Attenuation by Phosphodiesterase Inhibitors of Lipopolysaccharide-Induced Thromboxane Release and Bronchoconstriction in Rat Lungs

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ABSTRACT

Exposure of perfused rat lungs to lipopolysaccharides (LPS) causes induction of cyclooxygenase-2 followed by thromboxane (TX)-mediated bronchoconstriction (BC). Recently, phosphodiesterase (PDE) inhibitors have received much interest because they not only are bronchodilators but also can suppress release of proinflammatory mediators. In the present study, we investigated the effect of three different PDE inhibitors on TX release and BC in LPS-exposed perfused rat lungs. The PDE inhibitors used were motapizone (PDE III specific), rolipram (PDE IV specific), and zardaverine (mixed PDE III and IV specific). At 5 μM, a concentration at which all three compounds selectively block their respective PDE isoenzyme, rolipram (IC50 = 0.04 μM) and zardaverine (IC50 = 1.8 μM) largely attenuated the LPS-induced BC, whereas motapizone was almost ineffective (IC50 = 40 μM). In contrast to LPS, BC induced by the TX-mimetic U46619 was prevented with comparable strength by motapizone and rolipram. In LPS-treated lungs, the TX release was reduced to 50% of controls by rolipram and zardaverine but was unaltered in the presence of 5 μM motapizone. Increasing intracellular cAMP through perfusion of db-cAMP or forskolin (activates adenylate cyclase) also reduced TX release and BC. We conclude that PDE inhibitors act via elevation of intracellular cAMP. Although both PDE III and PDE IV inhibitors can relax airway smooth muscle, in the model of LPS-induced BC, PDE IV inhibitors are more effective because (in contrast to PDE III inhibitors) they also attenuate TX release.

Exposure of blood-free perfused rat lungs to LPS from Gram-negative bacteria causes bronchoconstriction that is independent of blood-derived leukocytes (Uhlig et al., 1995). We have recently shown that this effect of LPS depends on induction of the enzyme COX-2 and subsequent formation of TX (Uhlig et al., 1996). Because Gram-negative septicemia is frequently associated with bronchoconstriction (Pulosi et al., 1995; Wright et al., 1994), perfusion of LPS in isolated rat lungs may serve to study possible therapeutic interventions aimed at preventing bronchoconstriction in Gram-negative sepsis. However, it is emphasized that perfusion of rat lungs with LPS is only an incomplete model of the acute respiratory distress syndrome or sepsis in general because it lacks a number of important characteristics of this syndrome, such as neutrophil infiltration, edema formation and pulmonary hypertension. Still, its advantage is that a defined set of parameters allows an assessment of a response under controlled conditions followed by an interpretation of the results that is not confounded by interactions of the lung with other organs such as the liver, central nervous system or blood.

PDE inhibitors have long been known for their potential to relax airway smooth muscle (Rabe et al., 1995). In addition, they have some anti-inflammatory properties, a finding that further stimulated the interest in these compounds as drugs for the treatment of asthma (Schudt et al., 1995) or for prevention of the overactivation of the nonspecific immune system such as in septic shock (Fischer et al., 1993) or acute respiratory distress syndrome (Turner et al., 1993). At least seven major groups of PDE enzymes are classified (Beavo et al., 1993). Of these, PDE type III and type IV appear to have an important role in regulation of the cellular inflammatory response. The present study investigates the effect of selective or combined PDE III and PDE IV inhibition on endotoxin-induced TX release and subsequent bronchoconstriction in isolated perfused rat lungs.

ABBREVIATIONS: COX, cyclooxygenase; IC50, median inhibitory concentration; LPS, lipopolysaccharide; PDE, phosphodiesterase; PKA, protein kinase A; PLA2, phospholipase A2; db-cAMP, di-butyryl; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; RT, reverse transcription (transcriptase); PCR, polymerase chain reaction; PBS, phosphate-buffered saline; TNF, tumor necrosis factor; TX, thromboxane.
**Methods**

**Materials.** Female Wistar rats (220–250 g, Zentralinstitut, Hannover, Germany) were used as lung donors. Pentobarbital sodium (Nembutal) was purchased from the Wirtschaftsgenossenschaft Deutscher Tierärzte (Hannover, Germany). Bovine albumin (fraction V) from Serva (Heidelberg, Germany), db-CAMP and forskolin were from Sigma (Deisenhofen, Germany). Rolipram was a gift from Schering (Berlin, Germany), and zardaverine was from Byk Gulden (Konstanz, Germany). Motapizone was from Nattermann-Rhone Poulenc-Rorer (Cologne, Germany).

