Differential Regulation of Endothelial Cell Permeability by cGMP via Phosphodiesterases 2 and 3

James Surapisitchat, Kye-Im Jeon, Chen Yan, Joseph A. Beavo

Abstract—Endothelial barrier dysfunction leading to increased permeability and vascular leakage is an underlying cause of several pathological conditions, including edema and sepsis. Whereas cAMP has been shown to decrease endothelial permeability, the role of cGMP is controversial. Endothelial cells express cGMP-inhibited phosphodiesterase (PDE)3A and cGMP-stimulated PDE2A. Thus we hypothesized that the effect of cGMP on endothelial permeability is dependent on the concentration of cGMP present and on the relative expression levels of PDE2A and PDE3A. When cAMP synthesis was slightly elevated with a submaximal concentration of 7-deacetyl-7-(O-[N-methylpiperazino]-γ-butryl)-dihydrochloride–forskolin (MPB–forskolin), we found that low doses of either atrial natriuretic peptide (ANP) or NO donors potentiated the inhibitory effects of MPB–forskolin on thrombin-induced permeability. However, this inhibitory effect of forskolin was reversed at higher doses of ANP or NO. These data suggest that cGMP at lower concentrations inhibits PDE3A and thereby increases a local pool of cAMP, whereas higher concentrations cGMP activates PDE2A, reversing the effect. Inhibitors of PDE3A mimicked the effect of low-dose ANP on thrombin-induced permeability, and inhibition of PDE2A reversed the stimulation of permeability seen with higher doses of ANP. Finally, increasing PDE2A expression with tumor necrosis factor-α reversed the inhibition of permeability caused by low doses of ANP. As predicted, the effect of tumor necrosis factor-α on permeability was reversed by a PDE2A inhibitor. These findings suggest that the effect of increasing concentrations of cGMP on endothelial permeability is biphasic, which, in large part, is attributable to the relative amounts of PDE2A and PDE3A in endothelial cells. (Circ Res. 2007;101:811-818.)

Key Words: phosphodiesterase ■ cGMP ■ endothelial permeability ■ TNF-α

The endothelium plays an important role in maintaining normal vascular function. In addition to maintaining a nonthrombogenic surface, secreting anticoagulation factors, responding to and participating in inflammatory signaling and regulating vascular tone, the endothelium also acts as an active barrier between the blood and the underlying tissue. Endothelial dysfunction caused by injury or inflammatory signals has been shown to lead to numerous pathological conditions. For example, inflammatory cytokines can induce adhesion molecule expression in endothelial cells, thereby promoting the adherence and migration of immune cells into the vessel wall, leading to atherosclerosis.1 Dysfunctional endothelium also leads to decreased barrier function or increased permeability. Increased endothelial permeability is characteristic of many diseases and pathological conditions, including atherosclerosis, asthma, tumor growth, edema, and sepsis.2,3

Several inflammatory mediators, including vascular endothelial growth factor, tumor necrosis factor (TNF)-α, histamine, and thrombin, are known to increase endothelial permeability.2–4 TNF-α increases permeability, at least in part, via activation of protein kinases such as Fyn and p38, whereas vascular endothelial growth factor, histamine, and thrombin all signal via increasing intracellular Ca2+. An increase in Ca2+ in endothelial cells leads to activation of myosin light chain kinase and endothelial cell contraction, resulting in decreased barrier function. Whereas Ca2+ increases endothelial cell permeability, the second messenger, cAMP, has, in general, been shown to decrease permeability.2 β-Adrenergic agonists, adenylate cyclase activators such as forskolin, and cAMP-phosphodiesterase inhibitors all have been shown to improve endothelial barrier function basally and under stimulated conditions. Although it is has been shown that cAMP can decrease endothelial cell permeability, the effects of cGMP are controversial.2 Both barrier-enhancing and -impairing effects of cGMP have been reported.2,5,6 These differences most commonly have been attributed to the differences in the types of endothelial cells and vascular beds studied and/or to whether or not cGMP-dependent protein kinase (PKG) was expressed. In this study, we test another possible mechanism, namely that the effect of cGMP signaling on permeability is dependent on the relative

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expression of cGMP-regulated cAMP-hydrolyzing phosphodiesterases in the endothelial cells that are differentially sensitive to the concentration of cGMP that is generated.

Cyclic nucleotide phosphodiesterases (PDEs) play a critical role in regulating a variety of cellular functions. By catalyzing the breakdown of cyclic nucleotides, PDEs control the amplitude, duration, and compartmentalization of cyclic nucleotide signaling in the cell. The PDE superfamily consists of 11 different family members distinguished by their unique regulation, enzymatic characteristics, structure, and pharmacological inhibitory profiles, as well as by their tissue, cellular, and subcellular expression.7 Endothelial cells have been shown to express cGMP-stimulated PDE2, cGMP-inhibited PDE3, cAMP-specific PDE4, and cGMP-specific PDE5.8 Although the presence of these PDEs in most endothelial cells has been shown, their relative expression is variable depending on the endothelial cells being studied.8 Because endothelial cells express both cGMP-stimulated PDE2 and cGMP-inhibited PDE3, endothelial responses to increases in cGMP might be expected to vary because of the differential effects cGMP has on these PDEs. cGMP can activate PDE2 by binding to the regulatory domain of PDE2, thereby increasing its rate of hydrolysis of both cGMP and cAMP.7 On the other hand, cGMP also can act as a high-affinity competitive inhibitor of PDE3 because the Vmax for cGMP is much less than the Vmax for cAMP.7 Thus the outcome of cGMP signaling on endothelial functions may vary depending on the degree to which PDE2 is activated and/or PDE3 is inhibited.

