Role of Phosphodiesterases in the Regulation of Endothelial Permeability In Vitro

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Abstract

Neutrophil-derived hydrogen peroxide (H_2O_2) is believed to play an important role in the pathogenesis of vascular injury and pulmonary edema. H_2O_2 time- and dose-dependently increased the hydraulic conductivity and decreased the selectivity of an endothelial cell monolayer derived from porcine pulmonary arteries. Effects of H_2O_2 on endothelial permeability were completely inhibited by adenylate cyclase activation with 10^{-12} M cholera toxin or 0.1 μM forskolin. 10^{-8} M Sp-cAMPS, a cAMP-dependent protein kinase A agonist, was similarly effective. The phosphodiesterase (PDE) inhibitors motapizone (10^{-4} M), rolipram (10^{-6} M), and zardaverine (10^{-8} M), which specifically inhibit PDE-isoenzymes III, IV, and III/IV potently blocked H_2O_2-induced endothelial permeability when combined with 10^{-6} M prostaglandin E_1. Overall cellular cAMP content and inhibition of H_2O_2 effects on endothelial permeability were poorly correlated. H_2O_2 exposure resulted in a rapid and substantial decrease in endothelial cAMP content. The analysis of the PDE isoenzyme spectrum showed high activities of isoenzymes II, III, and IV in porcine pulmonary endothelial cells. The data suggest that adenylate cyclase activation/PDE inhibition is a powerful approach to block H_2O_2-induced increase in endothelial permeability. This concept appears especially valuable when endothelial PDE isoenzyme pattern and PDE inhibitor profile are matched optimally. (*J. Clin. Invest.* 1993. 91:1421–1428.) Key words: cultured pulmonary endothelial cells • hydraulic conductivity • albumin reflection coefficient • adenylate cyclase activation • phosphodiesterase isoenzymes

Introduction

The endothelium provides a major permeability barrier of the vessel wall. In the course of inflammation, polymorphonuclear leukocytes may damage endothelial cells resulting in an increased vascular permeability. Hydrogen peroxide (H_2O_2) is an important polymorphonuclear leukocyte–derived mediator of endothelial cell injury (1–3). Intracellular events after H_2O_2 exposure are manyfold and include a drop in ATP, NADH, and glutathione levels, an increased hydrolysis of inositol phospholipids, as well as an enhancement of the intracellular Ca^{2+} concentration (4–9). As a result endothelial permeability may increase, possibly caused by Ca^{2+}-mediated endothelial cell contraction (10–12).

Studies using isolated lungs and endothelial cell monolayers grown on filter membranes have shown a protective effect of cAMP-increasing reagents on increased vascular permeability (13–19). Endothelial adenylate cyclase can be stimulated by β-receptor agonists or experimentally by cholera toxin or the diterpene forskolin (20). In addition, 3-isobutyl-1-methylxanthine, an unselective and weak inhibitor of phosphodiesterases (PDE),[1] has been used frequently to increase cellular cAMP content (21).

Five PDE isoenzymes have been identified so far in mammalian tissue (22). The PDE classification is based on substrate specificity and regulation characteristics (22). Selective inhibitors are known for most of the PDE isoenzymes (PDE III selective motapizone, PDE IV selective rolipram, and PDE III/IV dual selective zardaverine) (22–24). PDE isoenzyme pattern differs among tissues and cells. For endothelial cells, the expression of at least two PDE isoenzymes have been described (25, 26). The identification of class-specific PDE inhibitors probably will allow a more efficient intervention in different target tissues in many pathophysiological situations (22, 23).

In the present study, H_2O_2-induced alterations of endothelial cell monolayer permeability were examined. Using this well-characterized system, effects of isoenzyme specific PDE inhibitors were studied. In addition, the PDE isoenzyme pattern in pig pulmonary artery endothelial cells was analyzed and correlated to the functional studies.

Methods

Materials. Tissue culture plasticware was obtained from Becton-Dickinson (Heidelberg, Germany). Medium 199, FCS, HBSS, trypsin-EDTA solution, Hepes, and antibiotics were from Gibco (Karlsruhe, Germany) Collagenase (type CLS II) was purchased from Worthington Biochemical Corp. (Freehold, NJ). H_2O_2 was from Merck (Darmstadt, Germany). Gelatin from porcine skin type I, cholera toxin, forskolin, PGE_1, and catalase were purchased from Sigma Chemical Co., Munich, Germany). The two cyclic adenosine monophosphorothioates Sp-cAMPS and Rp-cAMPS were a gift of Sandoz Research Institute (East Hanover, NJ). 4-(3′-cyclopentyl-oxy-4′-methoxyphenyl)-2-pyrrolidone (rolipram) was kindly provided by Schering AG, (Berlin). 4,5-dihydro-6-(4-(1H-imidazol-1-yl)-2-thienyl)-5-methyl-3-(2H)-pyridazinone (motapizone) and 2-*O*-propoxyphenyl-8-azapurine-6-one (zaprinast) were kindly provided by Rhone-Poulenc Rorer GmbH (Cologne, Germany). 6-(difluormethoxy-3-methoxyphenyl)-3-(2H)-pyridazinone (zardaverine) was synthesized by Byk Gulden GmbH (Konstanz, Germany). ^3H_2O (1 mCi/g) and labeled cyclic nucleotides were obtained from New England Nuclear (Dreieich, Germany). Methyl-^{14}C-albumin (0.026 mCi/mg) and the

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1. *Abbreviations used in this paper:* LDH, lactate dehydrogenase; PDE, phosphodiesterases.

^{125}I-cAMP assay system were from Amersham Buchler (Braunschweig, Germany). Polycarbonate micropore filter membranes (25 mm diameter, 5 μm pore size) were purchased from Nucleopore GmbH (Tübingen, Germany). All other chemicals used were analytical grade.

Botulinum C2 toxin was prepared and activated as reported previously (11). *Staphylococcus aureus* alpha toxin was purified as described by Bhakdi et al. (27).

