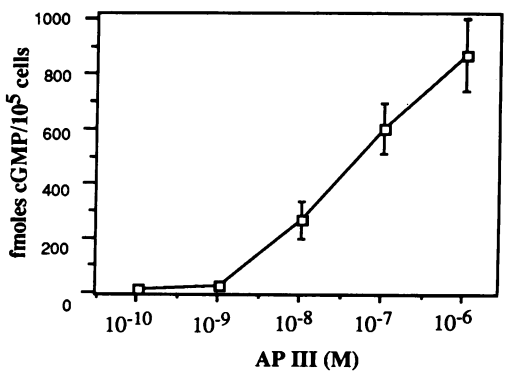

FIG. 1. Effect of AP-III on cGMP formation by BAEC. AP-III (10^{-10}–10^{-6} M) was added to cells in culture for 5 min at 37°C with 0.5 mM isobutylmethylxanthene. Values represent the mean ± SEM (n = 4). cGMP levels were measured by RIA.

indicating that AP-III under these conditions does not alter the permeability of the monolayer to ^{125}I-labeled BSA (data not shown). However, thrombin (1 unit/ml) caused a 2-fold increase in the ^{125}I-labeled BSA that passed across the monolayer in 60 min. This response was completely blocked by 5-min pretreatment of the monolayer with AP-III (10^{-6} M) (Fig. 2). Examination of the concentration dependency of this inhibitory effect of AP-III revealed that 10^{-8} M AP-III significantly inhibited the thrombin response, and 10^{-7} M AP-III completely abolished the effect of thrombin (Fig. 3). Comparison of the concentration-dependent increase in AP-elicited cGMP levels (Fig. 1) and the effects of AP on permeability showed that 10^{-8} M AP-III resulted in the first significant changes in each of these parameters. However, increases in cGMP continued at 10^{-7}–10^{-6} M AP-III, whereas these concentrations failed to elicit further decreases in permeability to ^{125}I-labeled BSA.

By Hoffman modulation contrast microscopy, BAEC monolayers grown to confluency in plastic wells revealed a typical cobblestone pattern of a contact-inhibited confluent monolayer (Fig. 4*A*), in which intercellular gaps were rarely present. A 35-min incubation with AP-III (3×10^{-7} M) did not alter this morphological profile. After a 30-min incubation with thrombin (1 unit/ml) many cells became spindle shaped, increased in height, and retracted from one another, creating intercellular gaps (Fig. 4*B*). Preincubation for 5 min with AP-III (3×10^{-7} M) before thrombin addition prevented the evolution of morphologic changes elicited by thrombin (Fig. 4*C*). These results were confirmed in cross-sections of the same monolayers illustrated in Fig. 4: untreated monolayers, as well as monolayers exposed to AP-III (3×10^{-7} M) alone (data not shown) appeared as ribbon-like structures in which the perinuclear regions were slightly thicker than perijunctional regions (Fig. 5*A*). In response to thrombin, many cells of the monolayer appeared hemicircular in profile and attenuated in the perijunctional areas (Fig. 5*B*). Gaps between cells were also seen (Fig. 5*B*, *Inset*). Preincubation with AP-III, as before, prevented these changes except in occasional cells (Fig. 5*C*). Many sections were indistinguishable from control monolayers.

Untreated monolayers stained for F-actin with rhodamine–phalloidin disclosed the presence of numerous stress fibers, some of which traversed the length of the cell and many others of which traversed the cell periphery to the perinuclear region (Fig. 6*A*). In response to thrombin, the intensity of the fluorescence as well as the frequency of stress fibers was

