

Full Length Research Paper

Different effects of dibutyryl cAMP on monolayer permeability in human aortic and coronary arterial endothelial cells

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Recent studies have demonstrated species- and/or organ-specific differences in the effects of dibutyryl cAMP (DBcAMP) on vascular permeability. In this study, we investigated the permeability of monolayers of human coronary arterial endothelial cells (HCAECs) and human aortic endothelial cells (HAECs) in the presence of DBcAMP using monolayer permeability models of endothelial cells and a double incubation chamber for measurement of FITC-labeled albumin or FITC-dextran (4 kDa) transfer through the monolayer. DBcAMP significantly lowered monolayer permeability in the HCAECs but had no effect in the HAECs when compared with untreated cells ($P < 0.01$). A cAMP-dependent protein kinase (PKA) inhibitor, Rp-8-Br-cAMPS (10^{-5} M), significantly reversed the DBcAMP induced decrease in permeability of the HCAEC monolayer to the level of untreated cells. Rp-8-Br-cAMPS (10^{-5} M) and Sp-cAMPS (a PKA activator) had no significant effect on the permeability of the HAEC monolayer after treatment with DBcAMP. These results indicate that DBcAMP diminishes the permeability of an HCAEC monolayer via a cAMP-mediated PKA pathway but does not do so in HAECs, suggesting that the effects of cAMP on permeability of endothelial cell monolayers are dependent on the origin of the cells.

Key words: Dibutyryl cAMP, endothelial cells, permeability.

INTRODUCTION

The endothelium forms a physical barrier that separates blood from tissue, and the endothelial cells that line blood vessels regulate endothelial barrier function, thereby restricting the direct transport of circulating substances across the endothelial barrier.

Endothelial cell dysfunction, which involves cell membrane damage and increase permeability, is considered to be an early event that subsequently leads to vascular wall disorders. Agents, e.g., as escin (Carrasco et al., 2007), may perturb endothelial cell integrity by directly triggering inflammatory signaling cascades, thereby increasing endothelial cell damage (Tefamariam et al., 2007). Monolayer permeability

models have been established with characteristics of blood vessel endothelial cells. These models are important for the study of intracellular signal transduction pathways that influence monolayer permeability.

Sodium *N*-6, 2'-*O*-dibutyryl adenosine-3', 5'-cyclic phosphate (DBcAMP) is widely used in clinical practice to facilitate wound healing (Zhou et al., 2000). DBcAMP penetrates cell membranes to exert its intracellular effects via 3', 5'-cyclic adenosine monophosphate (cAMP) (Ma et al., 2007; Le Varlet et al., 1995), and almost all of the actions of DBcAMP are mediated by cAMP-dependent mechanisms (Iceta et al., 2006). Intracellular cAMP is a second messenger that is not only an integral part of the signaling pathway but is also important in maintaining normal endothelial barrier function (Borland et al., 2009; Vandenbroucke et al., 2008); it also improves the barrier function of lymphatic microvessels and human microvessels *in vitro*

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(Belkacemi et al., 2008; Price et al., 2008; Wong et al., 2010). However, cAMP does not have the same impact on all endothelial cells and may either increase or decrease monolayer permeability in various cell types. Because the effects of DBcAMP in human endothelial cells have not yet been examined, the current study was aimed to investigate the different effects of DBcAMP on monolayer permeability of human coronary arterial endothelial cells (HCAECs) and human aortic endothelial cells (HAECs).

MATERIALS AND METHODS

Cell culture

Frozen HCAECs and HAECs at 3 passages were obtained from Cambrex Bio Science, Inc. (Walkersville, MD, USA). HCAECs or HAECs at 7 – 8 passages were incubated in endothelial growth medium-2 (EGM-2) within a humidified atmosphere (5% CO₂ + 95% air) at 37°C.

Establishment of endothelial cell monolayer for permeability assays

The incubation culture plates used in the experiments had two chambers, and the base of the upper chamber was a sieve with a 0.45 µm pore size (Intercell, Kurabo Inc. Osaka, Japan). We used 24-well microplates for the lower chamber. Prior to use, the upper chamber plate was coated with 50 µL of 50 µg/mL collagen IV and left to dry overnight in a laminar air-flow cabinet. Cells were detached from culture plates with trypsin/ethylene diamine tetra acetic acid (EDTA), washed once with fresh EGM-2 culture medium, and seeded at a density of 2×10^5 cells per well in 300 µL of EGM-2 culture medium. They were then incubated at a temperature of 37°C in 5% CO₂ + 95% air for 4 days. The EGM-2 culture medium (300 µL) was changed every day during the culture period.