Preparation of endothelial cells. Pulmonary artery endothelial cells were obtained from freshly slaughtered pigs by exposure to 0.1% collagenase for 12–15 min. Endothelial cells were isolated, characterized, maintained, and dispersed as previously described (28–30).

All studies performed (permeability, cAMP content, PDE activity, lactate dehydrogenase (LDH) release) were done on endothelial cells in their third passage. In addition, all parameters (except PDE activity) were determined on the same batch of cells. There were, however, differences with respect to matrix (cells on polycarbonate membranes, flasks, or well plates).

Endothelial cell monolayers were grown on polycarbonate filter membranes that were first coated with gelatin, exposed to glutaraldehyde, and sterilized as described (10, 11). The coated filter membranes were placed in the bottom of a petri dish, about 2×10^6 cells were seeded on the filter in medium 199/10% FCS and allowed to adhere for 3 h. Additional 10 ml of medium was then added to the petri dish. Thereafter, the monolayers were fed every other day and used ~ 6 d after plating.

Analysis of endothelial permeability. A confluent filter membrane was mounted in a modified chemotaxis chamber. Hydraulic conductivity of cell monolayers was determined as follows: The upper and lower compartments were filled with HBSS supplemented with 0.25% albumin. One port to the upper compartment is for substitution of filtrated volume and for application of pressure and one port is for the addition of reagents. The lower compartment of the system is part of a semiclosed perfusion system (total vol = 10 ml). A roller pump provides a flow of 10 ml/min. The circulating fluid drips into a 1-ml capillary; the height of the fluid level in this capillary directly corresponds to the volume filtrated through the cell monolayer. A filtrated volume of 10 μl can reliably be measured. The entire system is kept at 37°C by a heated water bath. A hydrostatic pressure of 10 cm H_2O was continuously applied to the upper side of the cell monolayer. The filtration rate across the endothelial monolayer was continuously determined (10, 11).

Albumin reflection coefficient. For calculation of this parameter 100 nCi ^3H_2O and 100 nCi ^{14}C-albumin were added to the upper compartment. The amount of ^3H_2O and ^{14}C-albumin in the lower compartment was continuously measured in a radioactivity monitor (Ramona LS-5; Raytest, Heidelberg, Germany) consisting of a flow through cell, a splitter/mixer, and an IBM-PC for data calculation (for details, see references 10 and 11). The albumin reflection coefficient (RC) of the endothelial cell monolayer was calculated every 5 min on the basis of ^3H_2O and ^{14}C-albumin in the upper and lower compartments of the filter system according to the formula: $RC = 1 - (A/B \times D/C)$, as outlined (10, 11). A and C are the differences (in cpm/ml) of ^{14}C-albumin and ^3H_2O, respectively, in the lower compartment of the system between time points x and $x + 5$ min, multiplied by the volume of the lower compartment. B and D are the sums (in cpm/min) of ^{14}C-albumin and ^3H_2O, respectively, in the upper compartment at time points x and $x + 5$ min, divided by 2. Calculation of A/B (C/D) yields the albumin (water) clearance.

Overall cellular cAMP content was measured by a radioimmunoassay using a commercially available ^{125}I assay system as described (24). Endothelials cells were grown in 24-well plates to confluence in complete medium with 20% FCS (about 250,000 cells/well). At the end of the experiments, the medium was aspirated and cells were washed twice with HBSS; buffer contained 0.5 mM isobutyl-methylxanthine to inhibit phosphodiesterase and prevent subsequent cAMP breakdown during cell solubilization and sample processing. Cells were extracted twice with 500 μl ice-cold 65% ethanol. Extracts were evaporated under a stream of nitrogen at 60°C and dissolved in assay buffer. An aliquot of the extracts and of the standards were acetylated by addition of acetic anhydride and triethylamine (1:2) to enhance the sensitivity of cAMP detection.

Release of LDH was used as a marker of cytotoxicity. Endothelial cell monolayers grown on the polycarbonate filter membranes were exposed to 1 mM H_2O_2 for 90 min. The medium was removed and centrifuged with 8,000 g for 2 min. LDH activity in the supernatant was determined by the colorimetric measurement of the reduction of sodium pyruvate in the presence of NADH as described (11). Enzyme release was expressed as the percentage of total enzyme activity liberated from endothelial cells in the presence of 100 μg/ml mellitin (10, 11).

Assay of phosphodiesterase. PDE activity was determined as described (23). A standard reaction mixture containing 40 mM Tris (pH 8.0), 5 mM $MgCl_2$, 0.1 mg/ml BSA and 0.5 μM cyclic nucleotide/^{3}H-labeled cyclic nucleotide (about 50,000 cpm) was used in a total volume of 200 μl. The reaction was initiated by addition of the enzyme solution (5–20 μg) and was carried out at 37°C for 10 min. The reaction was stopped by addition of 50 μl 0.2 M HCl followed by cooling on ice for 10 min. Crotalus atrox snake venom (50 μl; 1 mg/ml in 0.2 M Tris, pH 8.0) was added. After 10 min incubation at 37°C the reaction was stopped on ice. A 0.2 ml aliquot of the assay volume was applied to a small column (Econo; Bio-Rad Labs, Richmond, CA) containing 1 ml QAE-A25-sephadex followed by 2 ml of 30 mM ammonium formate (pH 6.0). The effluent was collected directly in scintillation vials.

Determination of PDE isoenzyme activities. Activities of the five PDE isoenzymes were evaluated directly in the supernatant and particulate fraction of cell homogenates derived from (with respect to cAMP) determinations and permeability studies, passage-matched endothelial cultures. 3.5×10^7 endothelial cells grown in flasks were washed and scraped into buffer A (20 mM Bis-Tris, 5 mM dithioerythritol, 2 mM EGTA, and 50 mM sodium acetate, 50 μM phenyl-methylsulfonyl-fluoride, 5 μM pepstatin, 40 μM leupeptin, 20 μM aprotinin, and 2 mM benzamidine). Cells were disrupted by sonication (Branson Sonifier; Branson Instruments, Danburg, CT) three times for 10 s on ice at a power output of 70 W and the homogenate was centrifuged at 100,000 g for 20 min at 4°C. Supernatant and resuspended pellet (i.e., cytosolic and particulate fractions) were used immediately for PDE assay.