**Isolated perfused rat lung preparation.** The rat lungs were prepared and perfused essentially as described recently (Uhlig and Wollin, 1994). Briefly, lungs were perfused at constant hydrostatic pressure (12 cm H₂O) through the pulmonary artery, which resulted in a flow rate of ~20 ml/min. As a perfusion medium, we used Krebs-Henseleit buffer (37°C), which contained 2% albumin, 1% glucose and 3% HEPES. The total amount of recirculating buffer was 100 ml. The lungs were suspended by the trachea and were ventilated by negative pressure ventilation with 80 breaths/min and a tidal volume between 1.6 and 2 ml. Every 5 min, a hyperinflation (~16 cm H₂O) was performed. Artificial thorax chamber pressure was measured with a differential pressure transducer, and air flow velocity was measured with a pneumotachograph tube connected to a differential pressure transducer. The lungs inspired humidified air. The perfusate flow and arterial and venous pressures were continuously monitored. The pH of the perfusate before entering the lung was kept at 7.35 by automatic bubbling of the buffer with CO₂ as soon as the pH exceeded this value. All data were transmitted to a computer (Compaq Deskpro 286) via an A/D converter (Metrabyte 16) or an RS232 serial interface and analyzed by self-written software (programming language ASYST 3.1). Simultaneously, chamber pressure, tidal volume (by electronic integration), perfusate flow were (programming language ASYST 3.1) and analyzed as described previously (Uhlig et al., 1995, 1996).

**Measurement of TX.** Samples taken from the perfusate were stored at −20°C. TXA₂ was measured as the stable byproduct TxB₂ via enzyme immunoassay (Cayman, Ann Arbor, MI). The cross-reactivity of the detecting antibody was TxB₂, 100%; 2,3-dinor TxB₂, 8.2%; and prostaglandins (2,6-dinor TXB₂, 0.5%.

**RT-PCR analysis.** RNA was isolated from lung tissue by using Chaosolv solution (Biotecx Laboratories, Houston, TX; supplier's manual) and analyzed as described previously (Uhlig et al., 1996). Then, 4 μg of RNA were used for target-specific RT with Superscript RT and specific primers (GC content of 50–60%). The primers used have been described previously (Uhlig et al., 1996). The reactions were cycled 32 times (30 sec at 94°C, 30 sec at 56°C and 30 sec at 72°C after a 5 min denaturing step at 95°C). Products were analyzed by 2% agarose gel electrophoresis and ethidium bromide staining. Without specific primer or with the PCR reaction lacking the template, no amplification products were found. Samples were assayed in various dilutions to ensure proportionality in the yield of PCR products. The identity of the fragments was evaluated by their molecular mass and restriction enzyme analysis.

**PDE inhibition.** PDE activity was determined with some modifications (Bauer and Schwabe, 1980) as described by Thompson and Appleman (1979). PDE I (Ca²⁺/1 mM)/calmodulin (100 nM) dependent) from bovine brain was kindly provided by Dr. Gietzen (Ulm, Germany). PDE II (cAMP stimulated (5 μM) was purified from rat heart (Schudt et al., 1991b). PDE III (cGMP inhibited) and PDE V (cGMP specific) were assayed in homogenates of human platelets as described by Schudt et al. (1991a). PDE IV (cAMP specific) was tested in the cytosol of human polymorphonuclear cells as described by Schudt et al. (1991a). IC₅₀ values were calculated from concentration-inhibition curves by nonlinear regression analysis using Graph-PAD software (GraphPAD, Sorrento Valley, CA).

**Experimental design.** db-CAMP, rolipram and motapizone were dissolved in 0.9% NaCl. Forskolin was dissolved in warmed cremophor EL (Sandoz AG, Basel, Switzerland) and further diluted in PBS; LPS was dissolved in PBS/0.005% hydroxyamine and subsequently sonified for 1 min; and zardaverine was dissolved in 1 M NaOH at 70°C and further diluted in PBS. U46619 was prepared as 1 mM stock solution in ethanol. None of the solvents alone had any effect on the LPS-induced bronchoconstriction.