Measurement of endothelial cell permeability

Endothelial cell monolayer permeability was determined by comparing the concentrations of the FITC-labeled albumin or FITC-dextran (4 kDa) between the upper and the lower chamber, as previously described (Chang et al., 2000; Lum et al., 1999). Next, 300 µL of 1% FITC-labeled albumin or 1 mg/ml FITC-dextran EGM-2 culture medium was added to the upper chamber, and 1 mL of EGM-2 culture medium containing 1% bovine serum albumin (BSA) was introduced into the lower chamber. Soon after addition of FITC-labeled albumin, 10^{-3} M – 10^{-7} M of DBcAMP was injected into the upper chamber medium and the cells were incubated for 1 – 4 h. One-hundred µL aliquots were aspirated from the lower chamber and diluted 1:4 with PBS before measurement of the concentration of FITC-labeled albumin or FITC-dextran using a fluorescent spectrophotometer (Fluoro Light FP-3000, Nition Corp., Chiba, Japan) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Diffusive flux (P_o) of FITC-dextran was assessed in these model systems, and the rate of diffusive flux (P_o) was calculated using the following formula:

$$P_o = [(F_A/\Delta t)V_A] / (F_L A)$$

where P_o is measured in centimeters per second, F_A is basolateral

fluorescence, F_L is apical fluorescence, Δt is change in time, A is the surface area of the filter (in square centimeters), and V_A is the volume of the basolateral chamber (in cubic centimeters).

Untreated endothelial cells were considered to be the control, and their obtained value was set at 100%. Pretreatment with 10^{-5} M of cAMP-dependent protein kinase (PKA) inhibitor Rp-8-Br-cAMPS or PKA activator Sp-cAMPS was performed 10 min prior to incubation with 10^{-3} M of DBcAMP for 2 h.

Measurement of intracellular cAMP in endothelial cells

Intracellular cAMP was measured using a Bridge-It cAMP designer fluorescence assay kit (Mediomics, St. Louis, MO, USA) according to the manufacturer's instructions. Briefly, 25,000 cells in 100 µL medium/well were placed on a 96-well culture microplates and incubated overnight at 37°C in 5% CO₂ + 95% air. Cells were treated with DBcAMP for 2 h, and the medium was subsequently carefully removed from the wells. The cells were then washed gently with 100 µL serum-free buffered saline such that the cell layer was not disturbed. Next, the buffer was removed from the attached cells on the 96-well microplate and replaced by cAMP designer assay solution. The microplates were covered with tinfoil to avoid exposure to light, placed on a rotator and gently mixed for 30 min at room temperature. Aliquots of 100 µL from each well were then placed into the corresponding well of a 96-well black polypropylene microplate, and the fluorescence intensity was measured with a fluorescence plate reader at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. The cAMP content in the cultured cells was obtained from a standard curve.

Materials

Plates were purchased from Becton Dickinson (Two Oak Park, Bedford, MA, USA). Other materials were acquired as follows: trypsin/EDTA was obtained from GIBCO Life Technologies (Eggenstein, Germany); collagen IV, FITC-labeled albumin and FITC-dextran (4 kDa) were purchased from Sigma (St. Louis, MO, USA); 96- and 24-well cell culture plates and poly-lysine were obtained from Becton Dickinson Biosciences (Franklin Lakes, NJ, USA); Rp-8-Br-cAMPS and Sp-cAMPS were purchased from Biolog Life Science Institute (Bremen, Germany); milrinone and rolipram were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); and DBcAMP was purchased from Daiichi Pharmaceutical Co. (Tokyo, Japan).

Statistical analysis

All measurements were conducted using two observers blinded to the treatment of the mice. Data were expressed as means ± standard error of the mean (SEM). SPSS version 17.0 as used to conduct the student's *t*-test (for comparison between 2 groups) or 1-way ANOVA (for comparison of 3 or more groups) followed by Tukey's post-hoc test (SPSS Inc, Chicago, Ill). A *P*-value of <0.05 was considered statistically significant.