Contributions made by PDE III and IV to total PDE activity were determined by quantifying the inhibition of PDE activity in the presence of 1 μM motapizone and 10 μM rolipram, respectively. PDE I and PDE II fractions of total PDE activity were calculated from the activity increase induced by 50 μM calcium chloride/15 nM calmodulin or 5 μM cGMP, respectively (simultaneous presence of 1 μM motapizone and 10 μM rolipram). PDE V fraction was determined from inhibition induced by 10 μM zaprinast in the presence of 0.5 μM cGMP as substrate. The concentrations of the selective inhibitors that were used blocked > 95% of the activity of the respective PDE isoenzyme.

Experimental protocol. "Sealed" endothelial cell monolayers were used in this study; i.e., monolayers which showed a final hydraulic conductivity of $< 5\text{–}10 \cdot 10^{-6}$ cm · s^{-1} · cm H_2O^{-1} and an albumin RC > 0.65 in the presence of a hydrostatic pressure of 10 cm H_2O. In all experiments, H_2O_2 was added as a bolus into the upper compartment at time 0 and then was infused continuously. Inhibitors were added as a bolus 15 min before H_2O_2 and were then infused continuously into the upper compartment. Possible effects of all reagents added on H_2O_2 concentration were checked and excluded spectrophotometrically at 230 nmM according to Beers and Sizer (31).

Statistical methods. Depending on the number of groups (A) and the number of different time points studied (B), data of Figs. 1–5 and Table I were analyzed by an A × B ANOVA. The data in Table III were analyzed using a one-way ANOVA. Main effects were then compared by an F probability test (32). Otherwise, data were analyzed by Student's t test for unpaired samples. $P < 0.05$ was considered significant.

Results

Effect of H_2O_2 on endothelial permeability. Resting endothelial cell monolayers showed a hydraulic conductivity of 2×10^{-6} $\times$ s^{-1} $\times$ cm H_2O^{-1} and an albumin RC of 0.70–0.75. H_2O_2

increased the hydraulic conductivity of a sealed endothelial cell monolayer dose (0.05–5 mM) and time dependently (15–75 min). This H_2O_2 effect was abolished in the presence of catalase, an enzyme that metabolizes H_2O_2 to O_2 and H_2O (Fig. 1). Addition of H_2O_2 resulted also in a dramatic decrease in the albumin reflecting properties of the endothelial cell monolayer (Fig. 2). 1 mM H_2O_2 was chosen for all subsequent studies using different inhibitors.

Effects of cAMP-modifying reagents. Preincubation of endothelial cell monolayers for 60 min with 1 pM cholera toxin or more made them resistant to a subsequent H_2O_2 exposure while 10^{-13} M cholera toxin only slightly reduced H_2O_2-induced increase in endothelial permeability (Table I). 10–50 nM forskolin reduced and 0.1–1 μm forskolin inhibited the effect of H_2O_2 on endothelial permeability (Table I). These results clearly indicated that an activation of adenylate cyclase antagonizes the permeability increasing effect of H_2O_2.

PGE_1 and isoproterenol, two receptor-operated adenylate cyclase activators, blocked the effects of H_2O_2 on endothelial permeability at 10^{-4} M (not shown). 5 mM of the membrane permeable cAMP analogue dibutyryl-cAMP were also inhibitory in this system (not shown). 10 nM Sp-cAMPS, a cAMP-dependent protein kinase agonist (15), blocked H_2O_2-induced increase in endothelial permeability (Table I). Rp-cAMPS, a putative cAMP-dependent protein kinase antagonist (15), was used as a control to Sp-cAMPS. 100 nM Rp-cAMPS were without effect in the experimental system used.

Effects of PDE inhibitors. Next, PDE III selective motapizone, PDE IV selective rolipram, and PDE III/IV dual selective zardaverine were tested (22–24). The effects of H_2O_2 on endothelial permeability were completely inhibited by 10^{-4} M zardaverine, rolipram, and motapizone (Fig. 3). A 50% reduction of the hydraulic conductivity was noted by 10^{-5} M zardaverine, 10^{-6} M rolipram, and 10^{-5} M motapizone.

The combination of a receptor-operated stimulus (PGE_1) and a PDE inhibitor was tested. When 1 μM PGE_1, which, per se, was slightly inhibitory was used, a complete inhibition of the H_2O_2-induced increase in hydraulic conductivity was noted

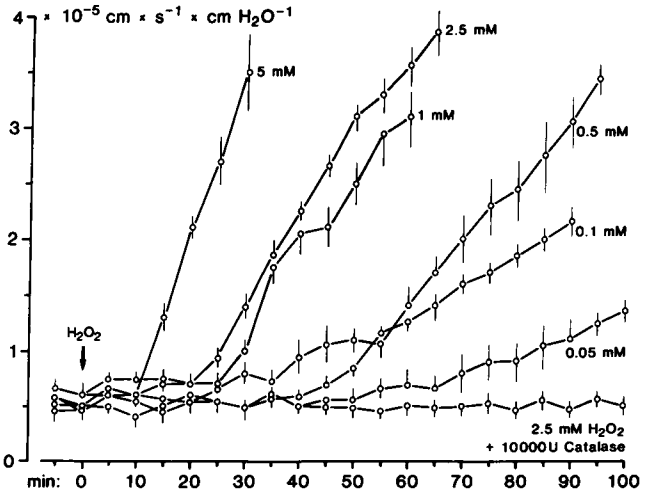

Figure 1. Hydrogen peroxide dose dependently (0.05–5 mM) increased the permeability of endothelial monolayers. Permeability is indicated as hydraulic conductivity ($cm \cdot s^{-1} \cdot cm\ H_2O^{-1}$). H_2O_2 was added as a bolus at 0 min and then infused continuously. Catalase that detoxifies H_2O_2 was added one minute before H_2O_2. Data presented are mean±SE of six separate studies.

