Dibutyryl cAMP Inhibits Endotoxin-Induced Increases in Pulmonary Vascular Resistance and Fluid Filtration Coefficient in the Perfused Rat Lung

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In sheep, intravenous infusion of endotoxin increases both pulmonary vascular permeability and pulmonary arterial pressure (Brigham et al. 1983). Cyclic
nucleotides including cyclic AMP (cAMP) and cyclic GMP (cGMP) have been shown to play a role in the control of diarrhea caused by E. coli (Hughes et al. 1978). cAMP is an intracellular messenger, mediating diverse physiological processes in many cells (Rasmussen and Goodman 1977), and is a vasodilator in both the pulmonary and systemic circulation (Sakai and Voelkel 1988). Drugs that elevate intracellular cAMP levels have been shown to attenuate endotoxin-induced lung injury (Foy et al. 1979; Welsh et al. 1988; Chang et al. 1989). This protective effect may be mediated by inhibition of phospholipase A2 (Lapetina et al. 1977), and/or cyclooxygenase (Malmsten et al. 1976). cGMP-dependent reduction of endothelial permeability in human aortic vein was reported (Draijer et al. 1995).

To determine whether dibutyryl cAMP (db-cAMP), a cell-permeable analogue of cAMP, or cGMP protect against endotoxin-induced lung injury, we studied the effects of db-cAMP or cGMP pre-treatment on endotoxin-induced alterations in the pulmonary arterial and venous vascular resistances of isolated perfused rat lungs. We also measured pulmonary microvascular fluid filtration coefficient (Kf) and lung wet-to-dry (W/D) weight ratio in endotoxin-treated rats with or without db-cAMP or cGMP pre-treatment. In addition, the effects of db-cAMP post-treatment on endotoxin-induced lung injury were investigated to determine whether db-cAMP can be used for treatment of acute pulmonary vascular injury.

**Materials and Methods**

*Pre-treatment with db-cAMP and cGMP in endotoxin-induced lung injury*

Thirty male Sprague-Dawley rats (281 ± 13 g; means ± s.e.) were allowed free access to food and water. They were divided into 5 groups; control group, endotoxin group, db-cAMP group (db-cAMP + endotoxin), cGMP group (cGMP + endotoxin), and 2cGMP group (two doses of cGMP + endotoxin). Control rats were injected with saline instead of endotoxin (*Salmonella enteritidis*, Sigma Co., St. Louis, MO, USA). For the endotoxin, db-cAMP, cGMP, and 2cGMP groups, the rats were injected with 2 mg/kg of endotoxin intraperitoneally (i.p.) 90 minutes before lung perfusion. In addition, saline (for endotoxin group), 5 mg/kg db-cAMP (1 mg in 1 ml saline; a gift of Daiichi Seiyaku, Tokyo, for db-cAMP group), 5 mg/kg cGMP (1 mg in 1 ml saline; Sigma Co., for cGMP group) or 10 mg/kg cGMP (for 2cGMP group) was injected i.p. every 30 minutes for a total of four doses in these four groups, starting 30 minutes before endotoxin injection (Chang et al. 1989).

Lung isolation was performed under pentobarbital anesthesia (70 mg/kg) as described by Chen et al. (1990). After endotracheal cannulation, the lungs were ventilated with a gas mixture containing 21% O₂, 5% CO₂ and 74% N₂ using a small animal ventilator (SN-480-7; Sinano Seisakuju, Tokyo) maintaining a tidal volume of 3 ml, a ventilation rate of 50 breaths/minute, and a positive end-
expiratory pressure of 2.21 mmHg. After injection of heparin into the right ventricle, cannulas were placed in the pulmonary artery and left atrium. The lungs were perfused at a constant flow (\( \dot{Q} \)) of 0.03 ml•g\(^{-1}\) body weight•min\(^{-1}\) (Chang et al. 1989; Chen et al. 1990; Czartolomna et al. 1991) using a peristaltic perfusion pump (Masterflex 7520–00; Cole Parmer Instrument, Chicago, IL, USA). The perfusate was a physiological salt solution containing (in mM): 119 NaCl, 4.7 KCl, 1.17 MgSO\(_4\), 22.6 NaHCO\(_3\), 1.18 KH\(_2\)PO\(_4\), 3.2 CaCl\(_2\), 2H\(_2\)O, 5.5 glucose, and 4\% (w/v) of albumin (Sigma Co.). The perfusate contained no cAMP, cGMP or endotoxin.