In the present study, we tested the hypothesis that the effect of cGMP on thrombin-induced permeability may be attributable to its differential regulation of PDE2 and PDE3 on cAMP hydrolysis. Specifically, we examined whether cGMP can potentiate the inhibitory effect of forskolin on thrombin-induced permeability by inhibiting cAMP hydrolysis by PDE3 and/or reverse the effect of forskolin by activating PDE2 cAMP hydrolysis. We found that low doses of guanylyl cyclase agonists, such as atrial natriuretic peptide (ANP) or NO donors, potentiated the effects of forskolin via cGMP inhibition of PDE3, whereas higher doses reversed this potentiation by cGMP activation of PDE2. Furthermore, as TNF-α induces PDE2 expression in endothelial cells,9 we show that TNF-α also decreases PDE3A expression in endothelial cells. Increasing PDE2 and decreasing PDE3 expression reverses the response of endothelial cells to low concentrations of cGMP. These findings suggest that the result of cGMP signaling on endothelial cell permeability is highly dependent on the concentration of intracellular cGMP, at least in part, because of the relative expression and activity of PDE2 and PDE3 in a particular endothelial bed.

Materials and Methods

Permeability Assays

Permeability assays were performed as previously described.10 Briefly, ~1×10⁵ human umbilical vein endothelial cells (HUVECs) were seeded on gelatin-coated transwell units (6.5-mm diameter, 3.0-μm pore size; Corning). After 48 hours, media±10 ng/mL TNF-α was changed, and measurements were performed 24 hours later. Before agonist stimulation, cells were rinsed once with medium 199 containing 1% BSA, followed by incubation in medium 199+1% BSA for 45 minutes. PDE inhibitors or vehicle were then added for an additional 15 minutes before stimulation. Permeability was measured by adding 1 mg of fluorescein isothiocyanate–dextran (M, 42,000) per milliliter, together with agonists to the upper chamber. After incubation for 30 minutes, 50 μL of sample from the lower compartment was diluted with 150 μL of PBS and measured for fluorescence at 520 nm when excited at 492 nm with a Beckman DTX880 Multimode Detector. Results are presented as either fold increase in permeability over nonstimulated control or as percentage of thrombin maximum. Paired t test was performed using Prism v4.00.

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Results

cGMP Has a Biphatic Effect on cAMP Inhibition of Thrombin-Induced Permeability

The consequence of cGMP signaling on endothelial barrier function has been controversial, as both increases and decreases in endothelial cell permeability in response to cGMP have been reported.2 We first tested whether the guanylyl cyclase agonists, ANP (a particulate guanylyl cyclase agonist) and S-nitroso-N-acetylpenicillamine (SNAP) (a NO donor and soluble guanylyl cyclase agonist) would affect permeability. ANP alone at 0.3 and 30 nmol/L and SNAP at 0.001 and 10 μmol/L had no significant effect on basal permeability (Figure IA and IB in the online data supplement). We then investigated whether these agonists could have an effect on thrombin-induced permeability. A low dose of ANP (0.3 nmol/L) significantly inhibited thrombin-induced permeability (supplemental Figure IA). However, this effect was completely reversed at a higher dose of ANP. Similarly SNAP at 0.001 μmol/L had no effect on thrombin-induced permeability, whereas 10 μmol/L potentiated the effect of thrombin (supplemental Figure IB). Because the guanylyl cyclase activators had differential effects on thrombin-induced permeability and we have confirmed that cAMP inhibits thrombin-induced permeability (supplemental Figure IIIA and IIIB), we hypothesized that cGMP may be differentially regulating cAMP PDEs. To try to mimic the in vivo condition of a modest adenylyl cyclase tone and test this hypothesis, we first increased endothelial permeability with thrombin (1 U/mL) and a submaximal dose of the water-soluble adenylyl cyclase activator 7-deacetyl-7-(O-[(N-methylpiperazino)-γ-butyryl]-dihydrochloride–forskolin (MPB–forskolin) (0.1 μmol/L) was then used to slightly increase intracellular cAMP and inhibit thrombin-induced permeability by ~10% to 15% of the thrombin maximum (100%). In vivo, one would expect that the effects of PDE regulation would be more pronounced when a cAMP tone is present, for example, from PGE2 release. We then examined the ability of cGMP generated in response to ANP (Figure 1A) and SNAP (Figure 1B) to either potentiate or reverse this inhibitory effect. As shown in Figure 1A, low doses of ANP (0.003 to 0.3 nmol/L) potentiated the inhibitory effect of cAMP on thrombin-induced permeability. The potentiating effect of low doses of ANP was reversed at higher doses (3 to 300 nmol/L). These concentrations of ANP dose-dependently increased intracellular cGMP under similar conditions (supplemental Figure IV). Similar results were seen with
SNAP, which increases cGMP via activation of soluble guanylyl cyclase (Figure 1B). Furthermore, the effect of SNAP was inhibited by oxadiazolo quinoxalin-1-one (1H-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one), a specific soluble guanylyl cyclase inhibitor (Figure 1C). These results suggest that cGMP has a concentration-dependent biphasic effect on cAMP inhibition of thrombin-induced permeability.