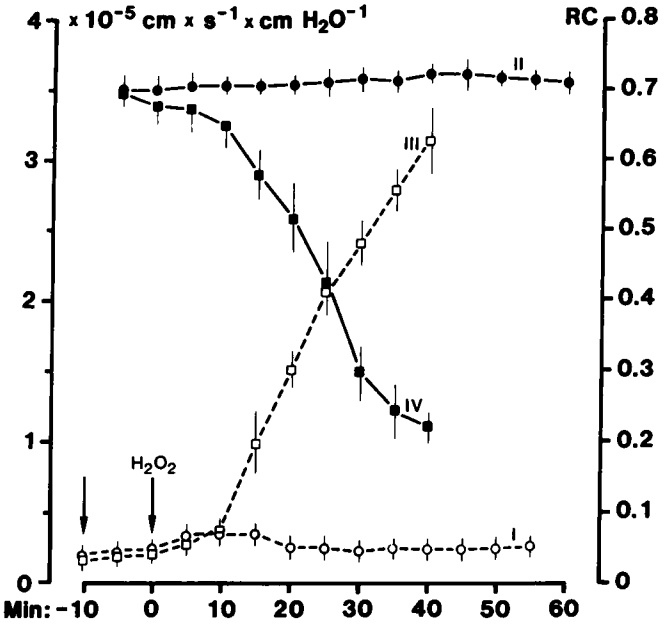

Figure 2. Resting endothelial monolayer had a hydraulic conductivity of 2×10^{-6} $cm \cdot s^{-1} \cdot cm\ H_2O^{-1}$ and an albumin reflection coefficient (*RC*) of 0.7. H_2O_2 (1 mM) increased Hydrolyzing Capacity (*III*) and decreased RC (*IV*). 10^{-6} M PGE_1 plus 10^{-7} M zardaverine blocked the effects of H_2O_2 on Hydrolyzing Capacity (*I*) and RC (*II*). Data presented are mean±SE of four separate experiments.

in the presence of 10^{-8} M zardaverine, 10^{-6} M rolipram, or 10^{-4} M motapizone (Fig. 4). Thus, PGE_1 increased the effectiveness of zardaverine by a factor of 10,000 and of rolipram by a factor of 100, while the effectiveness of motapizone remained unchanged. When 10^{-7} M PGE_1 was used zardaverine was completely inhibitory at 10^{-7} M (instead of 10^{-8} M) (data not shown).

The combination of 10^{-7} M zardaverine and 10^{-6} M PGE_1 prevented the H_2O_2-induced increase in hydraulic conductivity (Fig. 4 and Fig. 2, line *1*) and, in addition, the H_2O_2-induced decrease in the albumin reflection coefficient (Fig. 2, line *2*).

An increased permeability was noted at low concentrations of rolipram (10^{-7} M), zardaverine (10^{-6} M), or zardaverine (10^{-9} M)/PGE_1. This phenomenon may be related to the known protein kinase A induced phosphorylation and activation of PDE III (33, 34); in the presence of low concentrations of a PDE IV or PDE III/IV inhibitor the activity of the cAMP-dependent protein kinase will increase. PDE III, one target of this reaction, will increase its activity, thereby enhancing overall endothelial cAMP hydrolyzing capacity. Thus, it is conceivable that over a narrow concentration range of PDE-inhibitors cAMP decreases and permeability increases "paradoxically."

Zardaverine plus PGE_1 (10^{-6} M, each) were effective only when added 5 or 2.5 min before H_2O_2 addition (Fig. 5). A slight reduction in H_2O_2-induced endothelial permeability was noted when H_2O_2 and treatment (10^{-6} M zardaverine and 10^{-6} M PGE_1) were added simultaneously (Fig. 5, line *3*). No "therapeutic" intervention was possible after H_2O_2 addition.

An increase in endothelial permeability provoked by 10 μg/ml *S. aureus* alpha toxin, a transmembrane channel-forming protein (27), or by 100 ng/ml botulinum C2 toxin, an inhibitor of actin polymerization (11), was not blocked by rolipram at a concentration that reduced H_2O_2-induced increases in hydraulic conductivity (data not shown).

Table I. Modification of H_2O_2-induced Increase in Endothelial Monolayer Permeability by Cholera Toxin (CHT), Forskolin (FSK), Sp-cAMPS, and Rp-cAMPS

Reagents added	Time			
	0	20	40	60
			min	
No additions	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
H_2O_2 (1 mM)	0.8 ± 0.2	2.3 ± 0.4	4.6 ± 0.5	6.0 ± 0.7
H_2O_2 + CHT 0.1 pM	0.6 ± 0.2	1.3 ± 0.3	3.6 ± 0.7	5.1 ± 0.6
H_2O_2 + CHT 1.0 pM*	0.5 ± 0.1	0.7 ± 0.2	0.7 ± 0.2	0.8 ± 0.2
H_2O_2 + CHT 10.0 pM*	0.4 ± 0.1	0.3 ± 0.2	0.3 ± 0.2	0.2 ± 0.1
H_2O_2 (1 mM)	0.8 ± 0.3	1.7 ± 0.3	3.8 ± 0.4	5.8 ± 0.4
H_2O_2 + FSK 10 nM*	0.7 ± 0.1	1.7 ± 0.2	2.0 ± 0.3	4.1 ± 0.7
H_2O_2 + FSK 50 nM*	0.6 ± 0.1	1.3 ± 0.2	1.5 ± 0.3	1.4 ± 0.4
H_2O_2 + FSK 100 nM*	0.5 ± 0.1	0.7 ± 0.2	0.7 ± 0.2	0.7 ± 0.2
H_2O_2 + FSK 1.0 μM*	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.2
H_2O_2 (1 mM)	0.8 ± 0.2	1.7 ± 0.4	3.8 ± 0.3	6.0 ± 0.4
H_2O_2 + Sp-cA 1 nM	0.4 ± 0.1	0.8 ± 0.3	3.9 ± 0.5	6.1 ± 0.8
H_2O_2 + Sp-cA 5 nM*	0.7 ± 0.1	1.5 ± 0.3	2.7 ± 0.3	3.0 ± 0.8
H_2O_2 + Sp-cA 10 nM*	0.4 ± 0.1	0.5 ± 0.1	0.8 ± 0.2	1.0 ± 0.3
H_2O_2 + Sp-cA 100 nM*	0.2 ± 0.1	0.2 ± 0.2	0.4 ± 0.1	0.3 ± 0.1
H_2O_2 + Rp-cA 100 nM	0.6 ± 0.1	1.2 ± 0.3	2.6 ± 0.4	5.5 ± 0.4