Lung weight was monitored with a counterbalanced force-displacement transducer (SB-1T; Nihon Kohden, Tokyo) from which the isolated instrumented lung was suspended and was recorded with an electronic polyrecorder (TOA EPR-241-A; Toa, Tokyo).

The mean pulmonary artery perfusion pressure (Ppa) was monitored using a pressure transducer (model 37152; Nihon Kohden) and an amplifier (AP-630G; Nihon Kohden). Pulmonary microvascular pressure (Pmv) was measured by the double occlusion technique (Dawson et al. 1982; Townsley et al. 1986). When the perfusion circuit was occluded, Ppa and pulmonary venous pressure (Ppv) equalized and this pressure was defined as Pmv. Total pulmonary vascular resistance (TPR) was calculated as (Ppa-Ppv)/\( \dot{Q} \). TPR was partitioned into the arterial (Ra) and venous (Rv) resistances as follows (Johnson et al. 1989):

\[
Ra = \frac{(Ppa - Pmv)}{\dot{Q}}
\]
\[
Rv = \frac{(Pmv - Ppv)}{\dot{Q}}
\]

At the beginning of perfusion, the venous reservoir level was elevated 3 cm above the heart (meaning the Ppv 2.21 mmHg). Ppa and Pmv were measured after 10 minutes of perfusion (stage I) when the preparation was in an iso-

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**Fig. 1.** Protocol of the experiment. Pmv, pulmonary microvascular pressure.
gravimetric state, and then measured again at 39 minutes (stage II). At 40 minutes, the venous reservoir was rapidly elevated a further 10 cm (Ppv 7.35 mmHg), and 20 minutes later, Ppa and Pmv were measured again (stage III, Fig. 1). Kf was calculated from Ppa and Pmv at stage II and stage III.

Elevation of the venous reservoir caused a two-component weight increase; an initial rapid increase due to recruitment and distention in the vascular bed, and a second slow and constant increase due to fluid filtration (ΔW). The Kf was calculated by dividing the weight change extrapolated to time 0 (ΔW) by the change in Pmv (ΔPmv) after elevation of the venous reservoir, and then normalized to the dry lung weight:

$$Kf = \Delta W(g) \cdot \Delta Pmv(mmHg)^{-1} \cdot 20(min)^{-1} \cdot dry\ lung\ W(g)^{-1}$$

ΔW was calculated by extrapolating back to time 0 using the y-intercept of the log-linear-regression line of the dW/dt vs. time curve, and then taking the anti-log10 of the y-intercept. ΔPmv was the difference between Pmv measured at stage II and at stage III.

After perfusion the lung was dried in a 50°C desiccator for 2 weeks and the W/D weight ratio was calculated.

**Post-treatment with db-cAMP in endotoxin-induced lung injury**

Twenty-seven male Sprague-Dawley rats (262 ± 19 g) were divided into 3 equal groups; control group, endotoxin group, and db-cAMP group (endotoxin + db-cAMP). Control rats were injected with saline instead of endotoxin. In the endotoxin and db-cAMP groups, the rats received 2 mg/kg of endotoxin i.p. 4 hours before lung perfusion. In addition, either saline and db-cAMP (5 mg/kg) was injected i.p. every 30 minutes for a total of four doses in these two groups, respectively, starting 2 hours after endotoxin injection. The remainder of the protocol was the same as for the pre-treatment experiments.

**Data analysis and statistics**

Data are expressed as means ± s.e. One-way ANOVA was used to compare Kf and lung W/D ratio values. For comparison of the means of pulmonary vascular resistance, a two-way ANOVA followed by post hoc (Fisher) comparison was performed. Differences were considered significant when $p$ was $< 0.05$.

**Results**

**Pre-treatment with db-cAMP and cGMP in endotoxin-induced lung injury**

Elevation of the venous reservoir by 10 cm (7.35 mmHg) increased both Ppa and Pmv ($p < 0.01$; Stage II vs. Stage III), and caused significant decreases in TPR and Rv ($p < 0.01$ for both).