PKG Does Not Mediate the Effect of cGMP on cAMP Inhibition of Thrombin-Induced Endothelial Cell Permeability

There are several known effector molecules for cGMP, including PKG, cyclic nucleotide–gated (CNG) ion channels, and PDEs. To determine whether or not PKG or CNG channels mediated the effects of cGMP on endothelial permeability, we used 2 cGMP analogs, 8-Br-cGMP and 8-pCPT-cGMP, known to activate PKG and CNG channels but not regulate either PDE2 or PDE3.11 Neither 8-Br-cGMP nor 8-pCPT-cGMP had any significant effect on cAMP inhibition of thrombin-induced permeability (Figure 2). Therefore, PKG and CNG channels are not likely to mediate the effects of cGMP on endothelial permeability. Furthermore, we found that 8-Br-cAMP, a cAMP analog shown to activate PKA, Epac (exchange proteins activated directly by cAMP), and CNG channels, potentiated the inhibitory actions of MPB–forskolin on thrombin-induced permeability. This indicates that cyclic nucleotide analogs can permeate HUVECs and further demonstrates the barrier-enhancing effects of cAMP on endothelial permeability. Furthermore, we found that 8-Br-cAMP, a cAMP analog shown to activate PKA, Epac (exchange proteins activated directly by cAMP), and CNG channels, potentiated the inhibitory actions of MPB–forskolin on thrombin-induced permeability. This indicates that cyclic nucleotide analogs can permeate HUVECs and further demonstrates the barrier-enhancing effects of cAMP on endothelial permeability.
cGMP on endothelial cell permeability is not likely to be via PKG.

Roles of PDE2 and PDE3 in Mediating the Effects of cGMP on cAMP Inhibition of Thrombin-Induced Permeability

Because cGMP has a biphasic effect on cAMP-inhibited thrombin-induced permeability and this effect is not likely to be mediated by PKG or CNG, we hypothesized that the biphasic effect of cGMP is mediated via the inhibition of PDE3 at low doses of cGMP, followed by activation of PDE2 at higher doses of cGMP, thus altering the levels of cAMP in the cell in opposite directions. To test this hypothesis, we used the PDE2- and PDE3-specific inhibitors Bay 60-7550 (PDE2) and trequinsin (PDE3). Trequinsin (30 nmol/L) alone mimicked the actions of 0.03 nmol/L ANP on cAMP inhibition of thrombin-induced permeability (Figure 3A). This demonstrates that inhibition of PDE3 can potentiate the effect of MPB–forskolin on thrombin-induced permeability and suggests that the mechanism of low concentrations of cGMP signaling may be via PDE3 inhibition. If the effect of low concentrations of cGMP were attributable to a mechanism other than PDE3 inhibition, we might have expected an additive or synergistic effect with simultaneous addition of low concentrations of ANP with PDE3 inhibitors. When HUVECs were pretreated with trequinsin before addition of ANP, there was no additional potentiation of ANP on cAMP inhibition of thrombin-induced permeability (Figure 3A). Similar results were seen with cilostamide, a structurally different but selective PDE3 inhibitor (supplemental Figure VI). This suggests that the mechanism by which low doses of ANP improves barrier function is via cGMP inhibition of PDE3.

In other experiments, the PDE2 inhibitor Bay 60-7550 (100 nmol/L) blocked the actions of higher (30 nmol/L) concentrations of ANP on cAMP inhibition of thrombin-induced permeability (Figure 3B). Similar results were seen with EHNA (erythro-9-[2-hydroxy-3-nonyl]adenine), a structurally different but selective PDE2 inhibitor (supplemental Figure VI). This indicates that as cGMP concentrations increase its ability to potentiate the effects of cAMP by inhibiting PDE3 at lower concentrations is reversed by activation of PDE2.