Data represented are hydraulic conductivities (10^{-5}cm · s^{-1} · cm H_2O^{-1}). Cells were preincubated with different reagents for 15 min (60 min for cholera toxin). * Indicates significant difference of this group to cells treated with H_2O_2 alone.

Next, we examined the effect of adenylate cyclase activation/PDE-inhibition on endothelial cytolysis: LDH-release of control cells (no H_2O_2, 0.1 μM forskolin alone, 1 μM PGE1/1 μM zardaverine alone) was < 5% of total cellular LDH at 1, 3, and 6 h. LDH release in the presence of 1 mM H_2O_2 amounted to 4%, 63%, and 95% at 1, 3, and 6 h. LDH data in the H_2O_2 + 0.1 μM forskolin group were 3%, 31%, and 91%; LDH data in the H_2O_2 + 1 μM PGE1/1 μM zardaverine group were 3%,

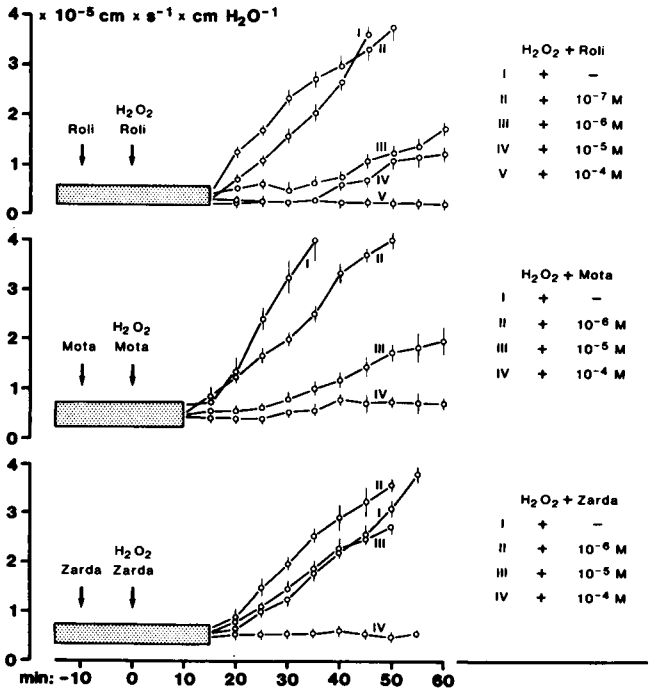

Figure 3. Modification of H_2O_2-induced increase in endothelial permeability by reagents that selectively inhibit different PDE isoenzymes. Rolipram (PDE IV), motapizone (PDE III), and zardaverine (PDE III/IV) were used at 10^{-4}–10^{-7} M. All PDE inhibitors blocked H_2O_2 effects at 10^{-4} M. Data presented are mean±SE of at least four separate experiments.

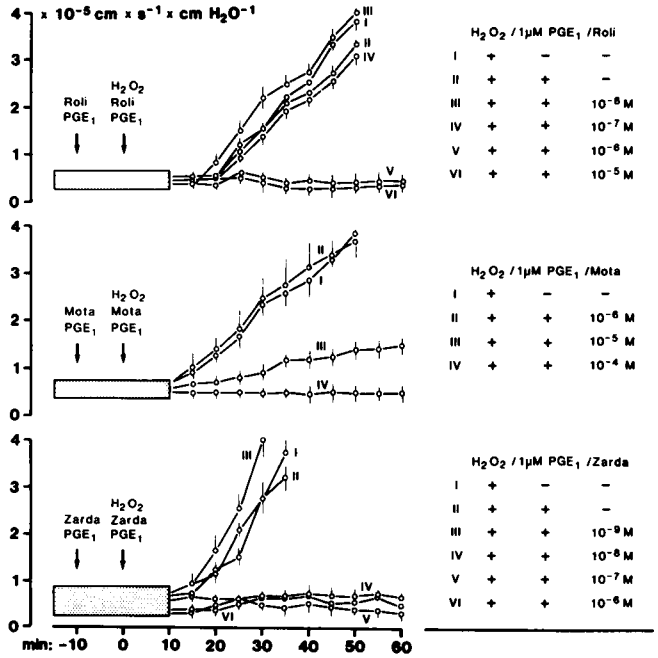

Figure 4. Simultaneous adenylate cyclase activation and PDE inhibition potently blocked H_2O_2-induced increase in endothelial permeability. Cell monolayers were incubated with 10^{-6} M PGE_1 plus one of the PDE inhibitors. 10^{-6} M PGE_1 alone only had a small effect in this system. In the presence of PGE_1, 10^{-8} M zardaverine, 10^{-6} M rolipram, or 10^{-4} M motapizone completely blocked H_2O_2-related enhanced endothelial permeability. Data presented are mean±SE of at least four separate experiments.