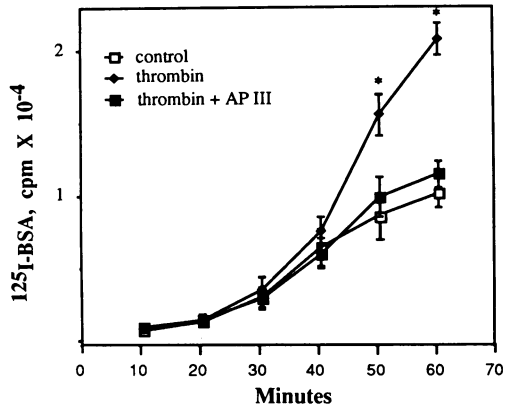

FIG. 2. Effect of AP-III on thrombin-induced increases in the permeability of BAEC monolayers to ^{125}I-labeled BSA. The experiment consisted of four groups: control, thrombin (1 unit/ml), preincubation with AP-III (10^{-6} M) for 5 min followed by thrombin (1 unit/ml), and AP-III (10^{-6} M) (data not shown, not different from control). Values represent the mean ± SEM (n = 7). Asterisks indicate values significantly different from control values ($P < 0.05$).

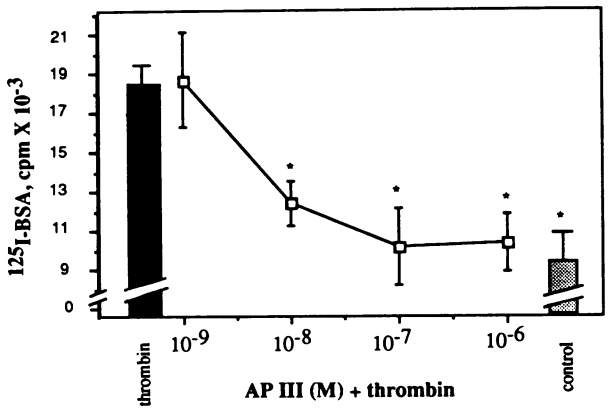

FIG. 3. Effect of 10^{-9}–10^{-6} M AP-III on thrombin-induced increases in the permeability of BAEC monolayers to ^{125}I-labeled BSA. The bars labeled thrombin (1 unit/ml) and control represent the mean values, 18,446 ± 590 cpm and 9447 ± 1506 cpm, respectively, after 60-min incubation (n = 4). Squares represent the mean ± SEM (n = 4) at each concentration of AP-III added 5 min before incubation with thrombin (1 unit/ml) for 60 min. Asterisks indicate values significantly different than for thrombin alone ($P < 0.05$).

decreased (Fig. 6*B*). Preincubation with AP-III diminished greatly the thrombin-induced changes in the actin cytoskeleton (Fig. 6*C*). The intensity of fluorescence and frequency and

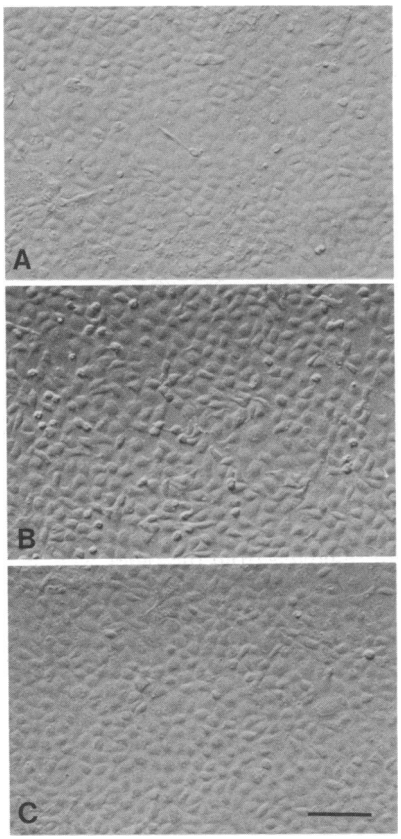

FIG. 4. Morphological analysis by Hoffman modulation contrast microscopy of BAEC monolayers grown in plastic wells. (*A*) Control monolayer. Cells are confluent and organized into an irregular hexagonal array. (*B*) Monolayer incubated for 30 min with thrombin (1 unit/ml). Many cells are spindle shaped and retracted from one another resulting in prominent intercellular gaps. (*C*) Monolayer treated with AP-III (3×10^{-7} M) 5 min before 30-min incubation with thrombin (1 unit/ml). Cells are similar in appearance to those in *A*. Some cells appear to be slightly thickened in the perinuclear region. Intercellular gaps are not evident. (Bar = 100 μm.)