During stage I and II, no significant differences were observed in Ppa or Pmv in lungs isolated from saline or endotoxin-treated animals. However, after
Table 1. *Pulmonary microvascular pressure (PmV) in the control, endotoxin-treated, and ab-cAMP and cGMP pre-treated rat lungs*

<table>
<thead>
<tr>
<th></th>
<th>PmV (mmHg)</th>
<th>Stage I</th>
<th>Stage II</th>
<th>Stage III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.18 ± 0.11</td>
<td>1.80 ± 0.07</td>
<td>7.75 ± 0.16*</td>
<td></td>
</tr>
<tr>
<td>Endotoxin</td>
<td>1.72 ± 0.53</td>
<td>1.88 ± 0.52</td>
<td>6.60 ± 0.46</td>
<td></td>
</tr>
<tr>
<td>db-cAMP + Endotoxin</td>
<td>1.78 ± 0.33</td>
<td>2.03 ± 0.31</td>
<td>7.90 ± 0.10*</td>
<td></td>
</tr>
<tr>
<td>cGMP + Endotoxin</td>
<td>2.15 ± 0.58</td>
<td>2.27 ± 0.52</td>
<td>7.02 ± 0.43</td>
<td></td>
</tr>
<tr>
<td>2cGMP + Endotoxin</td>
<td>1.50 ± 0.08</td>
<td>1.70 ± 0.15</td>
<td>7.38 ± 0.39</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± s.e.; n = 6 for all groups; Stage I, II and III are shown in Fig. 1.
*p < 0.05 compared with values of endotoxin.

Fig. 2. Effects of db-cAMP or cGMP pre-treatment on endotoxin (E)-induced changes in pulmonary vascular resistances. TPR, total pulmonary resistance; Ra, pulmonary arterial resistance; Rv, pulmonary venous resistance. Stage I, Stage II and Stage III were indicated in Fig. 1.
○, control; ●, E; □, db-cAMP + E; ▲, cGMP + E; ■, 2cGMP + E.
Values are means ± s.e.; n = 6 for all groups. *p < 0.05, **p < 0.01 compared with values of E.
elevation of the venous reservoir, \( P_{mv} \) was low in endotoxin-treated rat lungs compared with controls \( (p < 0.05, \text{Table 1}) \) and this effect was prevented by \( \text{db-cAMP} \) pre-treatment \( (p < 0.05 \text{ vs. endotoxin}) \). Neither cGMP nor 2cGMP normalized \( P_{mv} \).

No differences were observed in TPR, \( R_a \) or \( R_v \) between lungs isolated from endotoxin and saline-treated rats during stages I and II. However, in stage III, \( R_a \) decreased in controls but increased in the lungs from endotoxin-treated animals \( (p < 0.01) \). Pre-treatment with \( \text{db-cAMP} \) inhibited this increase in \( R_a \) due to endotoxin \( (p < 0.01 \text{ vs. endotoxin, Fig. 2}) \). Pre-treatment with 2cGMP attenuated the endotoxin-induced increase in \( R_a \) \( (p < 0.05, \text{Fig. 2}) \) after elevation of the venous reservoir.

Endotoxin-induced vascular damage was indicated by the increased \( K_f \) value (control group: \( 1.77 \pm 0.11 \left(10^{-2} \text{mmHg}^{-1} \text{min}^{-1}\right) \), endotoxin group: \( 3.27 \pm 0.29 \left(10^{-2} \text{mmHg}^{-1} \text{min}^{-1}\right) \), \( p < 0.01 \)), and this increase in permeability was inhibited by \( \text{db-cAMP} \) \( (1.98 \pm 0.23 \left(10^{-2} \text{mmHg}^{-1} \text{min}^{-1}\right) \), \( p < 0.01 \), Fig. 3, upper). Of the determinants of \( K_f \), \( \Delta P_{mv} \) decreased slightly and \( \Delta W_1/(\text{dry lung weight}) \) increased significantly \( (p < 0.05) \) after endotoxin treatment. These changes were attenuated by pre-treatment with \( \text{db-cAMP} \). Treatment with \( \text{db-cAMP} \) inhibited \( (p < 0.05) \) the endotoxin-induced increase in lung \( W/D \) ratio (control group: \( 6.08 \pm 0.13 \), endotoxin group: \( 6.80 \pm 0.17 \), db-cAMP group: \( 6.17 \pm 0.18 \), Fig. 3, lower). Treatment with either cGMP or 2cGMP did not show any effect on endotoxin-induced increases in \( K_f \) or lung \( W/D \) ratio (Fig. 3).