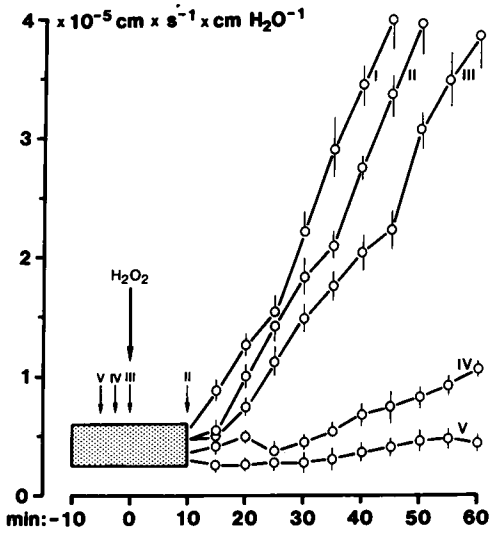

Figure 5. Pretreatment versus posttreatment: PGE_1 and rolipram (10^{-6} M each) were added to the endothelial cell monolayer 5 min before (*V*), 2,5 min before (*IV*), simultaneously with (*III*), or 10 min after (*II*) addition of 1 mM H_2O_2 (*I* = H_2O_2 alone). Simultaneous addition of stimulus and "therapy" slightly reduced H_2O_2-induced enhanced endothelial permeability. Pretreatment was very effective, posttreatment almost without effect in this system. Data presented are mean±SE of at least three separate experiments.

Table II. cAMP Content of Pulmonary Artery Endothelial Cells after Incubation with Different cAMP-modifying Reagents

Reagents added	Time	cAMP	*n*
	min	*pmol*	
None	10	4.35±0.6	10
Zardaverine (100 μM)	10	5.12±0.6	5
Motapizone (100 μM)	10	4.74±0.7	4
Rolipram (100 μM)	10	5.76±1.7	4
PGE_1 (1 μM)	10	5.50±1.3	6
PGE_1 + zardaverine (1 + 100 μM)	10	7.78±1.2*	8
PGE_1 + motapizone (1 + 100 μM)	10	8.51±2.2*	4
PGE_1 + rolipram (1 + 100 μM)	10	7.33±2.4*	4
Forskolin (1 μM)	10	33.28±2.5*	4
Forskolin (0.1 μM)	10	33.60±5.2*	3
Forskolin (0.01 μM)	10	14.40±3.0*	3
Cholera toxin (1 nM)	60	39.36±1.8*	4
Cholera toxin (1 pM)	60	12.80±1.9*	2
Cholera toxin (0.1 pM)	60	6.72±1.4	2

Data given are pmol cAMP/10^6 cells, mean±SE. * Indicates significant difference to untreated cells ($P < 0.05$).

13%, and 96% (SE of LDH data always < 10%, $n = 2$). Thus, adenylate cyclase activation/PDE-inhibition appears to delay (for 1–2 h) but does not prevent H_2O_2-induced endothelial cytolysis.

Endothelial cAMP content. Zardaverine, rolipram, and motapizone (10^{-4} M) did not increase endothelial cAMP content significantly. 1 μM PGE_1 combined with 100 μM of the PDE inhibitors tested nearly doubled endothelial cAMP (Table II). At 1 μM PGE_1 plus low concentrations of PDE inhibitors (≤ 10^{-6} M) endothelial cAMP content remained unchanged (not shown). Thus, PGE_1 plus PDE inhibitor reduced H_2O_2-related increases in endothelial permeability at concentrations which did not increase overall cellular cAMP. Forskolin (10^{-6}–10^{-8} M) and choleratoxin (10^{-9}–10^{-12} M) drastically increased endothelial cAMP concentration from 4.3 to 40 pmol/10^6 cells.

Exposure of endothelial cells to H_2O_2 resulted in a rapid decline of cAMP content, which was significant after 5 min (Table III). Within 10–15 min, cAMP decreased by > 50%. High endothelial cAMP content after preincubation with forskolin fell with similar kinetics after H_2O_2 exposure (Table III). Pretreatment of endothelial cells with PGE_1 and zardaverine did also not slow down the H_2O_2 induced cAMP fall. The relative rate of cAMP fall was even increased in the cholera toxin/H_2O_2 group (Table III).

PDE isoenzymes in porcine pulmonary endothelial cells. The PDE isoenzyme profile of porcine pulmonary artery endothelial cells was analyzed in cytosolic and particulate fractions of cell homogenates. Activity levels of each isoenzyme were quantitated using the selective inhibitors motapizone (PDE III), rolipram (PDE IV), zardaverine (PDE III + IV), zaprinast (PDE V), and the allosteric effectors cGMP (PDE II) and Ca^{2+}/calmodulin (PDE I).

Concentration inhibition curves by zardaverine were monophasic, whereas those of motapizone and rolipram were biphasic indicating the presence of PDE III and IV (Fig. 6). Two-site analysis of the data of the cytosolic fraction revealed an IC50 value for PDE III and IV of 9 nM and 42 μM (13 nM and 65 μM in particulate fraction) for motapizone and of 83 μM and 93 nM (110 μM and 110 nM in particulate fraction) for rolipram, respectively (Fig. 6). IC50 for zardaverine was 0.3 μM (0.5 μM) in cytosolic (particulate) fraction (Fig. 6). Zaprinast

Table III. cAMP Content of Endothelial Cells Incubated with H_2O_2 and cAMP-modifying Reagents

Reagents added	Time				
	0	5	15	30	60
			min		
H_2O_2 (1 mM); $n = 6$	4.9	3.3	2.2	1.7	1.2
	±0.3	0.2	0.2	0.1	0.1
Percent of initial		67	45	35	24
0.1 nM cholera toxin for	38.4	23.7	12.8	5.1	ND
60 min, then H_2O_2; $n = 3$	±1.9	1.0	1.9	1.4	ND
Percent of initial		62	33	13	
0.1 μM forskolin for	19.8	14.1	11.4	5.3	ND
5 min, then H_2O_2; $n = 2$	±0.9	0.9	0.4	2.4	ND
Percent of initial		71	58	27	
PGE_1 + zardaverine (1 + 100 μM)	6.0	4.2	2.5	1.4	1.1
10 min, then H_2O_2; $n = 5$	±0.6	0.5	0.4	0.2	0.1
Percent of initial		70	42	23	18

Data indicate a substantial fall of cAMP in endothelial cells exposed to 1 mM H_2O_2. The cAMP-drop is significant ($P < 0.05$) for all groups at 5 min and later. ND, not determined. Data presented are pmol cAMP/10^6 cells (mean±SE). The relative rate of cAMP fall is significantly enhanced in the cholera toxin/H_2O_2 group as compared to H_2O_2 alone.

