Expression of PKA inhibitor (PKI) gene abolishes cAMP-mediated protection to endothelial barrier dysfunction

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Lum, Hazel, H. Ari Jaffe, Irena T. Schulz, Anwar Masood, Amlan Raychaudhury, and Richard D. Green. Expression of PKA inhibitor (PKI) gene abolishes cAMP-mediated protection to endothelial barrier dysfunction. Am. J. Physiol. 277 (Cell Physiol. 46): C580–C588, 1999.—We investigated the hypothesis that cAMP-dependent protein kinase (PKA) protects against endothelial barrier dysfunction in response to proinflammatory mediators. An E1–, E3–, replication-deficient adenovirus (Ad) vector was constructed containing the complete sequence of PKA inhibitor (PKI) gene (AdPKI). Infection of human microvascular endothelial cells (HMEC) with AdPKI resulted in overexpression of PKI. Treatment with 0.5 μM thrombin increased transendothelial albumin clearance rate (0.012 ± 0.003 and 0.035 ± 0.005 μl/min for control and thrombin, respectively); the increase was prevented with forskolin + 3-isobutyl-1-methylxanthine (F + I) treatment. Overexpression of PKI resulted in abrogation of the F + I-induced inhibition of the permeability increase. However, with HMEC infected with ultraviolet-inactivated AdPKI, the F + I-induced inhibition was present. Also, F + I treatment of HMEC transfected with reporter plasmid containing the cAMP response element-directed transcription of the luciferase gene resulted in an almost threefold increase in luciferase activity. Overexpression of PKI inhibited this induction of luciferase activity. The results show that Ad-mediated overexpression of PKI in endothelial cells abrogated the cAMP-mediated protection against increased endothelial permeability, providing direct evidence that cAMP-dependent protein kinase promotes endothelial barrier function.

increases IN INTRACELLULAR levels of the second messenger cAMP in endothelial cells are known to protect against endothelial barrier dysfunction in response to proinflammatory mediators, including histamine, thrombin, oxidants, and tumor necrosis factor (5, 8, 22, 35, 40, 43, 48). This protective effect of cAMP has been demonstrated in several experimental systems with use of in vitro cell culture (8, 34, 40, 43, 48) and in intact organ models of barrier function such as ischemiareperfusion injury (2, 5, 6, 22, 35). Associated with the cAMP-mediated improvement of barrier function, endothelial cells become flattened (34), show decreased interendothelial gap formation (21, 43), and exhibit tighter adhesion to matrix and inhibition of migration (29). Furthermore, increased endothelial permeability in response to hypoxia exposure, tumor necrosis factor, or oxidant treatment was accompanied by decreased intracellular cAMP levels (21, 27, 41). Thus substantial evidence exists in support of the notion that increased cAMP levels in endothelial cells promote endothelial barrier function.

The mechanisms by which cAMP functions to regulate endothelial permeability are presumed to occur predominantly through activation of the cAMP-dependent serine/threonine protein kinase (PKA). Studies of PKA function have relied mostly on the use of cAMP analogs that increase intracellular levels of cAMP, the use of reagents that stimulate its production (i.e., forskolin), or inhibition of its metabolism (i.e., phosphodiesterase inhibitors). In the absence of cAMP, PKA is an inactive tetramer consisting of two regulatory and two catalytic subunits. Binding of cAMP to the regulatory subunit lowers the affinity for the catalytic subunits by four orders of magnitude, promoting dissociation into a dimer of regulatory subunit and two active monomers of the catalytic subunit (52).