cGMP Has Differential Effects on PDE2A and PDE3A cAMP Hydrolytic Activity

The functional studies of cGMP on thrombin-induced permeability suggest that cGMP biphasically regulates cAMP levels in HUVECs via inhibition of PDE3 at low concentrations and activation of PDE2 at higher concentrations. To demonstrate the effect of cGMP on PDE3 and PDE2 cAMP hydrolytic activity, we immunoprecipitated PDE2A and PDE3A from HUVECs and assayed for cAMP PDE activity at various concentrations of cGMP. As previously shown for PDE3A found in the heart and platelets, cGMP dose-dependently inhibited PDE3A activity with a calculated IC50 of ~59 nmol/L (Figure 4). PDE2A activity, on the other hand, was stimulated nearly 30-fold by cGMP with a calculated EC50 of ~1.1 μmol/L, which is similar to results found with PDE2A from the heart and adrenal gland (Figure 4). These results demonstrate that at low, nanomolar concentrations, cGMP inhibits PDE3A, whereas at higher, micromolar concentrations, cGMP activates PDE2A. Because the Vmax for PDE2A is much higher than that for PDE3A, one would expect that at high concentrations of cAMP, the PDE2A effect should dominate if both PDEs control pools of cAMP that can regulate endothelial barrier function.

cGMP Regulates cAMP in HUVECs via Inhibition of PDE3 and Activation of PDE2

These functional studies of cGMP on cAMP inhibition of thrombin-induced permeability suggested that cGMP can differentially regulate cAMP levels in HUVECs via PDE2 and PDE3. It is, however, not clear whether these effects could be detected on total cellular cAMP. When tested directly, we found little effect on whole cell cAMP (data not
shown). However, HUVECs have been shown to express high levels of PDE4 in addition to PDE2 and PDE3. Thus, we reasoned that the pool(s) served by PDE2 and PDE3 might be more easily detected in cells in which PDE4 had been inhibited. Therefore, we first pretreated HUVECs with rolipram (10 \( \mu \)mol/L), a PDE4-specific inhibitor, and used the same conditions and stimuli described for studying permeability. Under these conditions, we show that MPB–forskolin (0.1 \( \mu \)mol/L) causes a small but significant increase in cAMP after 5 minutes (supplemental Figure VIIA). MPB–forskolin plus inhibition of PDE3 by trequinsin or PDE2 by Bay 60-7550 increased cAMP in HUVECs compared with MPB–forskolin alone (supplemental Figure VIIA). Inhibition of both PDE2 and PDE3 had an additive effect on MPB–forskolin–stimulated cAMP increases. These results suggest that both PDE2 and PDE3 can regulate cAMP in HUVECs.

When PDE2 was inhibited by trequinsin, HUVECs stimulated with MPB–forskolin and increasing amounts of ANP displayed a decrease in cAMP levels, suggesting cGMP activation of PDE2 (supplemental Figure VIIB). On the other hand, in the presence of Bay 60-7550, which inhibits PDE2, ANP caused an increase in cAMP, suggesting cGMP inhibition of PDE3 (supplemental Figure VIIC). Together, these results strongly suggest that cGMP can regulate cAMP levels in HUVECs via inhibition of PDE3 and activation of PDE2.

Alterations in the Expression and Activity of PDE2 and PDE3 in Endothelial Cells Modifies cGMP Effects on Endothelial Cell Permeability

Recently, it was shown that TNF–\( \alpha \), an inflammatory cytokine known to increase endothelial cell permeability, also increased the expression of PDE2 in HUVECs. We confirmed an \( \approx 7 \)-fold increase in PDE2A mRNA and protein expression in HUVECs after 24 hours of TNF–\( \alpha \) stimulation (Figure 5A and 5B). Moreover, using quantitative real-time PCR, we found that PDE3A mRNA was decreased \( \approx 50\% \) in TNF–\( \alpha \)–stimulated HUVECs (Figure 5A). At the protein level, PDE3A expression decreased \( \approx 25\% \) in this time frame (Figure 5C). The apparent molecular mass of PDE3A expressed in HUVECs is \( \approx 94 \) kDa, corresponding to PDE3A3. The recombinant PDE3A1 used as a positive control in Figure 5C has a molecular mass of \( \approx 115 \) kDa.
Importantly cAMP PDE activity levels also changed after TNF-α stimulation. TNF-α increased basal PDE2 activity from ⊳16 pmol cAMP/min·mg⁻¹ to nearly 100 pmol cAMP/min·mg⁻¹ (Figure 5D). PDE3 activity, ⊳60 pmol cAMP/min·mg⁻¹, did not change significantly. Therefore, we used TNF-α treatment as a means to alter the expression and activity of PDE2A and PDE3A. As shown previously, 0.3 nmol/L ANP potentiated the inhibitory actions of cAMP on thrombin-induced permeability (Figure 6). In contrast, in HUVECs pretreated with 10 ng/mL TNF-α for 24 hours to induce PDE2 and decrease PDE3A expression, the same concentration of ANP was less effective in potentiating the inhibitory effects of cAMP (Figure 6). The PDE2 inhibitor Bay 60-7550 blocked this change in response to ANP (Figure 6). Again, these results are consistent with the hypothesis that agents able to increase the expression of PDE2 and decrease PDE3A expression can alter the response of endothelial cells to cGMP.