RESULTS

Effects of DBcAMP on the permeability of HCAEC and HAEC monolayers

FITC-labeled albumin concentrations were considered to

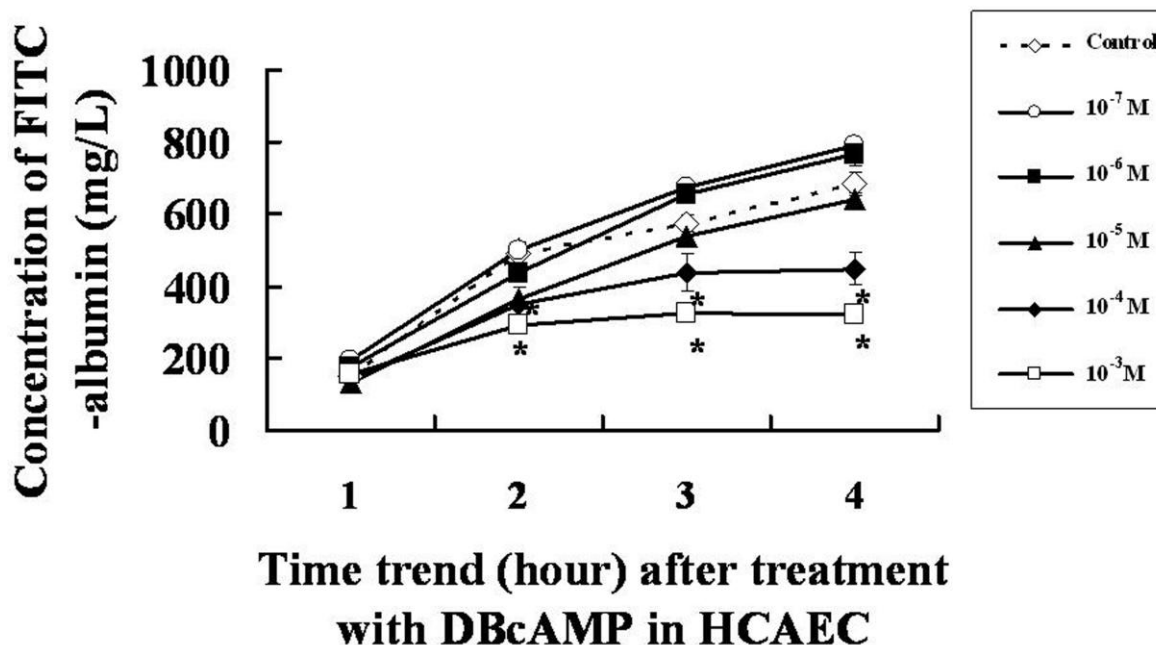


Figure 1. Changes in the FITC-labeled albumin concentration over time in HCAEC monolayers in the presence and absence of DBcAMP. The concentration of FITC-labeled albumin in the lower chamber was measured 1, 2, 3 or 4 h after the introduction of different concentrations of DBcAMP (10^{-7} - 10^{-3} M) into the upper chamber. The fluorescein dextran permeability was determined as described in the materials and methods section. The average P_o for the control samples was 1.23×10^{-4} cm/s. Data are shown as means \pm S.E.; $n = 8$; *: $P < 0.05$, respectively, vs. control cells (no treatment).