![Graph](image-url)

**Fig. 3.** Effects of db-cAMP or cGMP pre-treatment on endotoxin (E)-induced changes in pulmonary capillary filtration coefficient (Kf, upper) and lung wet-to-dry weight ratio (lower). Values are means ± s.e., *\( p < 0.05 \), **\( p < 0.01 \) compared with values of E.
**db-cAMP post-treatment in endotoxin-induced lung injury**

During stage III, Pmv in lungs from endotoxin-treated rats was lower than in controls \( p < 0.05 \) and was not affected by post-treatment with db-cAMP (Table 2). After elevation of the venous reservoir, treatment with endotoxin increased Ra \( p < 0.01 \) and post-treatment with db-cAMP attenuated the endotoxin-induced increase in Ra \( p < 0.05 \), Fig. 4).

<table>
<thead>
<tr>
<th>Table 2. <strong>Pulmonary microvascular pressure (Pmv) in the control, endotoxin-treated, and db-cAMP post-treated rat lungs</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pmv (mmHg)</strong></td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Endotoxin</td>
</tr>
<tr>
<td>db-cAMP + Endotoxin</td>
</tr>
</tbody>
</table>

Values are means ± s.e.; \( n = 6 \) for all groups. Stage I, II and III are shown in Fig. 1.

* \( p < 0.05 \) compared with values of endotoxin.

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**Fig. 4.** Effects of db-cAMP 2 hour-post-treatment on endotoxin (E)-induced changes in pulmonary vascular resistances. TPR, Ra, Rv are same as Fig. 2. Stage I, Stage II and Stage III were indicated in Fig. 1.

○, control; ●, E; □, E + db-cAMP. Values are means ± s.e.; \( n = 9 \) for all groups.

* \( p < 0.05 \), ** \( p < 0.01 \) compared with values of E.
Kf was elevated in the endotoxin-treated rat lungs compared with controls (2.58 ± 0.17 vs. 1.85 ± 0.06 [10⁻²•mmHg⁻¹•min⁻¹], \( p < 0.01 \)). Post-treatment with db-cAMP did not affect the endotoxin-induced increases in Kf or in lung W/D weight ratio.

**Discussion**

We used the isolated perfused rat lung preparations to determine whether cyclic nucleotides affect changes in permeability caused by endotoxin. Intraperitoneal injection of endotoxin increased pulmonary vascular permeability and W/D weight ratio. These changes were inhibited by pre-treatment but not post-treatment with db-cAMP. cGMP was not effective.

*Effects of db-cAMP on pulmonary vascular resistance*

In control rat lungs, elevation of the pulmonary vascular afterload increased Ppa and Pmv, whereas TPR, Ra and Rv decreased. Our results agree with those of El-Kashief et al. (1990) who reported that elevation of Ppv in the isolated dog lung perfused at constant flow caused a decrease in lobar vascular resistance by decreasing both Ra and Rv. When Ppa, Pmv and Ppv are elevated, the diameters of the pulmonary artery and vein, as well as the diameter of pulmonary microvasculature may increase and thus TPR decreases.

Following treatment with endotoxin, Ra increased after elevation of the venous reservoir, which was due to the smaller increase of Pmv in endotoxin treated rat lungs compare to controls. Pre-treatment with db-cAMP inhibited endotoxin-induced increase in Ra after venous reservoir elevation by the increase of Pmv.

db-cAMP freely diffuses across the plasma membrane and is an analogue of cAMP that is intracellularly deacylated to cAMP (Falbriard et al. 1967). cAMP is an important vasodilator in both the pulmonary and the systemic circulatory systems (Sakai and Voelkel 1988). Many pulmonary vasodilators such as isoproterenol, epinephrine and prostaglandin E1 raise the intracellular cAMP levels in alveolar macrophages. Various agents that increase intracellular cAMP have been shown to protect against endotoxin-induced lung injury (Welsh et al. 1988). db-cAMP may directly modulate the pulmonary vascular responses to endotoxin by stimulating vasodilation. An increase in cAMP may have a vasodilating effect due to the inhibition of the actions of intracellular calcium on vascular smooth muscle contraction (Rasmussen and Goodman 1977).