The function of PKA has also been studied using pharmacological PKA inhibitors such as Rp-cAMPS, a competitive inhibitor of cAMP for binding the regulatory subunit of PKA (55), and isoquinoline-based inhibitors, which target the ATP-binding domain on the catalytic subunit (13). However, limited studies have been performed using these PKA inhibitors to demonstrate a direct relationship between PKA activation and the protection against mediator-induced endothelial barrier dysfunction (50). Recent work reported that forskolin (an activator of adenylyl cyclase) inhibited voltage-sensitive Ca2+ channels in a cAMP-independent manner in ventricular myocytes and PC-12 cells (4, 42), suggesting possible nonspecific effects of forskolin. Furthermore, in bovine adrenal cells the inhibition of a K+ current by cAMP analogs and forskolin was not prevented by pharmacological inhibitors of PKA and PKA inhibitor peptide (PKI) (14). These studies indicate that cAMP-promoting agents such as forskolin, as well as cAMP, may have PKA-independent activity and question whether the enhanced barrier function associated with elevation of cAMP levels in endothelial cells is entirely mediated through activation of PKA. Furthermore, full activation of PKA appears to require phosphorylation of its catalytic subunit by a phosphoinositide-dependent protein kinase (9, 10). This latter observation raises the interesting possibility that PKA activity is regulated by phosphorylation-dephosphorylation, which in turn modulates the cell’s responsiveness to a variety of mediators.
to cAMP. Therefore, physiological activation of PKA is likely under multiple modulating signals.

The goal of the study is to test the hypothesis that PKA activation protects against mediator-induced increases in endothelial permeability. The endogenous PKA inhibitor (PKI), highly selective for PKA, has been isolated from rabbit muscle, and its primary amino acid sequence has been identified (46, 56). This heat-stable 75-amino acid protein has high affinity (0.2 nM) and specific binding for the peptide substrate binding site on the catalytic subunit of PKA (52), providing greater selective inhibition of PKA than pharmacological inhibitors. For this study, a replication-deficient adenovirus (Ad) containing the synthetic gene encoding the complete amino acid sequence of PKI (11) was constructed and used for introduction of the PKI gene into endothelial cells. The Ad-mediated gene transfer method was preferred over other transfection methods, since Ad vectors have been shown to have high transduction efficiency in endothelial cells and do not require host cell replication for gene expression (31).

The findings indicate that infection of endothelial cells with the E1−, E3−, replication-deficient recombinant Ad vector is highly efficient (>95%), does not alter baseline endothelial barrier function, and is not cytotoxic. Infection of endothelial cells with AdPKI resulted in overexpression of PKI, which abrogated the cAMP-mediated inhibition of the increased endothelial permeability in response to thrombin. Overexpression of PKI also inhibited the cAMP response element (CRE)-directed transcription of the reporter luciferase gene. Thus the results provide direct evidence that activation of the PKA pathway is critically important in conferring protection against mediator-induced endothelial barrier dysfunction.

METHODS

Cell culture. Human dermal microvascular endothelial cells (HMEC) (1) were maintained in culture in MCDB 131 medium (GIBCO BRL, Gaithersburg, MD) supplemented with 5% fetal bovine serum (FBS; HyClone, Logan, UT), 10 ng/ml human epidermal growth factor (Sigma Chemical, St. Louis, MO), 1 µg/ml hydrocortisone, and antibiotics. HMEC is an immortalized cell line transformed by simian virus 40 (SV40) large T antigen and has been shown to retain endothelial cell phenotypic and functional characteristics. They exhibit the expected morphological and functional endothelial phenotypes: they express and secrete von Willebrand’s factor, intercellular adhesion molecule-1, CD36, intercellular adhesion molecule-3, and take up acetylated low-density lipoprotein, form tubes when grown in Matrigel, and express CD31 (platelet endothelial cell adhesion molecule-1), CD36, intercellular adhesion molecule-1, and CD44 (1). They also bind purified T cells in a regulatable manner, and their response to cytokines is comparable to that of nontransformed endothelial cells. HMEC were passaged for 5–7 days when confluent and used for studies at passages 25–40. The 293 cells were maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 µg/ml penicillin, 100 U/ml streptomycin, and 10 mg/ml amphotericin B. All cell cultures were maintained at 37°C in a humidified CO2 incubator at 5% CO2.