Discussion

In the current study, we have demonstrated that cGMP-elevating agents such as ANP and NO have biphasic effects on cAMP inhibition of thrombin-induced endothelial permeability. Furthermore, we show that a major reason for the biphasic actions of cGMP on cAMP inhibition of thrombin-induced endothelial permeability is attributable to a dose-dependent differential regulation of PDE2 and PDE3. Finally, we demonstrate that altering the expression of PDE2 and PDE3 in endothelial cells can alter the effect of cGMP on endothelial permeability (see model in Figure 7). These results likely explain many of the conflicting results reported in the literature concerning the actions of cGMP on endothelial permeability and suggest important roles for PDE2 and PDE3 in regulating endothelial function.

Whereas the observation that cAMP produced by transmembrane adenylate cyclases decreases endothelial permeability is well accepted, the role of cGMP in regulating endothelial permeability has been controversial. There are several reports in which cGMP has been implicated in increasing endothelial permeability, although just as many demonstrate a barrier-enhancing effect of cGMP.²,⁵,⁶,¹⁵ For example, mice injected with high doses of ANP have been shown to have increased endothelial permeability, whereas others have shown that ANP decreases thrombin-induced permeability.⁵,¹⁶ The use of NO synthase inhibitors, endothelial NO synthase knockout mice, and our own results with soluble guanylyl cyclase inhibitors suggest that at basal levels, NO-derived cGMP inhibits increases in endothelial permeability.⁶,¹⁷ Conversely others have shown that endothelial NO synthase is required for inflammatory increases in permeability.¹⁸ The present results demonstrating a biphasic effect of cGMP caused by differential regulation of cAMP hydrolyzing PDE2 and PDE3 may help reconcile these differences.

Our results demonstrating the differential effects of cGMP on PDE2A and PDE3A cAMP hydrolytic activity are in agreement with previous studies and, to our knowledge, are the first to measure the effect of cGMP in a dose-dependent manner on PDE2 and PDE3 activity isolated from the same cell type (Figure 4).¹⁹,²⁰ Our findings that cGMP inhibits PDE3A with an IC₅₀ nearly 20-fold lower than the EC₅₀ for PDE2A activation supports our model of biphasic regulation of endothelial permeability by cGMP. It has been shown previously that small increases in cGMP can modulate cAMP-dependent processes, such as platelet aggregation via inhibition of PDE3.⁷ Furthermore, it has been shown that ANP, via increasing cGMP, can inhibit adrenocorticotropic hormone-induced aldosterone production from adrenal cells via activation of PDE2.⁷ Despite these reports, in cells expressing both PDE2 and PDE3, there have been few examples in which the ability of cGMP to differentially regulate cAMP signaling via these PDEs has been shown.
One exception is cardiomyocytes, in which both PDE2 and PDE3 are expressed. Vandecasteele et al demonstrated that cGMP at low concentrations inhibited PDE3, resulting in increased PKA stimulation of L-type Ca\(^{2+}\) current.\(^{21}\) However, higher concentrations of cGMP, via activation of PDE2, attenuated this stimulation. These results are consistent with our findings in endothelial cells that low concentrations of cGMP inhibit PDE3 to enhance barrier function, whereas higher concentrations activate PDE2 to increase permeability. Our results are, to our knowledge, the first to demonstrate opposing roles for PDE2 and PDE3 in the cGMP regulation of endothelial permeability.

Part of the reason for the lack of consistent information about the roles of PDEs 2 and 3 relates to a limitation of 1 of the major approaches used by many groups, namely the use of cGMP analogs.\(^{5,22}\) Although analogs such as 8-Br-cGMP and 8-pCPT-cGMP are useful in demonstrating the involvement of PKG, they are not useful in determining the possible contribution of cGMP-regulated PDEs because neither 8-Br-cGMP nor 8-pCPT-cGMP is an effective regulator of PDE2 or PDE3.\(^{11}\) Furthermore, several groups have reported effects of ANP and NO on permeability but did not see similar results with cGMP analogs.\(^{23,24}\) As a result, these findings were interpreted as being cGMP independent. The data presented in this report argue that the permeability effects seen with ANP and NO probably actually are cGMP dependent but work via regulation of PDE2 and PDE3 rather than PKG. We and others have found low levels of expression of PKG in HUVECs that can be activated by cGMP analogs (supplemental Figure V); yet these analogs have little or no effect on regulation of endothelial permeability (Figure 2).\(^{25,26}\) The finding that the actions of cGMP on endothelial permeability is attributable to its differential regulation of PDE2 and PDE3 further underscores the need to examine the role these PDEs play in cGMP signaling.

Until recently, it had been accepted that cAMP improves endothelial barrier function via its ability to activate PKA.\(^4\) Lately, another cAMP effector molecule, Epac, has also been shown to mediate at least part of the barrier-enhancing actions of cAMP.\(^{19}\) Furthermore, it was recently reported that the effect of cAMP on endothelial permeability is dependent on where in the cell cAMP is produced.\(^{27}\) In general, the concept of compartmentalization of cyclic nucleotide signaling is becoming well accepted.\(^{28}\) It is possible that there are several cAMP “pools” that are responsible for the regulation of endothelial permeability via PKA and/or Epac and controlled by specific PDEs. The role of cGMP, PDE2, and PDE3 in the regulation of PKA, Epac, and/or different cAMP pools in the regulation of endothelial barrier function requires further investigation.