Interestingly, pretreatment with cAMP, a vasodilating agent, offsets the effect of endotoxin on Pmv, suggesting a different vasodilatory mechanism or different vasodilating ability depending on the size or location of pulmonary vessels between endotoxin and cAMP.
Effects of db-cAMP on Kf and W/D ratio

In endotoxin-treated rat lungs, Kf was increased after elevation of the pulmonary vascular afterload. Pre-treatment but not post-treatment with db-cAMP inhibited this increase. These results corresponded to the changes in the W/D ratio.

Endotoxin has been reported to have direct effects on endothelial cells (Harlan et al. 1983; Meyrick et al. 1989) to increase Kf in isolated perfused rabbit lungs (Salzer and McCall 1990). Increased permeability of the pulmonary microvasculature has been reported to cause acute noncardiogenic pulmonary edema, and histological studies of edematous lungs show gaps between apparently healthy endothelial cells (Shasby et al. 1982). Primary alterations in endothelial cell cytoskeletons alter endothelial permeability to fluid and protein (Shasby et al. 1982).

db-cAMP treatment in vivo markedly attenuated the increases in lung albumin leak index and W/D weight ratio caused by endotoxin (Chang et al. 1989). Methylxanthine pentoxifylline, an inhibitor of the cyclic nucleotide phosphodiesterase that metabolizes cAMP, attenuated endotoxin-induced acute lung injury by preventing activation of polymorphonuclear leukocytes in guinea pigs (Hoffmann et al. 1991). Increased intracellular cAMP inhibits membrane phospholipase (Lapetina et al. 1977) and cyclooxygenase (Malmsten et al. 1976). db-cAMP may also alter the endothelial cell cytoskeleton. Changes in cAMP levels alter epithelial permeability, and simultaneously cause changes in the microfilaments (Duffy et al. 1981). It is also conceivable that an increase in cAMP may prevent the formation of endothelial intercellular gaps by inhibiting the accumulation of actomyosin fibrils of vascular endothelial cells (Shasby et al. 1982).

Koyama et al. (1992) reported that when db-cAMP was infused 30 minutes after endotoxin infusion, endotoxin-induced increases in lung lymph flow, lymph protein clearance, and plasma TXB₂ and 6-keto-PGF₁α were blocked, similarly to the effects observed with pre-treatment. Hoffmann et al. (1991) reported that early (30 minutes) post-treatment with db-cAMP attenuated E. coli-induced increases in lung W/D ratio and reduced the accumulation of ¹²⁵I-albumin in bronchoalveolar lavage fluid and lung tissue. db-cAMP also prevented endotoxin-induced activity of polymorphonuclear leukocytes (Hoffmann et al. 1991). Both of these groups concluded that 30 minutes post-treatment with db-cAMP may have therapeutic potential for endotoxin-induced lung injury. In our experiments, post-treatment with db-cAMP was performed 2 hours after endotoxin injection, and this delay may explain the differences between our results and those of these previous studies.
Effects of cGMP

Following pre-treatment with two different doses of cGMP, the endotoxin-induced increase in Ra observed after venous reservoir elevation was attenuated but endotoxin-induced increases in Kf and W/D were not affected.

Since an inhibitory effect of cGMP (Fujimoto et al. 1990) as well as db-cAMP (Sakai and Voelkel 1988) on hypoxic and angiotensin II-induced pulmonary vasoconstriction was reported, both of these cyclic nucleotides were used to investigate their effects on endotoxin-induced changes in pulmonary vascular resistance.

In unanesthetized sheep, peak cAMP concentrations in blood plasma and lung lymph were observed early in the endotoxin reaction during the period of marked pulmonary hypertension, whereas cGMP level increased in a gradual but persistent manner, reaching the peak during the late phase of the reaction corresponding to the period of increased permeability of the pulmonary capillary endothelium (Snapper et al. 1983). db-cAMP but not db-cGMP has inhibitory effects on the endotoxin-induced increase in thromboplasmin activity in human monocytes (Snapper et al. 1983), and aggregation of platelet-rich plasma induced by arachidonate (Malmsten et al. 1976). Therefore, the exact role of cGMP in endotoxicosis is not clearly understood and remains controversial.

It is concluded that pre-treatment with db-cAMP inhibited endotoxin-induced increases in Ra, W/D ratio and originally measured Kf. We speculate that db-cAMP which increases intracellular cAMP may be therapeutically useful in septic acute pulmonary vascular injury when given as pre-treatment.

Acknowledgment

We thank Daiichi Seiyaku for their generous gift of dibutyryl-cAMP.

References