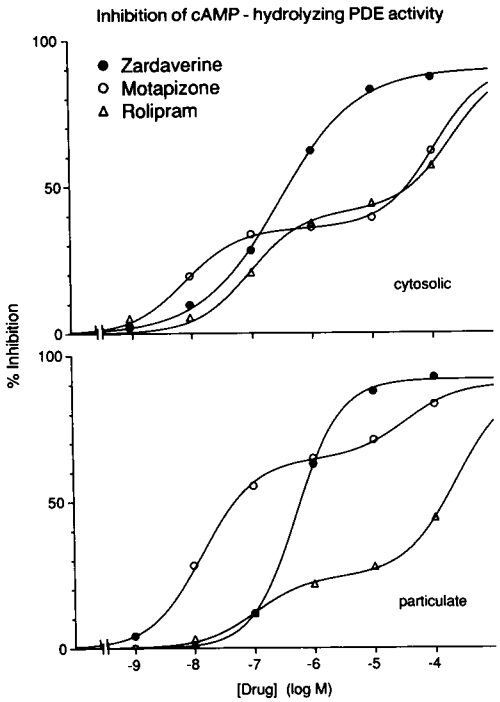

Figure 6. Analysis of PDE isoenzyme specific IC50 values for three different PDE inhibitors. Basal cAMP-hydrolyzing activity present in cytosolic and particulate fractions of endothelial cell lysates was assayed in the presence of 0.5 μM cAMP as substrate. Inhibition of this activity was determined in the presence of increasing concentrations (10^{-9}–10^{-4} M) of zardaverine, motapizone, and rolipram. Curves were analysed by linear regression using a normal (for zardaverine) or a two-site fitting model (for motapizone and rolipram). See Results for calculated IC50 values. Data given are the result of one (out of three) representative experiment.

inhibited cGMP-selective PDE V with an IC50 of 0.3 μM (not shown).

The contribution of each PDE isoenzyme made to overall basal cAMP-hydrolyzing activity (3.7 nmol/min per 10^8 cells in the presence of 0.5 μM substrate) was evaluated (Table IV): PDE III and IV activities amounted to 1.6 and 1.4 nmol/min per 10^8 cells. The combination of motapizone and rolipram inhibited the same fraction of basal activity as did zardaverine. Addition of 5 μM cGMP to cell homogenates which contained rolipram and motapizone or zardaverine almost completely restored cAMP hydrolyzing activity indicating the presence of a cGMP-stimulated PDE that has been termed PDE II, as outlined in Beavo (22). PDE II was the isoenzyme with the highest activity (2.3 nmol/min per 10^8 cells). No selective PDE II inhibitors are known. Ca^{2+}/calmodulin did not stimulate and zaprinast, a selective PDE V inhibitor, did not reduce cAMP or cGMP hydrolyzing activity, suggesting that porcine pulmonary artery endothelial cells contained negible amounts of PDE subtypes I and V.

Fig. 7, which is based on data of Table IV, presents the distribution of PDE isoenzymes in porcine pulmonary artery endothelial cells. PDE III appeared to be located preferentially in the particulate compartment (74%), whereas PDE IV was localized predominantly in the cytosol (62%).

Discussion

Hydrogen peroxide (H_2O_2) proved to be a potent mediator of enhanced endothelial permeability. It increased the porosity

Table IV. PDE Isoenzyme Activities in Cytosolic and Particulate Fractions of Endothelial Cell Homogenates

Reagents added	Activity	
	Cytosol	Particulate
	pmol substrate/min per 10^8 cells	
^{3}H-cAMP	1,719±77	2,008±187
+ Rolipram 10 μM	825±386	1,481±157
+ Motapizone 1 μM	1,296±130	810±303
+ Rolipram + motapizone	312±95	266±126
+ Zardaverine 10 μM	274±74	291±186
+ Rolipram + motapizone + cGMP 5 μM	1,940±103	967±49
+ Rolipram + motapizone + Ca^{2+}/CaM	421±57	186±34
^{3}H-cGMP	923±278	680±114
+ Zaprinast 10 μM	884±231	652±141

Basal PDE activity was assayed in the presence of 0.5 μM cAMP (PDE I-IV) or cGMP (PDE V) as substrates. Activation by cGMP or Ca^{2+}/calmodulin (CaM) for quantitation of PDE II or I was done while PDE III and IV were blocked by the simultaneous presence of rolipram and motapizone. Data are mean±SE of three separate experiments.

and decreased the selectivity of an endothelial monolayer in a time- and dose-dependent manner. A similar oxidant-induced increase in albumin transfer across cultured endothelium was noted previously using xanthine or glucose oxidase as a source of reactive oxygen species (35, 36). Leukocyte-derived H_2O_2 in a subcytolytic concentration apparently can, besides alterations of endothelial permeability, induce a broad range of metabolic and functional alterations in endothelial cells including stimulation of arachidonate metabolism, DNA strand break formation, potassium-efflux, etc. (1–5).

In the present study, endothelial permeability was investigated in the presence of a well defined transendothelial pressure gradient (10, 11). Under these conditions, resting endothelial cell monolayers displayed a hydraulic conductivity of $2 \cdot 10^{-6}$ cm · s^{-1} · cm H_2O^{-1} and an albumin reflection coefficient of more than 0.7; i.e., the permeability characteristics of our in vitro system are close to the in situ situation.