To obtain a stable baseline line, all lungs were perfused for 40 min before a bolus of 5 mg of LPS was injected into the pulmonary artery. The following values (n = 82, mean ± S.D.) were obtained after 40 min of perfusion and ventilation: tidal volume, 1.87 ± 0.21 ml; pulmonary resistance, 0.25 ± 0.03; and pulmonary compliance, 0.38 ± 0.15. All PDE inhibitors were added 10 min before the administration of LPS. Within these 10 min, none of them induced any alterations in lung function. After administration of LPS, the lungs were perfused and ventilated for an additional 110 min. To mimic the time course of the LPS-induced bronchoconstriction, the TX agonist U46619 was added as a bolus 70 min after the beginning of the experiment; also, in these experiments, PDE inhibitors were added 40 min before the administration of U46619.

**Statistics.** Values in the figures are given as mean ± S.E.M. For analysis of the pulmonary resistance data, the percentage data in the figures were transformed by the arcsin transformation (Zar, 1984) and multiple comparisons performed by the Tukey-Kramer Test (SAS software, release 6.11, SAS Institute, Cary, NC). The data for TX release were analyzed by repeated-measures analysis with orthogonal polynomials (SAS) and Dunnett's test against the LPS-induced TX release.

**Results**

The selectivity of the three inhibitors used in the present study on different PDE isoenzymes was quantitatively compared on the basis of their IC₅₀ values (table 1). These data confirmed that motapizone is predominantly PDE III specific and rolipram is PDE IV specific and that zardaverine acts on PDE III as well as PDE IV.

Perfusion of rat lungs with 50 μg/ml LPS increased pulmonary resistance (fig. 1 and see fig. 5) as described recently (Uhlig et al., 1995, 1996). Pretreatment of lungs with zardaverine (fig. 1A), motapizone (fig. 1B) and rolipram (fig. 1C) dose-dependently prevented the LPS-induced increase in pulmonary resistance. The IC₅₀ values were 1.8 μM for zardaverine, 40 μM for motapizone and 0.04 μM for rolipram.

Because it is known that the LPS-induced bronchoconstrictive...
tion in this model is mediated by TX (Uhlig et al., 1996), we investigated whether the PDE inhibitors acted by interfering with the release of this mediator. With respect to rolipram and zardaverine, we studied only concentrations that provided maximum protection against the LPS-induced bronchoconstriction. Both 5 μM rolipram and 50 μM zardaverine markedly reduced but did not completely prevent the LPS-induced TX release (fig. 2). Motapizone had no effect on TX release at 5 μM but attenuated it at 25 μM and completely prevented it at 250 μM (IC50 = 20 μM, fig. 3). By quantifying the area under the curve, we found that TX release was attenuated by 50% in the presence of 5 μM rolipram compared with control lungs perfused with LPS alone. The corresponding data were attenuation by 76% of LPS-induced TX release for 50 μM zardaverine, 3% for 5 μM motapizone, 76% for 25 μM motapizone and 93% for 250 μM motapizone. To further explore the mechanism(s) by which PDE inhibitors reduce the TX release in LPS-treated rat lungs, we checked whether they affected the expression of the message for COX-2, the enzyme responsible for TX formation in this model (Uhlig et al., 1996). Figure 4 shows that neither rolipram (5 μM) nor motapizone (250 μM) decreased the induction of COX-2 by LPS. In contrast, both rolipram and motapizone appeared to enhance COX-2 mRNA. We also examined whether rolipram or motapizone affected the activity of TX synthase or COX; however, neither rolipram nor motapizone had an effect on these enzyme activities (data not shown).

We further pursued the obvious hypothesis that the reduction in LPS-induced TX release and bronchoconstriction in the presence of PDE inhibitors is related to an increase in cAMP. We investigated the effects of db-cAMP and the adenylate cyclase activator forskolin. Both db-cAMP and forskolin prevented a major part of the increased airway resistance in lungs exposed to LPS (fig. 5). The LPS-induced TX release was reduced by 74% in the presence of db-cAMP and by 67% in the presence of forskolin (fig. 6).

