Phosphodiesterase 3 activity is reduced in dog lung following pacing-induced heart failure

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IN HUMANS WITH CONGESTIVE HEART FAILURE (CHF), the lung undergoes important adaptations such that these patients can tolerate the high pulmonary pressures that accompany CHF without the development of alveolar edema and respiratory distress (37). In a canine model of CHF induced by rapid ventricular pacing, Townsley et al. (45) observed a similar phenomenon: the incidence of alveolar edema was low despite significant pulmonary hypertension. This protective adaptation in CHF could be due to structural alterations in the capillary exchange barrier and/or to changes in specific intracellular signaling pathways that regulate endothelial barrier function. Thickening of the alveolar-capillary membrane is known to occur in various animal models of CHF (11, 45). Nonetheless, ablated permeability responses to specific agonists such as angiotensin II, thapsigargin, and 5,6-epoxyeicosatrienoic acid after CHF in the pacing model (13, 28) suggest an important contribution from adaptive changes in intraendothelial signaling pathways.

cAMP is an important signaling molecule that modulates endothelial permeability in the lung, and the pulmonary endothelial permeability response to injurious stimuli is reduced by agents that increase intracellular cAMP (14, 19). For example, inhibitors of cAMP hydrolysis have been shown to reverse increases in endothelial permeability induced by ischemia-reperfusion in the perfused rat lung model (3). cAMP has also been shown to reduce endothelial permeability in frog and rat mesenteric vessels (10) and increase the number of tight junction strands between endothelial cells (1). Cyclic nucleotides, including cAMP, are hydrolyzed by phosphodiesterases (PDEs), of which there are at least 11 families (4, 18, 24), each having their own specific physical and kinetic properties. More specifically PDE3, PDE4, and PDE5 predominate in the lung (25), providing mechanisms for hydrolysis of both cAMP and cGMP (26, 48). There is also evidence for PDE2 expression in pulmonary vascular smooth muscle (9). PDE isoforms have also been characterized in pulmonary endothelial and smooth muscle cells in culture (2, 43). Because an increase in cAMP is known to reduce endothelial injury responses in normal lung and intracellular cAMP can be modulated by PDEs, we hypothesized that the blunted response to endothelial injury observed in lungs isolated from paced dogs in CHF occurs as a result of increased intracellular cAMP, secondary to modulation of cAMP hydrolysis by PDEs. Our speculation was that CHF resulted in decreased activity and/or expression of cAMP-specific PDEs in the lung. Although changes in cardiac PDE expression and activity have been evaluated during CHF (30, 34, 47), changes in pulmonary PDE expression have not been studied.

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METHODS

Pacing Model of Heart Failure

Briefly, conditioned, microfilaria-negative dogs were anesthetized with pentobarbital sodium (30 mg/kg iv) for insertion of a transvenous pacing lead (Medtronic) into the right ventricle via the right jugular vein. A programmable generator was inserted subcutaneously anterior to the first rib and attached to the pacing lead. Several days postsurgery, pacing was induced at a rate of 245 beats/min. Pacing was continued for ~28 days or until the left ventricular shortening fraction (LVSF), measured via echocardiography in sinus rhythm, was reduced by ~50% from baseline. At this time point, in our hands, pacing results in a significant decrease in LVSF as well as significant increases in left ventricular end diastolic pressure, pulmonary arterial pressure, and pulmonary capillary wedge pressure (12, 13, 28, 45).

Lung Tissue Harvest

Control dogs and paced dogs in CHF were anesthetized with pentobarbital sodium (30 mg/kg iv in controls; <15 mg/kg iv in paced dogs) for a terminal experiment. The chest was opened at the fifth intercostal space, and the lung was exposed. Small pieces (250–500 mg) were sectioned from the peripheral lung parenchyma, snap-frozen in liquid nitrogen, and stored at −70°C.