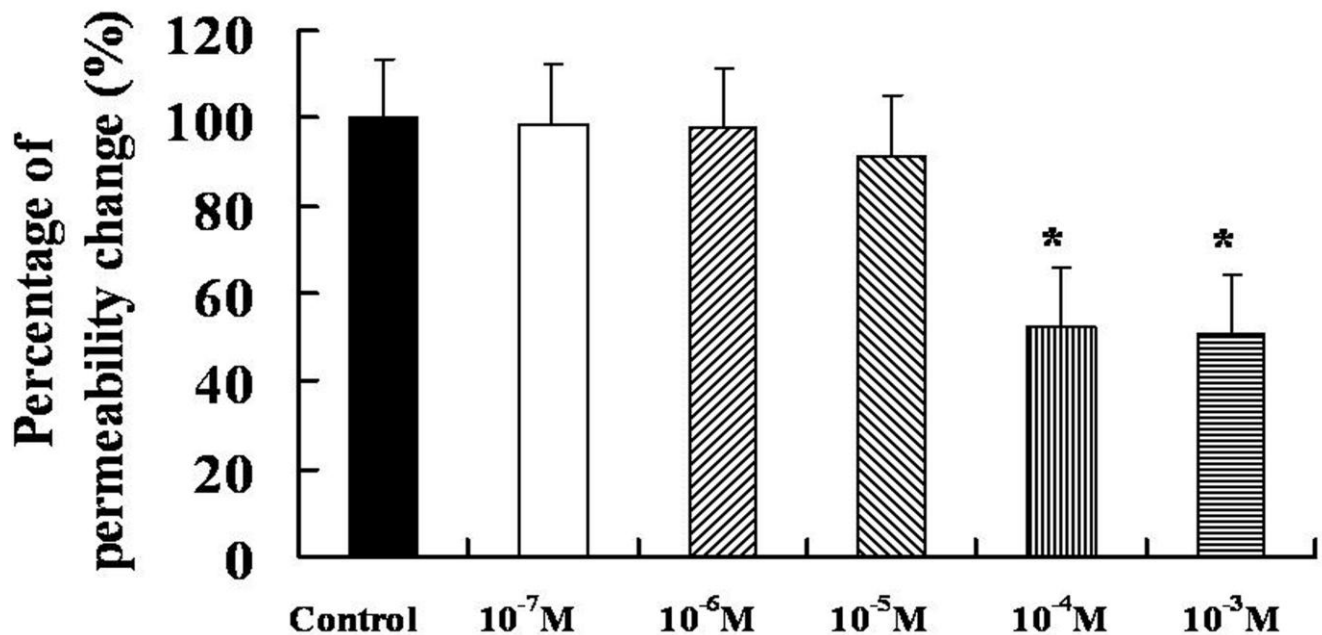
be an index of endothelial cell monolayer permeability. Treatment of HCAEC monolayers with 10^{-3} M DBcAMP significantly lowered their permeability when compared to the control cells (no DBcAMP). The FITC-labeled albumin concentrations obtained after 2, 3 and 4 h were 292.2 ± 20.2 , 322.8 ± 11.4 and 321.6 ± 21.9 mg/L, respectively, with each value being significantly lower than the corresponding values obtained without DBcAMP; they are as follows: 488.0 ± 34.2 , 570.5 ± 28.0 and 683.0 ± 33.7 mg/L, respectively ($P < 0.05$; Figure 1). In contrast, DBcAMP showed no effect on the permeability of HAEC monolayers at any concentration. After pretreatment of HAECs with 10^{-3} M DBcAMP, the FITC-labeled albumin concentrations after 1, 2, 3 and 4 h were 115.8 ± 23.8 , 387.3 ± 32.1 , 474.3 ± 19.4 and 510.6 ± 22.7 mg/L, respectively, and each value was not significantly different from the corresponding values obtained without DBcAMP, which are as follows: 139.8 ± 19.1 , 419.4 ± 21.6 , 516 ± 26.3 and 595.6 ± 36.5 mg/L, respectively (figure not shown). FITC-labeled albumin concentrations in the lower chamber were measured after 2 h incubations with 10^{-7} , 10^{-6} , 10^{-5} , 10^{-4} , and 10^{-3} M DBcAMP as well as the absence of DBcAMP (control). In HCAECs, these concentrations were 500 ± 33.4 , 435.6 ± 14.9 , 404.8 ± 8 , 350.6 ± 46.4 , 292.2 ± 20.2 and 488.0 ± 34.2 mg/L, respectively ($P < 0.05$ vs. control) (Figure 1). In

HAECs, the concentrations were 407.9 ± 26 , 440.3 ± 11.6 , 449.1 ± 20.6 , 372.2 ± 36.3 , 387.3 ± 32.1 and 419.4 ± 21.6 mg/L, respectively. Therefore, pretreatment of HCAEC monolayers with 10^{-4} M or 10^{-3} M DBcAMP significantly decreased permeability when compared to the control condition (figure not shown).

FITC-dextran concentrations were measured after 2 h incubations with 10^{-7} , 10^{-6} , 10^{-5} , 10^{-4} , and 10^{-3} M DBcAMP as well as the absence of DBcAMP (control). In HCAECs, these concentrations were 96 ± 0.8 , 90.3 ± 1.2 , 82.1 ± 1.8 , 74.8 ± 2.1 , 66.2 ± 1.8 and $100.0 \pm 13.6\%$, respectively ($P < 0.05$ vs. control); and in HAECs, concentrations were 96.5 ± 1.7 , 96.3 ± 2 , 89.9 ± 1.2 , 86.4 ± 6.2 , 84.6 ± 1.5 and $100.0 \pm 6.6\%$, respectively. Therefore, pretreatment of HCAEC monolayers with 10^{-4} M or 10^{-3} M DBcAMP significantly decreased permeability when compared to the control condition (Figure 2).