Finally, we examined whether inhibition of PDE as such prevents bronchoconstriction induced by the spasmogen TX using the stable agonist U46619. To create a time course comparable to the experiments shown above in which bronchoconstriction started after ~30 min after injection of LPS, 5 μM rolipram or 5 μM motapizone was present in the perfusate.
Elevation of cAMP reduces LPS-induced TX-release.

In our studies, we focused on TX as the principal mediator responsible for the LPS-induced bronchoconstriction (Uhlig et al., 1996). However, other COX metabolites, such as PGD<sub>2</sub> and PGF<sub>2α</sub>, could also be formed and act on TP receptors in LPS-treated lungs. Our data clearly show that increasing intracellular cAMP by various means reduced the LPS-induced release of TX. This finding is in line with the concept that an increase in intracellular cAMP reduces the release of various other proinflammatory mediators, such as TNF or eicosanoids (Rola-Pleszczynski and Stankova, 1992; Schudt et al., 1995). It was shown that db-cAMP can reduce the release of TX from endotoxin-treated (Koyama et al., 1992) or air-infused (Kobayashi et al., 1987) sheep in vivo and from murine hepatocytes (Mandl et al., 1988) in vitro. Similar effects are obtained by treatment with PDE inhibitors. For instance, PDE inhibitors prevented the LPS-induced formation of TNF in vivo (Fischer et al., 1993; Turnier et al., 1993; Kips et al., 1993), in perfused mouse liver (Leist et al., 1996) and in isolated peritoneal macrophages (Fischer et al., 1993). Interestingly, human monocytes contain almost only PDE IV (Schudt et al., 1995), and PDE III inhibitors did not suppress LPS-induced TNF-production from these cells (Seldon et al., 1995). However, it appears that further differentiation of monocytes induces other PDE isoenzymes, among them PDE III (Gantner et al., 1997; Schudt et al., 1995).

The value of 5 μM represents a concentration at which motapizone and rolipram provide nearly complete but still selective inhibition of PDE III and IV, respectively (Rabe et al., 1993, table 1). At this concentration, both PDE inhibitors attenuated U46619- and endothelin-1-induced (Held et al., 1997) bronchoconstriction, suggesting that this concentration was also effective under our conditions. However, at this concentration only rolipram, not motapizone, reduced the LPS-induced TX release. These findings suggest that the TX-producing lung cells, which are unknown in this model (Uhlig et al., 1996; Uhlig and Wendel, 1995), contain predominantly PDE IV. In agreement with these considerations, we observed that the mixed PDE III/IV inhibitor zardaverine was no more effective than rolipram alone. In addition, we recently found that only rolipram, not motapizone, suppressed the endothelin-1-induced TX release (Held et al., 1997). There are only a few reports in the literature on the effects of PDE inhibitors on TX synthesis. In piglets in vivo, amrinone (PDE III specific) had no effect on TX and TNF release in Streptococcus-induced pulmonary hypertension (Berger et al., 1993). Conflicting in vivo results were reported with two nonspecific PDE inhibitors: pentoxifylline and the closely related compound HWA 138. Although pentoxifylline was found to reduce LPS-induced TX formation in piglets (Li et al., 1995), HWA 138 failed to do so in sheep (Masouyé et al., 1992). Studies in perfused rat lungs demonstrated that milrinone (PDE III specific) attenuated TX release and bronchoconstriction elicited by antigen challenge (Post et al., 1989). However, the concentration of milrinone required, such as 100 μM, was high. On a cellular level, it was shown that milrinone diminished TX release from stimulated human platelets, although the efficacy of milrinone was stimulus dependent (Barradas et al., 1993; Jeremy et al., 1993). And, finally, rolipram and ibudilast (a nonselective PDE inhibitor) prevented the leukotriene B<sub>4</sub>-induced release of TX from guinea pig eosinophils (Souness et al., 1994).