DEAE-Trisacryl Fractionation of PDEs

Lung tissue was homogenized on ice in 10 vol of homogenization buffer (4 mM EDTA, pH 7.0) using a Polytron at half-maximum speed for three periods of 30 s. The homogenate was centrifuged at 100,000 × g for 45 min at 4°C, and the supernatant was applied to a DEAE-Trisacryl (10-ml bed vol) column preequilibrated with homogenization buffer. The column was washed with 100 ml of 0.007 M sodium acetate (NaAc) buffer containing 10 mM Na-p-tosyl-l-lysine chloromethyl ketone, 2-mercaptoethanol, and 30% ethylene glycol (pH 6.5). Eighty 2.0-ml fractions were eluted from the column using a linear NaAc gradient (0.007–1.0 M, pH 6.5) at a rate of 0.5 ml/min. Collected fractions were either stored at 4°C overnight for assay the following day or at −20°C for later analysis.

Cyclic Nucleotide PDE Assay

PDE activity was determined using the two-step radioisotope method (44). Aliquots of lung homogenate supernatant as well as every third fraction eluted from the DEAE-Trisacryl column were assayed. The first step in the assay involves the hydrolysis of radioactive 3',5'-cyclic nucleotides to 5' derivatives by the unknown PDEs contained in each sample (44). The total reaction volume of 0.4 ml contained 100 μM [3H]cAMP or [3H]cGMP (100,000 cpm). In the second step of the reaction, radioactive 5’ nucleotides were converted to radiolabeled adenosine or guanosine following 5'-nucleotidase treatment (King Cobra snake venom, Sigma). Radioactive adenosine or guanosine was separated from the unhydrolyzed substrate using a Dowex column and quantitated.

To confirm the identity of PDEs contributing to the major activity peaks, activity profiles were evaluated in the absence and presence of specific PDE inhibitors. For example, cAMP hydrolysis was assayed in the absence or presence of 5 μM cGMP (PDE3 inhibitor) or 10 μM rolipram (PDE4 inhibitor). Similarly, cGMP hydrolysis was assayed in the absence or presence of 10 μM sildenafil or zaprinast (PDE5 inhibitors). For each individual experiment, peak PDE3 and PDE4 activity (pmol/min) was divided by the total supernatant cAMP hydrolysis (pmol/min), resulting in an activity ratio. Similarly, the peak PDE5 activity (pmol/min) was divided by the total supernatant cGMP hydrolysis (pmol/min).

In parallel experiments, dose-response relationships were developed to determine whether the sensitivity to isoform-specific PDE inhibitors was altered after pacing-induced heart failure. The cytosolic fraction of homogenates of lung parenchyma was assayed for PDE activity in the presence of varying doses of isoform-selective inhibitors (10−6 M to 10−4 M); vinpocetine (a selective PDE1 inhibitor), erythro-9-(2-hydroxy-3-onyl)adenine (EHNA; a selective PDE2 inhibitor), milrinone (a selective PDE3 inhibitor), zaprinast, and rolipram. PDE1 requires the presence of calcium (2 mM) and calmodulin (0.25 mg/ml) in order for enzymatic activity to be observed; these were added to establish a baseline of activity before inhibition with vinpocetine. Concentrations of inhibitors required to reduce hydrolysis by 50% compared with that at baseline (IC50) were determined using Graphpad Prism software for groups with n = 5 or greater.

Isolated Canine Lung Preparation

Lungs were isolated for ex vivo perfusion as described previously (12, 13, 45). Briefly, the lower left or middle right lobe was isolated via a left thoracotomy. Lobes were rapidly excised, and wide-bore plastic cannulas were tied into the lobar artery, vein, and bronchus. The lobes were then suspended from a counterbalanced force transducer (Grass FT-10) and perfused at a constant flow with a mixture of 200 ml of autologus blood and 100 ml of Earle’s buffer solution (37°C). Lobes were ventilated with 30% O2–5% CO2, and perfusate pH was measured (Radiometer ABL 5) and corrected to 7.35–7.4 with the addition of sodium bicarbonate. Thin catheters were placed in the arterial and venous lines to measure pulmonary vascular pressures. Capillary pressure (Pc) was measured by the double vascular occlusion technique (46). All pressures were referenced to the level of the lung hila.