Ad-mediated expression of PKI gene. The 251-bp DNA fragment encoding the complete amino acid sequence of rabbit muscle PKI (11) was subcloned into the shuttle vector pACCMV.plP, creating pACCMV-PKI. This vector includes, in order, 0–1.3 map units from the left end of the Ad type 5 (Ad5) genome and, in place of E1a and part of E1b sequences (required for replication), the cytomegalovirus (CMV) immediate early promoter, pUC19 polylinker, SV40 small T antigen intron and polyadenylation signal sequences, and finally map units 9–17 of Ad5 (19). Equimolar amounts of pACCMV-PKI (0.8 µg) were cotransfected, by cationic liposomes (Lipofectamine, GIBCO/BRL, Grand Island, NY), with the plasmid pJM17 (0.8 µg), which contains the full-length Ad (Ad5) genome sequences (with a mutant E3 region), as well as ampicillin and tetracycline resistance sequences and a bacterial origin of replication (33), into 293 cells, a transformed renal embryonal kidney cell line (CRL 1573, American Type Culture Collection) (20). Homologous recombination between the two plasmids resulted in an E1−, E3− Ad genome that can replicate and be packaged into virions only in 293 cells in which E1 function is supplied in trans by integrated, constitutively expressed Ad E1 sequences. The vector AdPKI was amplified in 293 cells, and its genome was confirmed by PCR amplification of contiguous Ad/expression cassette sequences. The vector was purified by dialysis against virus suspension buffer (10 mM Tris, 10 mM MgCl2, 10% glycerol), titered, and stored at −80°C.

For control, the vector AdNull, expressing no transgene, was constructed in a similar manner but without subcloned gene sequences between the CMV promoter and the polyadenylation signal. The AdNull was constructed by subcloning the Escherichia coli lacZ gene sequences into pACCMV.plP shuttle vector. Additional control vectors were made by inactivating Ad vectors with use of heat (65°C overnight) or ultraviolet (UV) irradiation (254 nm for 60 min).

Northern blot analysis. Northern blot analysis was performed to determine the level of transcription of PKI in HMEC infected with AdPKI. Total RNA was isolated from control noninfected HMEC or HMEC infected for 24 h at 100 multiplicities of infection (MOI; equal to plaque-forming units/target cell) of AdPKI or AdNull with use of the RNA STAT-60 isolation kit (Tel-Test, Friendswood, TX). Equal amounts of total RNA were loaded in 2.2 M formaldehyde-1% agarose gel, electrophoresed, and transferred to nitrocellulose. The 32P-labeled PKI probe was generated by use of the Prime-a-Gene Labeling Kit (Promega, Madison, WI). The RNA blot was hybridized with the probe in hybridization solution containing 50% formamide at 42°C overnight, and then it was washed three times at room temperature with 0.1% SDS and 2× saline-sodium citrate solution (Fisher Scientific, Pittsburgh, PA). Equal loading of RNA samples was demonstrated by visualization of the fluorescence of ethidium bromide bound to the 28S rRNA subunit. Kodak X-Omat X-ray film was used to expose the blot at −80°C for 1–3 days.

β-Galactosidase activity. The efficiency of gene transfer into HMEC with use of the Ad vectors was determined by microscopic evaluation of the fraction of HMEC expressing β-galactosidase activity after infection with Adβgal. HMEC grown to confluency in six-well culture dishes were washed twice in Ca2+- and Mg2+-free PBS and incubated for 4–6 h at 37°C in the X-gal staining solution (50 mM Tris-HCl, pH 7.5, 2.5 mM ferricyanide, 15 mM NaCl, 1 mM MgCl2, 0.5 mg/ml X-gal). Blue-stained HMEC were positive for β-galactosidase.

Luciferase assay. A reporter plasmid containing the firefly luciferase gene under the transcriptional control of multiple
units of CRE (pADneo2-C6-BGL) was used as an index of the cAMP-dependent signaling pathway (24). HMEC were grown to 60–70% confluency in six-well culture dishes and incubated with pADneo2-C6-BGL in Lipofectamine at a ratio of 1:8 for 3 h at 37°C. The HMEC were washed and replaced with complete medium containing 10% FBS, and after incubation for 20 h the cells were treated with 10 µM forskolin + 1 µM 3-isobutyl-1-methylxanthine (F + I) overnight. The cells were washed twice with Ca2+- and Mg2+-free PBS and collected in reporter lysis reagent (Promega). Luciferase activity was determined from the cell extracts with use of the luciferase assay reagent system (Promega). Activity in relative light units was measured in a luminometer (model FB 12, Zylux, Maryville, TN) and reported as values normalized to protein (bicinchoninic acid kit; Pierce, Rockford, IL).