Effects of Rp-8-Br-cAMPS or Sp-cAMPS on the DBcAMP-induced decrease in the permeability of HCAEC and HAEC monolayers

FITC-dextran concentrations were measured after 2 h incubations with the following: 10^{-3} M DBcAMP; 10^{-5} M Rp-8-Br-cAMPS (PKA inhibitor); 10^{-5} M Sp-cAMPS (PKA



Treatment with DBcAMP after 2 hour in HCAEC

Figure 2. Percentage of permeability changes in HCAEC monolayers in the presence and absence of DBcAMP. The concentration of FITC-labeled albumin in the lower chamber was measured 2 h after the introduction of different concentrations of DBcAMP (10^{-7} - 10^{-3} M) into the upper chamber. The percentage of permeability change by FITC-dextran decreased in a dose dependent manner after incubation with different concentrations of DBcAMP for 2 h. Fluorescein dextran permeability was determined as described in the materials and methods section. The average P_o for the control samples was 1.23×10^{-4} cm/s. Data are shown as means \pm S.E.; $n = 8$; *: $P < 0.05$, respectively, vs. control cells (no treatment).

activator); 10^{-3} M DBcAMP following pretreatment with 10^{-5} M Rp-8-Br-cAMPS; 10^{-3} M DBcAMP following pretreatment with 10^{-5} M Sp-cAMPS; and no DBcAMP (control). In HCAECs, the concentrations were $54.7 \pm 7.4\%$, $97.8 \pm 3.1\%$, $91.4 \pm 4.1\%$, $52.5 \pm 8.7\%$, $50.6 \pm 11.6\%$, and $100.0 \pm 13.6\%$, respectively ($P < 0.05$ vs. control); and in HAECs, the concentrations were $80.7 \pm 5.2\%$, $93.7 \pm 2.2\%$, $95.8 \pm 2.5\%$, $83 \pm 7.6\%$, 76.1 ± 5.5 , and $100.0 \pm 6.6\%$, respectively. Therefore, pretreatment of HCAEC monolayers with Sp-cAMPS did not affect the DBcAMP-induced decrease in permeability to the level at which it affected the control (Figure 3).

Effects of DBcAMP, milrinone or rolipram on intracellular cAMP in HCAECs or HAECs

Intracellular cAMP levels were determined 2 h after addition of DBcAMP to the HCAEC and HAEC monolayers (Figure 4). Administration of 10^{-5} , 10^{-4} and 10^{-3} M DBcAMP increased the intracellular cAMP level in HCAECs and HAECs when compared to control cells (no DBcAMP). In HCAECs, cAMP levels were 6.5 ± 1.9 , 17.5 ± 1.5 ($P < 0.01$), and 25.2 ± 1.8 ($P < 0.01$), as compared to 3.2 ± 0.9 pmol/well in controls. In HAECs, cAMP levels were 6.5 ± 1.1 , 10.9 ± 1.8 , and 13.2 ± 2.3 ($P < 0.05$), as compared to 4.5 ± 1.2 pmol/well in controls. Interestingly,

we have observed that HCAECs had higher levels of cAMP induced by DBcAMP at the concentrations of used in this (10^{-5} , 10^{-4} , and 10^{-3} M) than in HAECs. Furthermore, the intracellular cAMP content was measured after the cells were incubated in the absence of DBcAMP (control), with 10^{-3} M DBcAMP, and with 10^{-5} M milrinone or rolipram. The results were as follows: in HCAECs, 3.2 ± 0.9 pmol/well, 25.2 ± 1.8 pmol/well, 19.5 ± 2.7 pmol/well, and 20.5 ± 2 pmol/well, respectively and in HAECs, the results were 4.5 ± 1.2 pmol/well, 13.2 ± 2.3 pmol/well, 12.9 ± 1.7 pmol/well, 14.2 ± 1.5 pmol/well, respectively.