Evaluation of Permeability and Transvascular Fluid Exchange

The capillary filtration coefficient (Kf,c) was determined as a measure of microvascular permeability, as previously described (45). The rate of lung weight gain (ΔW/Δt) measured at 13–15 min after increasing Pc by ~10 cmH2O was used in the following equation to determine Kf,c

\[ K_{f,c} = \frac{\Delta W/\Delta t}{\Delta P_c} \]

where ΔPc is the measured increment in Pc, Kf,c was expressed as milliliters per minute per centimeter of H2O per 100 g of wet lung weight.

Isolated Lung Protocol

After baseline hemodynamic and permeability measurements, and after the lungs had returned to an isogravimetric state, we tested the ability of agents that increase cAMP to prevent lung microvascular injury. Thapsigargin, a drug that activates capacitative Ca2+ entry and increases microvascular permeability in normal canine lung (12), was used as an experimental tool. Final measures of Kf,c obtained 60–75 min after addition of thapsigargin or vehicle were compared with...
the paired measurements obtained at baseline in each of the following experimental protocols.

**Forskolin-induced synthesis of cAMP.** Lobes (n = 5) were pretreated for 30 min with forskolin (100 nM) before addition of thapsigargin (150 nM) to the reservoir. Results were compared with those in lobes (n = 5) treated with thapsigargin alone (150 nM).

**Inhibition of PDE3 using cilostamide.** Lobes (n = 4) were pretreated with the PDE3-specific inhibitor cilostamide (100 nM) 30 min before addition of thapsigargin (150 nM) to the reservoir. In separate experiments, DMSO (30 nM) was administered (n = 8) 30 min before thapsigargin (150 nM). The effect of cilostamide alone was also determined: lobes (n = 3) were pretreated with cilostamide (100 nM) 30 min before DMSO (30 μl) administration.

All drugs were prepared as stock solutions in DMSO. The volumes of DMSO or drug added to the 300-ml circulating perfusate were limited to 30 μl. All concentrations noted are final circulating concentrations in the perfusate.

**RNA Isolation and Quantification**

RNA was purified from canine lung parenchyma using the RNeasy Maxi kit (Qiagen) following the manufacturer’s guidelines. RNA was quantified using the Ribogreen RNA quantitation kit (Molecular Probes) with a Perkin Elmer fluorimeter.

**RT-PCR**

Evaluation of steady-state mRNA levels was carried out using RT-PCR. First-strand cDNA was generated from 1 μg of RNA using the Superscript First-Strand Synthesis System (Invitrogen). Amplification reactions were performed using Taq DNA polymerase, 2 μl of the first-strand reaction, and 30 pmol of PDE3A or PDE3B sense and antisense oligonucleotide primers, as described by Liu and Maurice (15); predicted products were 508 and 499 bp, respectively. The following conditions were used: 30 s at 95°C (melting), 30 s at 58°C (annealing), and 1 min at 72°C (extension). For the nonlinear range of cDNA amplification, 35 cycles were used. Amplification in the linear range for PDE3A was obtained after 22 cycles and for PDE3B after 21 cycles.

**Statistical Analysis**

Numerical data are presented as means ± SE. Statistical differences between means were determined using either paired or unpaired Student’s t-test with P < 0.05 considered significant.

**RESULTS**

**PDE Activity Profile in Canine Lung**

**Overall hydrolysis of cAMP and cGMP by total homogenate supernatant.** Total cAMP hydrolysis in the cytosolic fraction of dog lung parenchyma was not different between the control (n = 6) and the paced (n = 5) group (211.5 ± 10.7 vs. 211.6 ± 18.6 pmol·min⁻¹·ml⁻¹). Similarly, there was no difference between the total cGMP hydrolysis in the cytosolic fraction of dog lung parenchyma in either experimental group (control: 133.6 ± 16.7 vs. pace: 148.5 ± 14.1 pmol·min⁻¹·ml⁻¹).

**Activity profiles of cyclic nucleotide hydrolysis.** Figure 1 shows analysis of cyclic nucleotide hydrolysis in fractions eluted from a DEAE-Trapacsyl column. Representative data obtained from control dog lung (Fig. 1A) and that from a dog paced to heart failure (Fig. 1B) are shown. A similar number of activity peaks was observed in the PDE activity profiles in lung of control and paced dogs. With the use of 0.5 μM [³H]cAMP as a substrate, we observed a profile of PDE activity with at least four distinct peaks of activity. Only one main peak of activity was observed when 0.5 μM [³H]cGMP was used as a substrate.