Transendothelial albumin clearance rate. The transendothelial albumin clearance rate across cultured monolayers of endothelial cells was determined using an in vitro system, as described previously (32). This system measures the diffusive flux of the tracer molecules across cell monolayers and consists of luminal and abluminal compartments separated by a polycarbonate microporous filter (0.8 µm pore diameter). Endothelial cells were seeded at 105 cells on each filter and grown for 3–4 days to attain confluency. Both compartments contained the same medium (DMEM, 20 mM HEPES, pH 7.4) at volumes of 700 µl and 25 ml, respectively, but only the luminal compartment contained Evans blue-labeled albumin tracer (0.67 mg/ml in 4% albumin) (44). The luminal compartment was fitted with a Styrofoam outer ring and floated in the abluminal medium so that fluid levels remained equal after repeated samplings from the abluminal compartment. The abluminal compartment was stirred continuously for rapid mixing, and the entire system was kept at 37°C by a thermostatically regulated water bath. Samples of 300 µl were removed from the abluminal compartment at 10-min intervals for 60 min, and optical density (OD) was read at 620 nm. The change in volume over time provided the clearance rate in microliters per minute, as determined by weighted least-squares nonlinear regression (BMDP Statistical Software, Berkeley, CA).

Cell viability assay. The effects of Ad infection of HMEC on cell viability were determined by reduction of the tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; Sigma Chemical) by mitochondrial dehydrogenases (25). HMEC were plated at 50,000 cells/well in a 96-well tissue culture dish and grown for 48 h. The cells were washed twice in Hanks’ balanced salt solution, infected with Ad vectors accordingly, and incubated with 0.5 mg/ml MTT for 3 h at 37°C. Subsequently, 0.04 M HCl in β-isopropanol was added, and the OD was read at 570 nm in a plate reader. The OD units provided an index of enzymatic activity of living cells.

Statistics. Single sample data were analyzed by the two-tailed t-test; a multiple range (Scheffé's) test was used for comparisons of experimental groups with a single control group (49).

RESULTS

Efficiency of Ad-mediated transgene expression. HMEC were infected with Adβgal at 10 or 100 MOI. As control, HMEC were also infected with heat-inactivated Adβgal at 100 MOI or AdNull at 100 MOI. At 2 days postinfection with Adβgal at 100 MOI, >95% of HMEC were stained with X-gal, indicating a high level of β-galactosidase activity (Fig. 1A); at 10 MOI, <30% of HMEC were stained (Fig. 1B). HMEC infected with heat-inactivated Adβgal at 100 MOI (Fig. 1C) or AdNull at 100 MOI (Fig. 1D) did not stain with X-gal, indicating lack of β-galactosidase activity. UV-irradiated Adβgal also showed inhibition of β-galactosidase activity (not shown).

Effects of Ad infection on endothelial permeability. The effects of Ad infection per se on normal endothelial barrier function were determined. The studies were made using Adβgal for infection, since expression of β-galactosidase in endothelial cells is not expected to affect barrier properties. HMEC were grown to confluency on microporous filters and infected with Adβgal (5–100 MOI) or UV-Adβgal (100 MOI) for 1, 2, or 3 days, and the transendothelial clearance rate of albumin was determined. The albumin clearance rate of

![Fig. 1. Efficiency of adenovirus (Ad)-mediated gene transfer into endothelial cells by use of recombinant Adβgal, an Ad expressing Escherichia coli lacZ gene. Human dermal microvascular endothelial cells (HMEC) were infected with Adβgal for 2 days at 100 multiplicities of infection (MOI; A) and 10 MOI (B), with heat-inactivated Adβgal at 100 MOI (C), or with AdNull at 100 MOI (D). Expression of β-galactosidase in cells is shown by blue X-gal stain. Original magnification ×200; n = 2.](http://ajpcell.physiology.org/).
control noninfected HMEC was 0.021 ± 0.004 µl/min; infection of HMEC at 5, 50, and 100 MOI for 2 days resulted in clearance rates not different from the control (Fig. 2A). As a positive control, treatment with 100 µM H₂O₂ increased the clearance rate to 0.056 ± 0.006 µl/min (Fig. 2A). Infection of HMEC at 100 MOI for 1, 2, and 3 days also did not change the clearance rate relative to control (Fig. 2B). HMEC infected with UV-irradiated Adβgal showed clearance rates not different from control (Fig. 2).