DISCUSSION

Recent studies have demonstrated a heterogeneous influence of cAMP on endothelial barrier function that is dependent on the vascular region, species, and state prior to stimulation (Bindewald et al., 2004; Dejana et al., 2008; Ikeda et al., 1999; Seybold et al., 2005). A direct correlation between the intracellular cAMP level and the increase in monolayer permeability has been reported in rat coronary endothelial cells (a cAMP-dependent increase in permeability), whereas other studies have shown an inverse relationship between cAMP level and the permeability of human umbilical vein endothelial cells

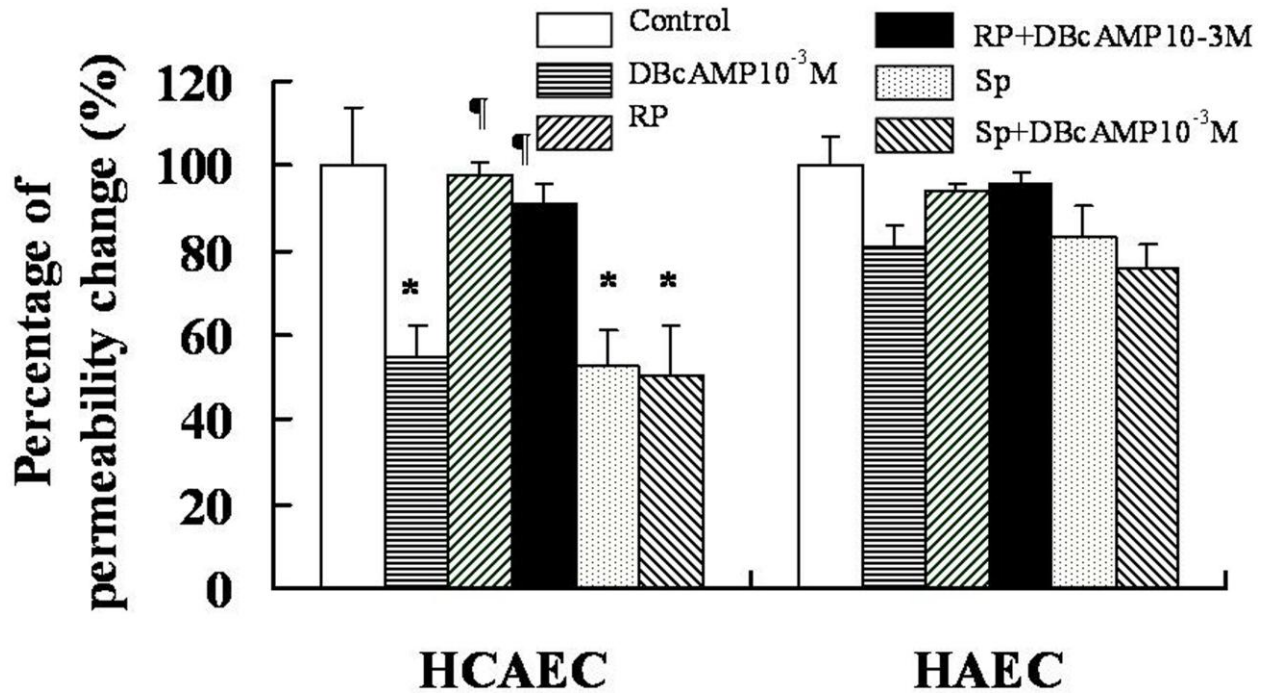


Figure 3. Effect of Rp-8-Br-cAMPS (Rp) and Sp-cAMPS (Sp) on the DBcAMP-induced decrease in permeability of HCAEC and HAEC monolayers. The fluorescein dextran permeability was determined as described in the materials and methods section. The average P_o for the control samples was 1.23×10^{-4} cm/s. Pretreatment with Rp-8-Br-cAMPS inhibited the DBcAMP-induced decrease in HCAECs only, but Sp-cAMPS caused inhibition in two cell-types. Data are presented as means \pm S.E.; $n = 8$; *: $P < 0.05$ vs. control cells (no treatment). †: $P < 0.05$ vs. 10^{-3} M DBcAMP in HCAECs.