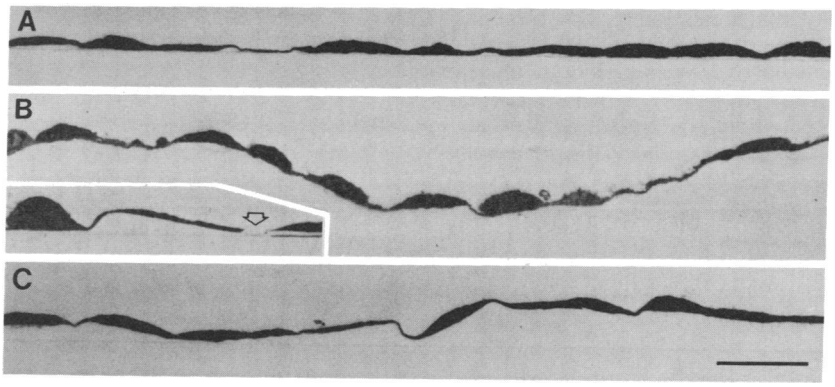

FIG. 5. Toluidine blue-stained cross-sections of BAEC monolayers grown in plastic wells. (*A*) Control monolayer. Cell sheet in cross-section appears as an uninterrupted ribbon-like structure. (*B*) Monolayer incubated for 30 min with thrombin (1 unit/ml). Cells appear hemicircular in profile and attenuated at their periphery. Gaps between cells are seen (*Insert*, arrow). (*C*) Monolayer treated with AP-III (3×10^{-7} M) 5 min before 30-min incubation with thrombin (1 unit/ml). Many cells are indistinguishable from control (*A*). Although occasional cells are thickened in the perinuclear region, intercellular gaps are not evident. (Bar = 25 μm.)

distribution of stress fibers were comparable to untreated and AP-III (3×10^{-7} M)-treated (data not shown) monolayers.

DISCUSSION

Our results provide direct evidence that AP acts on endothelial monolayers to prevent thrombin-mediated increases in macromolecular permeability and changes in endothelial cell shape. Thrombin-induced increases in endothelial macromolecular permeability are receptor mediated and independent of proteolytic activity (10). The increase in permeability is physically represented by intercellular gap formation that has been postulated to occur as a result of cytoskeletal reorganization; thrombin decreases the number and length of stress fibers and the ratio of F- to G-actin (13, 21). It is notable that pretreatment with AP prevents the thrombin-mediated rearrangements of the actin-containing stress fibers and supports the possibility that AP, directly or indirectly, stabilizes the endothelial cell cytoskeleton and intercellular junction.

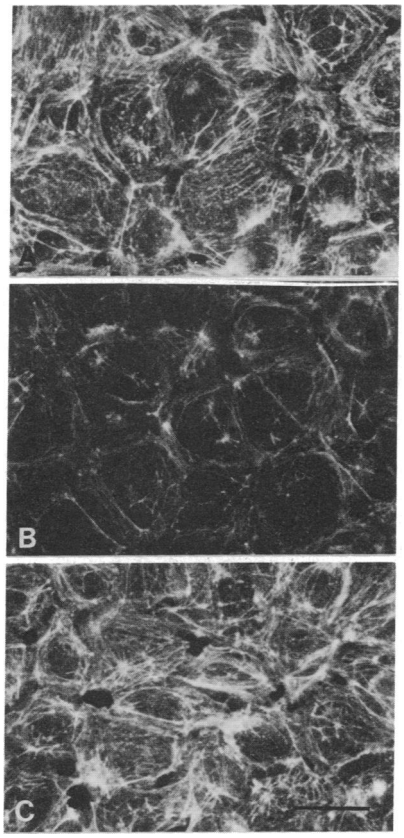

FIG. 6. Rhodamine–phalloidin fluorescence of BAEC monolayers grown on glass coverslips. (*A*) Control monolayer. The distribution of F-actin is primarily in stress fibers that extend from the cell periphery to the perinuclear region. (*B*) Monolayer incubated for 30 min with thrombin (1 unit/ml). The number of stress fibers and intensity of fluorescence is diminished. (*C*) Monolayer treated with AP-III (3×10^{-7} M) 5 min before 30-min incubation with thrombin (1 unit/ml). The number of stress fibers and intensity of fluorescence is comparable to control (*A*). All photographic exposures were 30 sec, and the resulting negatives were printed identically. (Bar = 25 μm.)