Potential cytotoxicity of Ad infection on endothelial cells was evaluated. HMEC were infected with Adβgal (5–200 MOI) for 2 days. Results indicated absence of cytotoxicity at up to 200 MOI (Fig. 3).

Ad-mediated overexpression of PKI. Northern blot analysis indicated that infection of HMEC with AdPKI at 100 MOI for 24 h resulted in a single transcript hybridized with the PKI-specific probe (Fig. 4, top, lane 2).

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Fig. 2. Effects of Ad infection of HMEC with Adβgal on transendothelial albumin clearance rate. A: HMEC grown to confluence on microporous filters were infected with Adβgal at 5, 50, or 100 MOI for 2 days, and clearance rate was determined. As control, ultraviolet-irradiated Adβgal at 100 MOI (UV 100) was used for infection; as a positive technical control for assay, oxidant H₂O₂ (100 µM) was used to treat cells to increase permeability. Each group contained 4–5 monolayers; n = 4. B: HMEC were infected with Adβgal at 100 MOI for 1, 2, or 3 days or with UV Adβgal for 3 days (UV 3). Each group contained 4–5 monolayers; n = 2. C, noninfected control.

Fig. 3. Effects of Ad infection on endothelial cell viability. HMEC were infected with Adβgal (5–200 MOI) for 2 days, and cytotoxicity was determined by ability of mitochondrial dehydrogenases to reduce substrate tetrazolium salt, detected colorimetrically by reading optical density (OD) at 570 nm. Each group contained 4–8 monolayers; n = 2.

Fig. 4. Northern blot of human microvascular endothelial cells: lane 1, control, noninfected; lane 2, infected with AdPKI at 100 MOI for 24 h; lane 3, infected with AdNull at 100 MOI for 24 h. Top: arrowhead indicates overexpression of protein kinase A inhibitor (PKI) mRNA from AdPKI-infected group (lane 2); bottom: membrane showing 28S rRNA subunit detected by ethidium bromide fluorescence of control, AdPKI, and AdNull groups.
However, mRNA was not detectable after 4 h of infection (data not shown). The control noninfected HMEC (lane 1) and HMEC infected with AdNull (lane 3) showed absence of PKI transcript. In Fig. 4, bottom, equal loading of RNA of the three groups is illustrated by the 28S rRNA band.

Effects of overexpression of PKI on endothelial permeability. Initial studies were carried out to determine the effects of increased intracellular cAMP on endothelial permeability in HMEC in response to thrombin activation. A combination of forskolin (direct activator of adenyl cyclase) and IBMX (phosphodiesterase inhibitor) was used to increase intracellular levels of cAMP in HMEC. Confluent monolayers of HMEC grown on microporous filters were pretreated with F+I (Sigma Chemical) for 15 min, human α-thrombin (0.5 µM) was added, and albumin clearance rate was determined. The control clearance rate was 0.015 ± 0.004 µl/min; thrombin treatment increased the clearance rate to 0.040 ± 0.009 µl/min (Fig. 5). Pretreatment with F+I inhibited the thrombin-induced increase in clearance rate (Fig. 5). The clearance rates from F+I alone and the DMSO vehicle were not significantly different from control.

In the subsequent studies the effects of infection with AdPKI on the cAMP-mediated inhibition of the increased clearance rate were determined. HMEC were grown to confluency on microporous filters and infected with AdPKI (100 MOI) for 2 days. The cells were treated with F+I as described above, thrombin was added, and albumin clearance rate was determined as described above. Results indicated that with HMEC infected with AdPKI, the thrombin-induced increased clearance rate was not decreased after F+I treatment (0.045 ± 0.0045 µl/min); the increased clearance rate was similar to that of the thrombin-alone group (absence of F+I; 0.040 ± 0.0035 µl/min; Fig. 6). In HMEC infected with the control UV-irradiated AdPKI, the thrombin-induced increased clearance rate was inhibited by F+I treatment (Fig. 6). Infection with AdPKI or UV-AdPKI alone did not alter baseline clearance rate (Fig. 6).