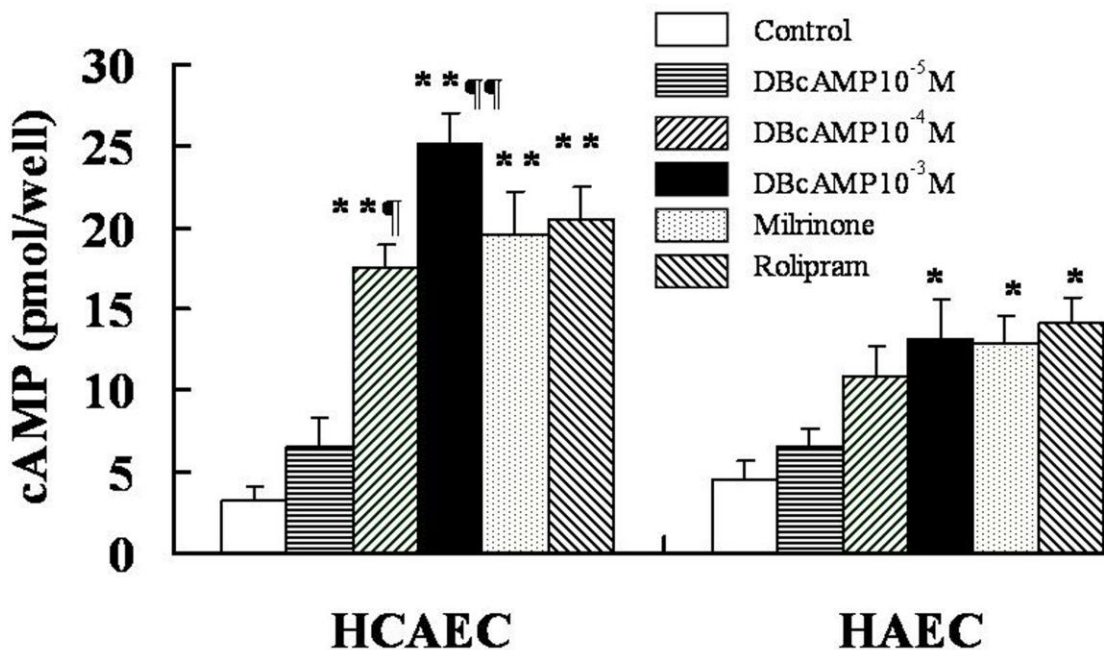


Figure 4. The effects of DBcAMP, milrinone and rolipram on intracellular cAMP in the HCAECs or HAECs. Intracellular cAMP content was measured after the cells were incubated with 10^{-5} , 10^{-4} or 10^{-3} M DBcAMP for 2 h. The intracellular cAMP content was measured after the cells were incubated without DBcAMP (control), with 10^{-3} M DBcAMP, and with 10^{-5} M milrinone or rolipram in HCAECs and HAECs. Data are shown as means \pm S.E.; $n = 8$; * and **: $P < 0.05$ and < 0.01 vs. control cells (no treatment). †: $P < 0.05$ vs. 10^{-4} M DBcAMP; ††: $p < 0.01$ vs. 10^{-3} M DBcAMP in HCAECs.

(HUVECs) (Seybold et al., 2005), porcine aortic endothelial cells (Bindewald et al., 2004) and rat aortic endothelial cells (Nan et al., 2004) (a cAMP-dependent decrease in permeability). The permeability of HAEC monolayers has previously been shown to be unaffected by the cAMP level, and in the present study, DBcAMP had no influence on HAEC monolayers at any concentration. In contrast, DBcAMP dose-dependently decreased the permeability of HCAEC monolayers. When used clinically, blood plasma concentrations reach 4.7 $\mu\text{g/mL}$ at 0.1 mg/kg/min and 10.8 $\mu\text{g/mL}$ at 0.2 mg/kg/min for 60 min after intravenous infusion of DBcAMP, which represents a concentration of approximately $1 - 2 \times 10^{-5}$ M. This is the lowest effective concentration of DBcAMP (10^{-4} M) used in the present study, and only five times higher than that of the concentrations used clinically.