**Activity profiles in the absence or presence of specific inhibitors of PDEs.** Figure 2 shows the hydrolysis profile of cAMP (Fig. 2A) and cGMP (Fig. 2B) in the absence and presence of selective PDE inhibitors in one representative control experiment. The first peak (fraction 12–21) of [³H]cAMP hydrolysis was attenuated in the presence of 5 μM rolipram, indicative of PDE4 activity (Fig. 2A). The single peak of [³H]cGMP hydrolysis was inhibited in the presence of sildenafl or zaprinast (10 μM), indicative of PDE5 activity (Fig. 2B). The average PDE activity profile for hydrolysis of [³H]cGMP in lung tissue removed from control dogs (n = 5) was not different from that in the paced group (n = 5, Fig. 3B). Indeed, the ratio of the peak cGMP PDE hydrolytic

![Fig. 1. Cyclic nucleotide phosphodiesterase (PDE) activity in supernatant from control (A) and paced (B) dog lung determined using the 2-step method with radioactive cAMP (●) and cGMP (○) 0.5-μM substrates. The two PDE activity profiles are similar; both have the same number of peaks of activity, and these peaks are eluted at similar sodium acetate (NaAc) salt concentrations, suggesting that the differences in PDE activity between the two experimental groups are subtle.](image-url)
activity at fraction 21 to total activity in controls was not different from that in the paced group (0.72 ± 0.16 vs. 0.96 ± 0.18, respectively). On the other hand, the average hydrolysis of \([^{3}\text{H}]\text{cAMP}\) (Fig. 3A) in fractions 15–27 (PDE3 activity) of activity in lung removed from paced dogs \((n = 5)\) was reduced compared with control \((n = 5)\). The ratio of the peak cAMP PDE hydrolytic activity in fraction 21 to that in total supernatant was significantly reduced from 0.26 ± 0.08 in control to 0.12 ± 0.02 in the pace group \((P < 0.05, \text{Student's } t\text{-test})\). The ratio of total cAMP PDE hydrolytic activity in fraction 66 was not different between the control \((0.26 ± 0.07)\) and pace group \((0.21 ± 0.05)\).

### Sensitivity to Specific PDE Inhibitors

These studies were performed to test whether CHF resulted in any alteration in the sensitivity of PDEs in lung to specific inhibitors. Table 1 shows IC50 data for the inhibitors vinpocetine, rolipram, and zaprinast. In both control and pace groups, cAMP hydrolysis showed similar sensitivity to inhibition by the PDE4 inhibitor rolipram. Comparable observations were noted with the PDE3 inhibitor milrinone \((n = 2)\), but due to the small sample number, IC50 data could not be calculated. With respect to cGMP hydrolysis, we found no differences in the sensitivity for the PDE5 inhibitor zaprinast or the PDE1 inhibitor vinpocetine. EHNA, a PDE2-specific inhibitor, resulted in only 25% inhibition of cGMP hydrolysis at 10^{-4}\, \text{M} in both groups \((\text{data not shown})\). In view of this, expression of the PDE2 isomorph appears to contribute little to overall cGMP hydrolysis in canine lung and does not appear to be impacted by CHF.

#### Forskolin and Cilostamide: Isolated Lung Data

At baseline, flow in all isolated lung lobes \((n = 25)\) averaged 1.19 ± 0.01 l\,\text{min}^{-1}\cdot100\, \text{g}^{-1} \text{ wet weight}, resulting in pulmonary arterial and venous pressures of 13.8 ± 0.1 and 4.9 ± 0.1 cmH$_2$O, respectively, and total vascular resistance of 7.7 ± 0.1 cmH$_2$O\,\text{l}^{-1}.$