Overexpression of PKI inhibits CRE-driven transcription of reporter gene activity. The function of PKI expressed in HMEC was investigated using a reporter plasmid containing the firefly luciferase gene under the transcriptional control of CRE (pADneo2-C6-BGL) (24). HMEC were transiently transfected with pADneo2-C6-BGL, and the activation of transcription was determined by measuring luciferase activity. The transfected HMEC were treated with F+I for 2, 24, or 48 h, and cells were collected for luciferase activity assay (see METHODS). Results indicated that luciferase activity was greatest after 1 day of F+I treatment and declined progressively by 48 h (Fig. 7A). The control HMEC group (absence of F+I treatment) was consistently lower in luciferase activity than the treated group.

To determine the effects of PKI on the induction of CRE-driven luciferase activity, HMEC were infected with AdPKI (100 MOI) by use of a protocol similar to that used for the permeability studies, and on the following day they were transfected with pADneo2-C6-BGL. Results indicated that expression of PKI in HMEC was accompanied by inhibition of the increase in luciferase activity in response to F+I treatment (Fig. 7B). HMEC infected with control UV-irradiated AdPKI or AdNull showed increased luciferase activity similar in extent to noninfected HMEC in response to F+I treatment (Fig. 7B).

DISCUSSION

Findings from this study indicate that overexpression of the PKI gene in endothelial cells abrogated the cAMP-mediated protective effects on the thrombin-
PKI GENE ABOLISHES PROTECTION TO BARRIER DYSFUNCTION

induced increase in permeability and directly support the notion that the PKA signaling pathway was a predominant regulator functioning to prevent endothelial barrier dysfunction. Although the protective effects of increased intracellular cAMP are generally accepted to occur through activation of PKA, direct evidence supporting this idea has been limited. In one study, Stelzner et al. (50) reported that the pharmacological PKA inhibitor Rp-cAMPS reversed ~50% of the endothelial barrier enhancement produced by forskolin, an activator of adenyl cyclase. They attributed this partial inhibition to nonspecific effects of Rp-cAMPS. However, an alternative explanation may be that forskolin (or increased cAMP) decreases endothelial permeability via PKA-dependent and -independent mechanisms. In the present study, overexpression of PKI in endothelial cells was effective in fully reversing the barrier-enhancing effects of F+I. PKI is an endogenous inhibitor of PKA and functions by competitive inhibition for the peptide substrate binding site on the catalytic subunit of PKA (52). The binding affinity of PKI is 0.2 nM and is highly selective and specific for PKA. The present findings provide strong support that cAMP-mediated barrier enhancement is regulated by predominantly PKA-dependent mechanisms.

The function of PKI was assessed by its capacity to inhibit induction of luciferase activity in endothelial cells transiently transfected with the reporter plasmid pADneo2-C6-BGL, which contains six heterologous CRE sequences upstream of the β-globin minimal promoter (24). Cell lines transfected with this plasmid showed luciferase induction that was specific to elevation of cAMP levels (24). In this study, treatment of endothelial cells transfected with the reporter plasmid with F+I induced a threefold increase in luciferase activity over control. The induction of activity has been shown to involve a sequence of events initiated by increased cAMP, which lead to activation of PKA, phosphorylation of CRE-binding protein, binding of phosphorylated CRE-binding protein to CRE, and activation of transcription. We found that overexpression of PKI in endothelial cells abrogated this transcriptional activation of luciferase activity, indicating that the Ad-mediated PKI expression in endothelial cells was functionally active in inhibition of PKA.