DBcAMP is an analog of cyclic AMP that stimulates cAMP-dependent protein kinase. Research shows that DBcAMP can stimulate the activity of Na^+, K^+ -ATPase (Na^+, K^+ -pump), bring about morphological differentiation of astrocytes, and block the contraction of the vas deferens in rat studies. This study showed that the cAMP concentration induced by DBcAMP is higher in HCAECs compared to HAECs, and a significant elevation in the cAMP level is consistent with a reduction in permeability. cAMP is produced by the enzyme adenylate cyclase from ATP and acts on the intracellular kinase PKA (Kammer et al., 1998). PKA then induces events, including phosphorylation of nuclear transcription factors, e.g., cAMP response element-binding protein, which affects cellular functions, including gene transcription (Bird et al., 2011). We believe that there must be a difference that occurs at some point that results in the induction of cAMP, e.g., the differences between HCAEC and HAEC. However, the definite distinction is still unclear. cAMP levels in the human ovarian carcinoma cell are 5.6 ± 1.1 pmol/mg protein (Parekh et al., 2006). When CD8 cells were stimulated with forskolin, maximal cAMP levels increased by approximately 15-fold (Procino et al., 2004). The approximately 8-fold increase in HCAEC in this study is comparable to that study's 15-fold maximal increase. This suggests that DBcAMP reduces cell-membrane permeability via a cAMP-mediated pathway in HCAECs. Stimulation of cAMP synthesis can increase junctional strands between endothelial cells of intact microvessels, thereby reducing gap formation induced by inflammatory mediators in cells (Shao et al., 2007; Wang et al., 2010). Seybold et al. (2005) reported that thrombin activates endothelial phosphodiesterase, causing a decline in intracellular cAMP levels and an increase in endothelial permeability in HUVECs. Furthermore, Liu et al. (2005) showed that genistein increased the cAMP level and significantly inhibited the thrombin-induced increase in avidin-FITC passage in BAECs, suggesting that genistein may stabilize barrier functions in the cAMP-dependent monolayer permeability system. Other studies have

demonstrated that elevations in intracellular cAMP improves barrier function by decreasing intercellular gap formation, thereby reducing endothelial permeability induced by thrombin (Ke et al., 2007; Patterson, 2007; Qiao et al., 2003).

DBcAMP reduced the permeability of HCAEC monolayers to albumin, and this effect was inhibited by Rp-8-Br-cAMPS, which is a PKA inhibitor. This suggests that DBcAMP inhibits cell-membrane penetration via a cAMP/PKA-mediated pathway in HCAECs. The protective effects of cAMP on inflammatory mediators that induce endothelial cell dysfunction occur primarily through PKA-dependent mechanisms (Patterson, 2007; Qiao et al., 2003), and PKA plays an important role in endothelial cell barrier regulation by modulating the balance between contractile and tethering forces on endothelial cells (Liu et al., 2001; Patterson, 2007). In some endothelial models, it has been reported that stimulation of cAMP/PKA signaling may reorganize the F-actin-based cytoskeleton and stabilize cell adhesion structures (Xu et al., 2008). It is well known that elevation of cAMP and PKA activity induces a decrease in permeability in endothelial cells through RhoA and myosin light chain (MLC) phosphorylation.

Endothelial cell barrier dysfunction is accompanied by F-actin cortical ring dissolution, stress fiber assembly, and intracellular gap formation, which are closely associated with increased MLC phosphorylation (Birukova et al., 2004; Verin et al., 2001), and the phosphorylation of MLC by MLC kinase also plays an important role in endothelial cell junction gap formation and vascular permeability (Patterson, 2007). Increased PKA activity results in the phosphorylation and inactivation of endothelial MLC kinase. This results in MLC dephosphorylation and leads to the inhibition of stress fiber assembly and a reduction in endothelial cell barrier protection (Chilcoat et al., 2008; Garcia et al., 1995). Based on these results, one possible mechanism is that the decreased permeability induced by cAMP may be attributable to the inhibitory action of cAMP/PKA on MLC kinase activity in HCAECs.

Clinically, cAMP plays a role in the control of blood pressure, intracranial pressure, and vascular permeability in vascular lung disease; thus, determining the appropriate cAMP altering drug is important. There are many drugs, including amrinone, milrinone, and olprinone, that increase cAMP. In this context, our results suggest that utilization of certain drugs may induce opposite effects in different cell types via changes in cAMP concentration.

In conclusion, DBcAMP increases the intracellular cAMP concentration in HCAECs, causing myosin relaxation and reducing the gaps between cells, which may lead to decreased monolayer permeability. Such an effect was not observed in HAECs, suggesting that different endothelial cell types respond differently to DBcAMP. Our results demonstrate that monolayer

permeability decreased with an increase in the intracellular cAMP concentration. DBcAMP also increases intracellular cAMP concentration in HAECs; however, the increase was insufficient to decrease monolayer permeability, suggesting that HAECs may not be sensitive to the cAMP/PKA pathway.

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