Normal physiological levels of ANP and NO are relatively low but increase significantly under pathological conditions.\(^{29,30}\) Our findings that picomolar concentrations of ANP enhance endothelial barrier function via inhibition of PDE3 are consistent with normal vascular function in healthy individuals. At higher ANP concentrations, as seen in heart failure, endothelial barrier function is compromised.\(^{29,30}\) Our results implicating activation of PDE2 by ANP fits well with those reported by Sabrane et al, in which mice lacking endothelial guanylyl-cyclase-A receptor for ANP had decreased permeability and exhibited hypertension and cardiac hypertrophy.\(^{15}\) Thus PDE2 regulation of endothelial permeability may play an important role in the physiological response to pathological conditions. NO has been traditionally thought of as an atheroprotective signal, and dysfunctional endothelium is reported to have decreased levels of NO.\(^{31,32}\) From the present results, low NO would be expected to lead to decreased inhibition of PDE3 by cGMP and thus increased permeability. At the same time, in pathological conditions such as acute inflammation, production of large amounts of NO by endothelial NO synthase and immune cells increases endothelial permeability in vitro and in vivo.\(^{18,33}\) The data reported here strongly suggest that this increased permeability is attributable to increased activity of PDE2. For example, TNF-α and other inflammatory cytokines stimulate several endothelial cell responses that lead to endothelial dysfunction and pathology.\(^3\) The upregulation of PDE2 and downregulation of PDE3α by TNF-α suggests that altering the expression and activity of PDE2 and PDE3, thus altering the effect of cGMP on endothelial cell barrier function, may be a major mechanism by which TNF-α causes endothelial dysfunction (Figure 5). In particular, the finding that the effect of cGMP on endothelial permeability is altered in HUVECs stimulated with TNF-α and that this change can be reversed by PDE2 inhibition supports this hypothesis (Figure 6). Thus PDE2 and PDE3 in the endothelium can act as a sensor or switch to detect normal versus pathological concentrations of cGMP and thus regulate endothelial permeability accordingly. Furthermore, the expression and activity levels of these PDEs control the sensitivity of this switch to cGMP.

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**Disclosures**

None.

**References**


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Materials and Methods

Reagents: Thrombin, Fluorescein Isothiocyanate-Dextran (FITC-Dextran), 8-br-cAMP, 8-br-cGMP and rolipram were purchased from Sigma. Forskolin 7-Deacetyl-7-[O-(N-methylpiperazino)-γ-butyryl]-Dihydrochloride (MPB-forskolin), atrial natriuretic peptide (ANP), S-nitroso-N-acetylpenicillamine (SNAP), ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), trequinsin, cilostamide, Erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) and anti-PKG1 antibody were purchased from Calbiochem. Bay 60-7550, Anti-phospho-Ser239 and Anti-phospho-Ser157 Vasodilator-Stimulated Phosphoprotein (VASP) antibody were purchased from Axxora. 8-pCPT-cGMP was obtained from Biolog. TNF-α was purchased from Research Diagnostics, Inc. Polyclonal anti-PDE2 antibody used for western analysis was purchased from Fabgennix. Monoclonal PDE2 antibody used for immunoprecipitation was generated as previously described.3

Cell culture: Endothelial cells were isolated from human umbilical veins (HUVEC) as previously described and used at passages 1-3.4 All cells were grown on tissue culture flasks coated with gelatin. At least four different HUVEC preparations were utilized for each of the experiments shown.

Western blot analysis: Western blot analysis was performed as described previously.4 PDE3A-specific antibody was created by immunizing rabbits with a portion of rat PDE3A sequence (aa 432-660) fused to glutathione S-transferase. PDE3A antibody does not cross-react with recombinant PDE3B (Data not shown). Immunoreactive bands were visualized by chemiluminescence (Amersham ECL) and analyzed by densitometry using ImageJ.

PDE2A and PDE3A immunoprecipitation and phosphodiesterase assay: For total cAMP PDE activity, HUVEC cell lysate was assayed for PDE activity in the presence of vehicle or PDE inhibitors with 0.3 µmol/L [3H]cAMP as substrate as described previously.5 To determine the effects of cGMP on PDE2 and PDE3 cAMP activity, HUVEC cell lysate were centrifuged at 16,000Xg for 20 minutes and supernatant was incubated overnight at 4°C with monoclonal PDE2A or polyclonal PDE3A specific antibodies followed by 30µl of a 1:1 slurry of protein A/G PLUS-agarose beads (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 2 hours at 4°C. The immunoprecipitate was then washed three times with lysis buffer and assayed for PDE activity in the presence of 1 nmol/L-10 µmol/L cGMP with 0.3 µmol/L [3H]cAMP as substrate for 10 min as described previously.5 The contribution of PDE2 and PDE3 to whole cell cAMP hydrolytic activity was determined by the use of 100 nmol/L Bay 60-7550 (PDE2 inhibitor) or 1 µmol/L Cilostamide (PDE3 inhibitor).

cAMP/cGMP Enzyme Immunoassay (EIA): cAMP and cGMP content of HUVECs was measured by using EIA kits from American Qualex (San Clemente, CA) as previously described.6 Cells were grown to confluence after which the media was changed. After 24 hours, cells were rinsed 1X with M199 containing 1% bovine serum albumin (BSA) followed by incubation in
M199+1% BSA for 45 min. PDE inhibitors or vehicle were then added for an additional 15 minutes before stimulation.