### Table 1. Sensitivity to specific PDE inhibitors

<table>
<thead>
<tr>
<th>PDE Inhibitor</th>
<th>Control IC50 (-logM)</th>
<th>Paced IC50 (-logM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vinpocetine</td>
<td>4.72 ± 0.12(6)</td>
<td>4.64 ± 0.08(7)</td>
</tr>
<tr>
<td>Rolipram</td>
<td>6.12 ± 0.07(6)</td>
<td>6.11 ± 0.05(6)</td>
</tr>
<tr>
<td>Zaprinast</td>
<td>6.22 ± 0.05(6)</td>
<td>6.36 ± 0.10(6)</td>
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Values are means ± SE. IC50, the dose of drug required to inhibit cyclic nucleotide hydrolysis by 50%, was determined in cytosolic fraction of lung homogenates. Numbers in parentheses represent the number of experiments performed.
min⁻¹·100 g. These data are similar to those reported previously from our laboratory (12, 13, 28, 45, 46). Thapsigargin has been shown to increase permeability in the isolated canine lung (12) and is known to induce this increase via the opening of store-operated Ca²⁺ channels and capacitative Ca²⁺ entry into endothelial cells (19). Figure 4A shows that the significant increase in endothelial permeability induced by thapsigargin in canine lung (P < 0.05) was completely blocked by a 30-min pretreatment with forskolin. As shown in Fig. 4B, pretreatment with cilostamide also prevented the permeability induced by thapsigargin, although cilostamide in and of itself had no effect on lung endothelial permeability.

RT-PCR Analysis

As shown in Fig. 5, PDE3A and PDE3B were amplified to a similar extent when 35 cycles of PCR reaction were used. Linear amplification of cDNA for PDE3A (22 cycles) and PDE3B (21 cycles) suggests that there is no difference between the message for these PDE isoforms in the control dog lung (n = 5) and that in lung parenchyma from dogs paced to heart failure (n = 6). As also shown in Fig. 5, mRNA expression of the housekeeping gene GAPDH was not different between the two experimental groups.

DISCUSSION

Our findings suggest that PDE3, PDE4, and PDE5 are the main cyclic nucleotide PDE isoforms expressed in the canine lung, as determined using anion-exchange chromatography and isoform-selective inhibitors. These findings in normal canine lung concur with those of Pyne and Burns (25) in guinea pig lung. Furthermore, although CHF does not alter this expression pattern, it does result in downregulation of PDE3 activity alone. The overall PDE activity profile in the cytosolic fraction was similar in lung parenchyma from control dogs and dogs paced to heart failure. However, the ratio of PDE3 activity to total cAMP hydrolysis in the lung parenchyma removed from dogs paced to heart failure was significantly reduced compared with that in controls. The activity ratios for other PDE isoforms were unchanged, suggesting a selective effect of CHF on PDE activity in lung parenchyma. Although CHF has been treated clinically using PDE3 inhibitors such as milrinone and indolidan, the positive inotropic effect of these PDE inhibitors is reduced in CHF (7, 8, 21, 22, 32). For example, Sato et al. (30) found that the increase in left ventricular dP/dt in response to milrinone was significantly attenuated in dogs paced to heart failure compared with that in control dogs. They attributed this attenuation to a decrease in cAMP levels in the endocardium and a decrease in PDE activity in the particulate fraction of the endocardium. Smith et al. (33) found that PDE3A mRNA and protein were reduced in canine right ventricle after 3 wk of pacing and CHF, although a similar decrement in PDE3 expression in the left ventricle was not seen until after 5 wk of pacing (34). Thus whereas decreased
PDE3 activity is not necessarily a novel finding in CHF, our study provides the first evidence of a decrease in PDE3 activity in lung after pacing-induced heart failure.

To further our findings, we needed to determine whether a decrease in protein expression was responsible for the decrease in PDE3 activity, as reported in ventricular myocardium (33). However, due to the unavailability of commercial antibodies to specifically probe for PDE3A and PDE3B in canine tissue using Western blotting, we turned to RT-PCR to investigate the level of message for these enzymes in canine lung parenchyma. The two PDE3 isoforms (PDE3A and PDE3B) have similar structural organization but are the products of distinct genes and can be differentially regulated in the same tissue (23). For example, PDE3B is elevated after prolonged increases in cAMP in rat vascular smooth muscle cells (15). However, our findings demonstrated similar amplification of PDE3A and PDE3B from control and pace canine lung cDNA, regardless of whether 35 cycles or 21–22 cycles (in the linear range) were utilized. Hence in contrast to the decrease in PDE3 activity in parenchyma from dogs paced to heart failure, there was no observed difference in expression of message for PDE3A or PDE3B between the two groups. This suggests that the reduced activity of PDE3 occurs as a result of altered posttranslational processing or altered regulation of catalytic activity.