Two potential target substrates of PKA associated with regulating barrier function include Ca\(^{2+}\)-calmodulin-dependent myosin light chain (MLC) kinase (MLCK) (15, 38, 43) and RhoA (12, 30). In endothelial cells, thrombin-induced barrier dysfunction is associated with cell rounding, intercellular gap formation, and increase in MLC phosphorylation (15, 39). Expression of the constitutively active catalytic domain of MLCK into Madin-Darby canine kidney epithelial monolayers resulted in a threefold increase in MLC phosphorylation, which was accompanied by increased paracellular permeability of solutes and decreased transepithelial resistance (23). Activated MLCK directly phosphorylates threonine-18 and serine-19 of MLC, which is related to isometric tension development and actin polymerization in endothelial cells (18). The specific target protein of RhoA, Rho kinase, is known to directly phosphorylate MLC as well as the myosin-associated MLC phosphatase, both processes contributing to the increased MLC phosphorylation (26, 28). In an in vitro study, inhibition of RhoA in endothelial cells by Clostridium botulinum C3 exoenzyme reduced the thrombin-induced barrier dysfunction and MLC phosphorylation (3), supporting the notion that the thrombin-induced barrier dysfunction occurred in part through activation of RhoA.

The recently cloned endothelial MLCK contains highly conserved phosphorylation sites for PKA, its phosphorylation resulting in reduced MLCK activity,
which presumably provides the basis for protection against barrier dysfunction (16). PKA also terminates RhoA signaling by mediating the phosphorylation of the COOH-terminal domain of RhoA (30), providing an additional/alternative pathway by which MLC phosphorylation and endothelial permeability can be reduced. Several reports confirmed that elevation of intracellular cAMP in endothelial cells inhibited MLC phosphorylation in response to thrombin (15, 43) and histamine (38, 48). Recently, however, Moy and coworkers (37) observed that increased cAMP inhibited the thrombin-mediated decrease in transendothelial resistance, but it did not inhibit the increased MLC phosphorylation and tension development, suggesting that the promotion of barrier function was independent of MLC regulation. Thus the mechanisms by which PKA activation inhibits the mediator-induced increase in endothelial permeability are yet to be resolved.

Evidence also indicates that increased endothelial permeability may not be entirely accounted for by increased MLC phosphorylation (17, 39, 43, 47). Interestingly, Patterson et al. (43) showed that increased cAMP inhibited the endothelial barrier dysfunction in response to phorbol ester activation of protein kinase C, which occurred in the absence of increased MLC phosphorylation. These observations suggest that the mechanisms by which PKA confers protection against barrier dysfunction involve phosphorylation of other target substrates in addition to MLCK and RhoA. Substrates containing known PKA phosphorylation sites implicated in endothelial barrier function regulation include inositol 1,4,5-trisphosphate receptors (36, 57), filamin (21), and serine/threonine protein phosphatases (54). It will be important for future studies to identify the target substrates for phosphorylation by PKA and to determine their role in regulation of endothelial permeability.

Our findings indicate that the use of recombinant Ad vectors for gene transfer in endothelial cells did not impair the barrier function, did not result in cell toxicity, and showed a high gene transfer efficiency and expression. The high efficiency of gene transfer is consistent with reports from other investigators who have used similar recombinant Ad vectors for in vitro infection (31, 51, 53) as well as for in vivo gene transfer to a wide variety of target tissues (7). The finding that infection of endothelial monolayers with Ad vectors did not impair barrier function or induce cell cytotoxicity indicated that Ad vectors provide a particularly advantageous tool for studies of the effect of gene expression on endothelial barrier function. Furthermore, Piedimonte et al. (45) also documented a lack of direct effect of an E1<sup>−</sup>, E3<sup>−</sup>, replication-deficient recombinant Ad on tracheal barrier function. Thus the use of Ad vectors for studies of barrier function is superior to other gene transfer methods, such as the use of liposomes, electroporation, CaPO<sub>4</sub>, and retroviral vectors, which typically result in lower gene transfer efficiencies in addition to cytotoxicity, which disrupts baseline barrier function.

In summary, infection of endothelial cells with the E1<sup>−</sup>, E3<sup>−</sup>, replication-deficient AdPKI resulted in overexpression of PKI, which abrogated the cAMP-mediated inhibition of the increased endothelial permeability in response to thrombin. Overexpression of PKI also inhibited transcription of the reporter gene luciferase, which is under the control of CRE, indicating that the expressed PKI in endothelial cells was functionally active. Furthermore, Ad-mediated transgene expression of endothelial cells was highly efficient (>95%), did not alter baseline endothelial barrier function, and was not cytotoxic. Thus the results provide direct evidence that activation of the PKA pathway is critically important in conferring protection against mediator-induced endothelial barrier dysfunction.

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