Other agents variously affect the endothelial cytoskeleton and either increase (histamine and thromboxanes) or decrease (serotonin, prostaglandin I_2, and norepinephrine) microvascular permeability, although responsiveness to particular substances depends on the source of endothelial cells (22). Preincubation with AP reverses thrombin-mediated increases in endothelial permeability to macromolecules; the ability of AP to produce this effect in endothelia of low basal permeability remains to be determined. Under conditions used in this study AP alone did not appear to affect either basal permeability or morphological characteristics of BAEC in monolayer culture.

The mechanism by which AP modulates endothelial permeability is unknown. Because AP binding to its receptor on endothelial cells markedly increases cGMP levels (4), increased cGMP levels could, as in smooth muscle, decrease intracellular calcium levels. cGMP administration to vascular smooth muscle cells has been shown to lower intracellular calcium levels by cGMP-dependent protein kinase activation of a Ca^{2+}-ATPase located in the plasma membrane (23). Regulation of intracellular calcium concentrations in the endothelial cells is probably of major importance in maintaining the cytoskeleton and intercellular tight junctions. Increases in intracellular free calcium have been associated with loss of stress fibers, a smaller ratio of F- to G-actin (24), and more intercellular gaps. These changes that occur in response to thrombin administration are thought to reflect the mechanism and pathway of increased macromolecular permeability. Therefore, in endothelial cells, agents that oppose the increase in free cytoplasmic calcium induced by thrombin might stablize the cytoskeleton. However, cGMP may act through a nonprotein kinase mechanism, as has been suggested for the actions of this second messenger in the retina (25, 26), in which cGMP is thought to bind to a membrane-bound protein to regulate membrane conductance. This mechanism is a plausible alternative in light of reports that endothelial cells lack cGMP-dependent protein kinase (27,

28). Alternatively, AP may regulate endothelial permeability through a mechanism independent of cGMP, as has recently been proposed for AP-mediated vascular smooth muscle relaxation (29).

This action of AP on endothelial macromolecular permeability may partially underlie the role of this peptide hormone in the homeostasis of extracellular fluid volume. AP has been reported to decrease thirst, salt appetite, intestinal fluid absorption, vasopressin secretion, and aldosterone synthesis and to increase fluid and salt excretion (1). These actions of AP would tend to decrease extracellular fluid volume. The effect of AP on the endothelial barrier would also tend to decrease the extracellular fluid volume by regulating macromolecular permeability and, ultimately, oncotic pressure. By maintaining capillary oncotic pressure, AP may modulate the flow of fluids across the endothelial barrier.

The finding that AP regulates endothelial macromolecular permeability and its relevance to what is considered to be a primary role of the hormone—i.e., the regulation of extracellular fluid volume, has clinical as well as therapeutic implications. Our results indicate that AP may act to antagonize the formation of edema resulting from endothelial cell injury. Indeed, AP can block the formation of edema in the isolated perfused guinea pig lung in response to a number of different agents (30, 31). The mechanism of this response has not been determined, but this action of AP could be related to its effects on the endothelial cell cytoskeleton and associated changes in endothelial macromolecular permeability. Complementing this work is our recent observation that AP inhibits oxidant-induced increases in endothelial permeability and associated changes in endothelial cell shape and cytoskeleton (32). In addition, pulmonary tissue levels of AP have been elevated in a model of congestive heart failure (33), and circulating levels of AP rise during conditions of increased vascular permeability associated with pulmonary hypertension and congestive heart failure (1, 34). From these collective data, we propose that AP may oppose edema formation in these and other disease states and suggest a role for AP in the treatment of pulmonary, cardiac, cerebral, and peripheral microvascular edema. These observations are clearly relevant, as well, to the determination of the mechanisms involved in endothelial cell motility and modulation of the barrier function of the endothelial tight junction.

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