Real-Time PCR analysis for PDE2A and PDE3A: HUVEC RNA was isolated using RNeasy Mini RNA isolation kit (Qiagen) as per the manufacturer’s instructions. Total RNA (1µg) was reverse transcribed into cDNA using oligo dT as a primer and Superscript III (Invitrogen) as per the manufacturer’s instructions. Primers were designed to detect human PDE2A or human PDE3A. Primers utilized are 5’GAGATGATGGACGGGAGAA3’ and 5’GGTCTGTCAACAGCTTGAGATG3’ for PDE2A, 5’CAGGAGATCTGTGACCCAGAA3’ and 5’TGTGTGGGAGATTCATTATTTTCCAG3’ for PDE3A and 5’CGACCATTTTGTCAAGCCTCA3’ and 5’TCTGTGTCCAGGAGGCCATGT3’ for GAPDH. Real-time PCR performed with various dilutions of cDNA using Power SYBR Green master mix (ABI) gave similar results. The pairs of primers showed similar amplification efficiency on cDNA isolated from HUVEC.
Supplemental Figure 1. Effects of cGMP elevating agents on basal and thrombin-induced permeability. A. HUVEC were stimulated with either vehicle, 0.3 nmol/L ANP, 30 nmol/L ANP, 1U/ml thrombin (Thr), 1U/ml thrombin and 0.3 nmol/L ANP or 1U/ml thrombin and 30 nmol/L ANP. Permeability was analyzed as described in Methods. Data represent means (mean±SEM) from 4 independent experiments using HUVEC isolated from 4 different cords. *P=0.0483, vs. Thrombin alone. B. HUVEC were stimulated with either vehicle, 0.001 μmol/L SNAP, 10 μmol/L SNAP, 1U/ml thrombin (Thr), 1U/ml thrombin and 0.001 μmol/L SNAP or 1U/ml thrombin and 10 μmol/L SNAP. Permeability was analyzed as described in Methods. Data represent means (mean±SEM) from 3 independent experiments using HUVEC isolated from 3 different cords.

Supplemental Figure 2. High concentrations of SNAP attenuate increases in cAMP. Since high concentrations of SNAP potentiated the effect of thrombin on endothelial permeability and we hypothesized that this is due to cGMP activation of PDE2 to decrease cAMP, we measured cAMP in HUVEC under these conditions. HUVEC were stimulated with 1U/ml Thrombin for 5 min with or without 10 μmol/L SNAP. cAMP levels were then measured as described in methods and presented as fold over control. As expected no significant changes in cAMP levels were seen unless PDE3 and PDE4 were inhibited, as PDE3 and PDE4 tend to be the dominant cAMP PDEs in HUVEC (Supplemental Fig 7A). However due to compartmentalization of cyclic nucleotides, presumably a pool of cAMP controlled by PDE2 is relatively small compared to the total. Thus to detect changes in cAMP levels at the whole cell level due to PDE2 activity, PDE3 and PDE4 inhibitors were used. Under conditions in which changes in cAMP due to PDE2 can be seen at the whole cell level due to PDE2 activity, PDE3 and PDE4 inhibitors were used. Under conditions in which changes in cAMP due to PDE2 can be seen at the whole cell level, thrombin increased cAMP levels approximately 1.48 fold over control. This small increase in cAMP by thrombin is not seen without PDE3 and PDE4 inhibition (Supplemental Fig 7A) and indicates that thrombin can increase cAMP in a pool that is controlled in part by PDE3 and/or PDE4. This increase was attenuated in HUVEC stimulated with 10 μmol/L SNAP. This suggests that 10 μmol/L SNAP, via increasing cGMP and activation of PDE2, decreases cAMP levels in HUVEC. Data represent means (mean±SEM) from 3 independent experiments using HUVEC isolated from 3 different cords.
Supplemental Figure 3. MPB-forskolin inhibits thrombin-induced permeability. A. HUVEC were stimulated with increasing concentrations of thrombin with or without 10 µmol/L MPB-forskolin. Permeability was analyzed as described in Methods. Solid line represents thrombin stimulated HUVEC. Dotted line represents thrombin + 10 µmol/L MPB-forskolin stimulated HUVEC. B. HUVEC were stimulated with 1U/ml thrombin with increasing concentrations of MPB-forskolin. *P=0.0146, vs. Thrombin alone.