Mechanism of action. As far as smooth muscle relaxation is concerned, the relaxant properties of cAMP-elevation are well known and understood (Rabe et al., 1995; Souness and Giembycz, 1994). The mechanism by which an increase in intracellular cAMP can affect the production of TX is far less clear. In the case of LPS-stimulated monocytes/macrophages, the cAMP-mediated suppression of TNF release has been ascribed to reduced transcription of the TNF gene (Gi- roir and Beutler, 1992; Scales et al., 1989; Spriggs et al., 1992). However, the LPS-induced induction/stabilization of COX-2 mRNA, which is mandatory for formation of TX in our model (Uhlig et al., 1996), was not diminished but rather...
increased by the PDE inhibitors. This finding is in agreement with the presence of a cAMP-responsive element in the COX-2 gene (Appleby et al., 1994) and with cellular studies on the effect of cAMP on COX-2 mRNA levels (Nüssing et al., 1996). We also excluded a direct effect of the PDE inhibitors on the activity of COX or TX synthase. Moreover, neither of these two enzymes appears to be regulated by PKA. Therefore, the target for cAMP, or rather PKA, probably is upstream of COX. In line with this, the liberation of arachidonic acid by PLA2 is suppressed in the presence of PDE IV inhibitors in neutrophils (Hichami et al., 1995) and monocytes (Nakashimura et al., 1995), an effect that appears to be mediated by PKA (Nakashimura et al., 1995). The substrate for PKA, however, is not known. The 85-kDa cytosolic PLA2 appears to be the enzyme responsible for agonist-induced arachidonic acid release (Mukherjee et al., 1994), although the involvement of other PLA2 isoenzymes cannot be excluded. Although phosphorylation of cPLA2 by mitogen-activated protein kinase, protein kinase C or G proteins appears to be a control mechanism for this enzyme (Mukherjee et al., 1994), we found no evidence in the literature that PKA may phosphorylate PLA2. Because PKA appears to not directly affect PLA2, alternative explanations must be considered: PKA could be regulating one of the kinases that phosphorylate PLA2, or, alternatively, PKA may control the increase in intracellular calcium that is essential for activation of cPLA2. Of relevance to the current discussion may be the recent findings that PDE inhibitors reduced the secondary influx of extracellular calcium in human neutrophils (Schudt et al., 1991a) and that in smooth muscle cells, calcium influx through L-type calcium channels was inhibited by cAMP-mediated PKA activation (Orlov et al., 1996).

Effect of PDE inhibitors on airway smooth muscle. Ample evidence exists that cAMP PDE inhibitors relax airway smooth muscle (Rabe et al., 1995; Souness and Giembycz, 1994). In general, it is thought that airway smooth muscle contains both PDE III and PDE IV (de Boer et al., 1992; Rabe et al., 1993). PDE inhibitors appear to be partic-
ularly effective in relaxing smaller airways (Souness and Giebysz, 1994). This is of interest to the present study because we have previously shown that the bronchoconstriction in response to LPS and TX occurs predominantly at smaller airways (Martin et al., 1996; Uhlig et al., 1995).

In the present study, we observed (1) that both rolipram and motapizone attenuated the increase in airway resistance induced by U46619 and (2) that in LPS-treated lungs, rolipram (and also forskolin and db-AMP) only partially prevented the release of TX but completely abolished the bronchoconstriction. Thus, in our model, PDE inhibitors exhibit dual activity: they reduce both the formation and the action of TX. Therefore, measuring the reduction in airway resistance by PDE inhibitors in LPS-treated lungs sums up these two actions. And in fact, both activities appear to be required because motapizone was almost ineffective at 5 μM, a concentration that clearly reduced U46619-induced bronchoconstriction but had no effect on LPS-induced TX release. Release of inflammatory mediators such as TX or TNF appears to be sensitive to PDE IV but not to PDE III inhibitors, whereas spasmylocytic activity may be obtained with inhibition of either PDE isoenzyme. In line with this, at a concentration of 5 μM, both PDE inhibitors attenuated the endothelin-1-induced bronchoconstriction ( Held et al., 1997).

Thus, our data demonstrate that in the present model, inhibition of PDE IV is more effective than inhibition of PDE III. Interestingly, rolipram was also more effective than a PDE III inhibitor in preventing antigen-induced bronchoconstriction in sensitized guinea pigs (Underwood et al., 1994). In summary, PDE IV inhibitors possess both antiinflammatory and spasmylocytic properties that encourage further studies in the treatment of bronchoconstriction in inflamed lung tissue.

References


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