A reduction in lung PDE3 activity after heart failure has potential implications with respect to regulation of endothelial permeability, assuming that this reduction in PDE3 activity occurs in lung endothelium. It is noteworthy that PDE expression in endothelial cells appears to be species and location dependent. For example, in pig and bovine aortic endothelial cells, there is evidence for the presence of PDE2 and PDE4 (16, 35), and in rat pulmonary microvascular endothelial cells, PDE4 is almost exclusively expressed (43). In human umbilical vein endothelial cells, there is evidence for PDE2, PDE4 (39), and PDE3 activity (6, 39). Reduced PDE3 activity in lung endothelium could be a beneficial modification of intracellular signaling in heart failure. It is well documented that an increase in cAMP decreases endothelial permeability (1, 10, 36) via increased cell-matrix and cell-cell tethering (1). Intracellular cAMP content in pulmonary microvascular endothelial cells is higher compared with that in pulmonary arterial endothelial cells (36), a factor that contributes to the resistance of microvascular endothelium to acute barrier dysfunction. Because lung parenchyma comprises not only pulmonary microvasculature but also small airways, lymphatic interstitial cells, and alveolar epithelial cells, we cannot specifically identify the cellular target accounting for the observed reduction in parenchymal PDE3 activity. Nonetheless, it is interesting to note that endothelium comprises ~30% of total cell number in the peripheral lung (5).

The results obtained by measuring endothelial permeability in the isolated lung model lend credence to our hypothesis that PDE3 plays an important role in preventing increases in endothelial permeability. Thapsigargin inhibits Ca2+ reuptake into intracellular stores and thus is used as a pharmacological tool to mimic store depletion, evoke Ca2+ influx through store-operated Ca2+ channels, and increase pulmonary microvascular permeability in normal canine lung lobes (12, 13). Pretreatment of lobes with forskolin, a selective agonist of adenylyl cyclases (20, 27, 29), prevented thapsigargin-induced increases in endothelial permeability in the isolated canine lung. Therefore, the forskolin-induced increase in intracellular cAMP accumulation serves to protect the lung from thapsigargin-induced injury. Inhibition of PDE3 should also increase intracellular cAMP by limiting degradation of the cyclic nucleotide. We found that pretreatment of the isolated lung with cilostamide, a selective PDE3 inhibitor (17, 42), prevented an increase in permeability after thapsigargin administration. Seeger et al. (31) found that administration of forskolin or zardaverine, an inhibitor of both PDE3 and PDE4, inhibited hydrogen peroxide-induced increases in permeability in isolated rabbit lungs possibly via increased intracellular cAMP from dual PDE inhibition. Sutterp et al. (38) carried out a similar study using thrombin and Escherichia coli hemolysin to perturb porcine pulmonary artery and human umbilical cord endothelial cell monolayers. In this study, inhibitors of PDE3 and/or PDE4 and agents that increased adenylyl cyclase activity abrogated increases in endothelial permeability. In a canine model of acute pulmonary hypertension and in a canine model of lung injury induced by administration of oleic acid, application of the selective PDE3 inhibitor milrinone was found to decrease endothelial permeability (40, 41). It is clear that PDE activity and message expression need to be confirmed in primary cultures of canine lung endothelial cells. Nonetheless, the previous studies, taken together with our observation that the PDE3 inhibitor cilostamide prevents thapsigargin-induced increases in lung endothelial permeability, provide support for our hypothesis that reduction in PDE3 activity in lung endothelium could contribute to the observed resistance to acute lung injury after CHF.

In summary, our results indicate that there is reduced PDE3 activity in lung parenchyma removed from dogs paced to heart failure, but this does not correlate with a decrease in expression of message for either the PDE3A or PDE3B isoforms. The fact that the PDE3 inhibitor cilostamide is effective in limiting endothelial injury in the canine lung highlights an important role for this PDE isoform in regulation of lung endothelial permeability. Further studies are needed to determine whether PDE3 expression in lung endothelial cells is specifically downregulated in heart failure.

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