Supplemental Figure 4. ANP dose-dependently increases cGMP levels in HUVEC. HUVEC were stimulated with either: vehicle, 1U/ml thrombin, thrombin plus 0.1 µmol/L MPB-forskolin or thrombin, MPB-forskolin and 0.003-300 nmol/L ANP as in Fig 1A. After 15 min, cGMP levels were determined by EIA as described in methods. Data represent mean of 6 replicates (mean±SEM) from 3 independent experiments using HUVEC isolated from 3 different cords. Results presented as fold increase over control. *P=0.0146, vs. Thrombin alone.
Supplemental Figure 5. PKG is present and can be activated by cGMP analogs in HUVEC. A. To demonstrate that PKG is present in HUVEC, cell lysates were subjected to western blot analysis for PKG expression. Representative data showing PKGI protein expression by Western blot analysis in HUVEC isolated from 2 different cords. Human uterine smooth muscle cells (HUMSC) was used as a positive control. Similar results were observed using HUVEC from passages 1-4. B. To demonstrate that PKG in HUVEC was active, phosphorylation of VASP, a well-characterized substrate for both PKA and PKG was analyzed using antibodies specific to phospho-Serine239 of VASP (preferred PKG site) and phospho-Serine157 of VASP (preferred PKA site).¹ Phosphorylation of Ser157 leads to a shift in the apparent molecular weight from 46 to 50 kDa. HUVEC were stimulated with vehicle, 1 mmol/L 8-br-cAMP or 100 µmol/L 8-pCPT-cGMP for 30 minutes. Western blot analysis for phospho-VASP Ser239 and phospho-VASP Ser157 was performed on cell lysates. Using an antibody specific for VASP phosphorylated at Ser239, HUVEC stimulated with 8-pCPT-cGMP exhibited increased phosphorylation of VASP over control cells and cells stimulated with 8-Br-cAMP. On the other hand, HUVEC stimulated with 8-Br-cAMP displayed increased phosphorylation at Ser157 of VASP compared to HUVEC stimulated with 8-pCPT-cGMP. These results indicate that PKG is present in HUVEC and can be activated by cGMP analogs.

Supplemental Figure 6. Structurally different PDE2 and PDE3 inhibitors have similar effects on endothelial permeability. HUVEC were stimulated with either vehicle, 1U/ml thrombin, 1U/ml thrombin and 0.1 µmol/L MPB-forskolin, thrombin, MPB-forskolin and 0.03 nmol/L ANP with or without PDE3 inhibitors Trequinsin (30 nmol/L) or Cilostamide (10 µmol/L) or thrombin, MPB-forskolin, 30 nmol/L ANP with or without PDE2 inhibitors Bay 60-7550 (100 nmol/L) or EHNA (30 µmol/L). Endothelial permeability was analyzed as described in Methods. Data represent mean of 4 replicates (mean±SEM) from 2 independent experiments using HUVEC isolated from 2 different umbilical cords. Results presented as percent of thrombin maximum.
Supplemental Figure 7. ANP regulates MPB-forskolin-induced cAMP levels in HUVEC via PDE2 and PDE3. The PDE4 inhibitor Rolipram (10 µmol/L) (all samples) and other PDE inhibitors were added 15 min prior to stimulation. A. The PDE3 inhibitor Trequinsin (Treq) 30 nmol/L, PDE2 inhibitor Bay 60-7550 (Bay) 100 nmol/L or both Treq and Bay were added and then HUVEC were stimulated with either: vehicle, 1U/ml thrombin or thrombin plus 0.1 µmol/L MPB-forskolin. After 5 min, cAMP levels were determined by EIA as described in methods. B. The PDE3 inhibitor Trequinsin 30 nmol/L or vehicle was added and then HUVEC were then stimulated with either: vehicle, 1U/ml thrombin or thrombin plus 0.1 µmol/L MPB-forskolin and 0.003-300 nmol/L ANP as in Fig 1A. After 5 min, cAMP levels were determined as in A. C. Same as in B but with Bay 60-7550 100 nmol/L instead of trequinsin. Data represent mean (mean±SEM) from 2 independent experiments using HUVEC isolated from 2 different cords. Results presented as fold increase over control.

Supplemental Figure 8. TNF-α alters whole cell cAMP but not cGMP levels. HUVEC were stimulated with or without 10ng/ml TNF-α for 24 hours after which cells were stimulated with or without 1U/ml Thrombin for 5 min. cAMP and cGMP levels were then measured as described in methods and presented as fold over respective controls. To detect changes in cAMP levels at the whole cell level due to PDE2 activity, PDE3 and PDE4 inhibitors were used. Under conditions in which changes in cAMP due to PDE2 can be seen, Thrombin increased cAMP levels approximately 1.48 fold over control (A). This increase was attenuated in HUVEC stimulated with TNF-α. This suggests that TNF-α, via increasing PDE2 expression, decreases cAMP levels in HUVEC. No significant changes in cGMP levels were seen in thrombin stimulated HUVEC treated with or without TNF-α over controls (B).
References