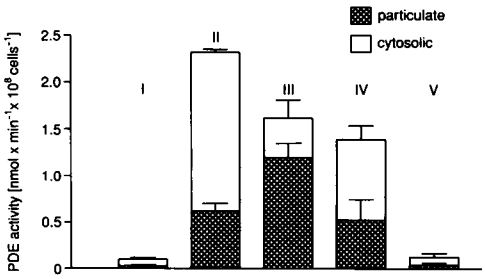

Figure 7. PDE isoenzyme activities in the cytosolic and particulate (criss-cross) fraction of porcine pulmonary artery endothelial cells were calculated from the inhibition induced by 1 μM motapizone, 10 μM rolipram, and 10 μM zaprinast. PDE III and IV were inhibited by the simultaneous presence of motapizone/rolipram to determine PDE II activity by activation with 5 μM cGMP and PDE I activity by activation with Ca^{2+}/calmodulin. PDE isoenzyme activities were determined in the presence of 0.5 μM cyclic nucleotides cAMP (PDE I–IV) and cGMP (PDE V). Data given are mean±SE of three separate experiments.

Activation of adenylate cyclase suppressed H_2O_2-induced endothelial permeability. Cholera toxin and forskolin were effective at extremely low (10^{-12} and 5×10^{-8} M) concentrations. The role of cAMP-dependent effects in regulating endothelial permeability was strengthened by studies with PGE_1, a receptor-operated adenylate cyclase activator, and with Sp-cAMPS, a cAMP-dependent protein kinase agonist. Our observations extend the demonstrated improvement of barrier properties in resting endothelial monolayers using norepinephrine, isoproterenol, or iloprost as cyclase activators (13–15).

PDE inhibition with motapizone, rolipram, or zardaverine blocked H_2O_2-related endothelial permeability at high concentrations (10^{-4} M), suggesting a low basal adenylate cyclase activity in these cells. As anticipated simultaneous adenylate cyclase activation/PDE-inhibition proved to be very potent. In the presence of 1 μM PGE_1, the effectiveness of zardaverine and rolipram increased 10,000 and 100 times, respectively, whereas that of motapizone remained unchanged. Thus, a maximum effect was observed when both isoenzymes (PDE III and IV) were blocked, suggesting that PDE III and IV were actively hydrolysing cAMP under the conditions used, whereas PDE II seemed to be inactive.

An analysis of the PDE isoenzymes in porcine pulmonary artery endothelial cells showed high activities of PDE II, III, and IV. Two previous studies reported on the preparation and identification of PDE isoenzyme II and IV in bovine and porcine aortic endothelial cells (25, 26). Most of the cAMP hydrolytic activity was, similar to the present study, detected in the cytosolic fraction. The identification of PDE III in endothelial cells represents a novel observation. Three quarters of this isoenzyme was detected in the particulate fraction. The discrepancy to previous studies may relate to analytical differences. Our approach used is fast, circumvents much of the proteolytic degradation, and compares isoenzyme activities in the cytosolic and particulate compartment at 0.5 μM as a substrate concentration.

Adenylate cyclase activation/PDE inhibition potently blocked H_2O_2-related enhanced endothelial permeability. This concept was especially effective when endothelial PDE isoenzyme pattern and profile of PDE inhibitors used were matched. While this concept appears clear, a straight correlation between endothelial cAMP-levels and protection against H_2O_2-related enhanced endothelial permeability was missing. Endothelial permeability was low and cAMP high in the presence of cholera toxin or forskolin. Zardaverine and PGE_1, however, were protective at concentrations that did not increase detectable cAMP. This poor correlation is probably caused by determination of overall cellular cAMP content. Dependent on intracellular PDE distribution and on mode of adenylate cyclase activation high local subcellular cAMP peaks are likely to occur, as has already been demonstrated in microwave-fixed cells (37). These cAMP peaks probably are easily missed by global cAMP determinations in cell lysates.

Matters are further complicated by the fact that cAMP levels dropped in H_2O_2-exposed endothelial cells by > 50% within 15 min, suggesting that an early cAMP peak is sufficient for providing protection against a H_2O_2 bolus. The observation of a H_2O_2-induced cAMP decrease made is new but not surprising, since a rapid ATP-fall is a well known phenomenon in H_2O_2-poisoned cells (7, 36, 38, 39). To our opinion, the low substrate concentration most likely explains the cAMP decrease in H_2O_2-exposed cells.

The mechanisms of the protection against H_2O_2-related enhanced endothelial permeability by adenylate cyclase activation/PDE-inhibition are not clear; an answer relates to possible mechanisms of H_2O_2-induced cell injury. Exposure of endothelial cells to H_2O_2 results in a complex metabolic response including a drop in ATP and NADP, an activation of the glutathione cycle, an increased hydrolysis of inositol phospholipids, and an increase in intracellular free Ca^{2+} concentration (1–9, 35, 36, 38). This Ca^{2+} signal most likely induces a Ca^{2+}-dependent actin/myosin-based endothelial cell contraction with subsequent interendothelial gap formation. Considerable evidence has been accumulated in support of this sequence (10–12, 40).

Adenylate cyclase activation/PDE inhibition with subsequent stimulation of protein kinase A favors cell relaxation probably by decreasing the intracellular Ca^{2+} signal and by reducing myosin light chain kinase activity (41, 42). Failure of PDE inhibitors to block the effects of staphylococcal alpha toxin on endothelial permeability would be compatible with this notion because intracellular Ca^{2+} probably cannot be decreased in cells carrying a high number of toxin-induced membrane pores. On the other hand, Carson et al. (18) did not note a reduction of the histamine-induced Ca^{2+} signal in theophylline or forskolin exposed umbilical vein endothelial cells suggesting that cAMP-related Ca^{2+} handling may be different between cells. Additional studies examining intracellular Ca^{2+}, protein kinase C activity, phospholipid hydrolysis, cytoskeleton, etc., will hopefully resolve the mechanisms of the therapeutic approach presented.

PMN-mediated vascular injury and oxidant-induced pulmonary edema are prominent features of the adult respiratory distress syndrome. Adenylate cyclase activation/PDE inhibition proved useful in blocking H_2O_2-related enhanced endothelial permeability in vitro. Further studies in more integrated systems are required to test this concept and to determine the optimal match between endothelial PDE isoenzyme pattern and PDE inhibitor profiles.

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