PKA delivery to the distal lung air spaces increases alveolar liquid clearance after isoproterenol-induced alveolar epithelial PKA desensitization

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Submitted 12 April 2004; accepted in final form 19 April 2005

Maron, Michael B., Hans G. Folkesson, Sonya M. Stader, and Jon M. Walro. PKA delivery to the distal lung air spaces increases alveolar liquid clearance after isoproterenol-induced alveolar epithelial PKA desensitization. Am J Physiol Lung Cell Mol Physiol 289: L349–L354, 2005. First published April 22, 2005; doi:10.1152/ajplung.00134.2004.—Isoproterenol (Iso) infusion for 48 h in rats decreases the ability of β-adrenoceptor (β-AR) agonists to increase alveolar liquid clearance (ALC). An impairment in protein kinase A (PKA) function appears to be critical in producing the desensitized ALC response. To test this hypothesis, we used a novel protein delivery reagent (Chariot, Active Motif) to deliver either the PKA catalytic subunit or the PKA holoenzyme to the distal lung epithelium of Iso-infused rats (400 μg·kg⁻¹·h⁻¹, 48 h). After this infusion, ALC was measured by mass balance over 2 h. ALC in Iso-infused rats was 27.9% (SD 5.8) of instilled volume absorbed. Delivery of the catalytic PKA subunit to Iso-infused rats increased ALC to 47.7% (SD 8.9) (P < 0.05). ALC in Iso-infused rats delivered the inactive PKA holoenzyme [29.6% (SD 2.5)] was not increased above baseline values. Subsequent holoenzyme activation by intravenous infusion of the stable cAMP analog Sp-8-Bromo-cAMPS increased ALC to 41.7% (SD 8.8) (P < 0.05). Immunohistochemical localization of Chariot-delivered PKA revealed staining in the alveolar and distal airway epithelium. These data indicate that protein delivery reagents can be used to rapidly deliver biologically active proteins to the distal lung epithelium and that PKA desensitization may be an important rate-limiting event in the development of Iso-induced desensitization of the alveolar epithelial β-AR signaling pathway.

pulmonary edema; β-adrenoceptor signaling pathway; protein kinase A; protein delivery reagents

IT IS NOW WELL ACCEPTED that β-adrenoceptor (β-AR) agonists can increase the rate at which excess fluid is removed from the distal air spaces of the lung. This ability suggests that β-AR agonist therapy might be useful for the treatment of alveolar edema (3, 5, 9, 16). We have recently shown, however, that isoproterenol (Iso) infusion for 48 h in rats produces a dose-dependent inhibition of the ability of β-AR agonists to increase alveolar liquid clearance (ALC) (18). The desensitized ALC response appears to involve impaired signaling at multiple steps in the alveolar epithelial type II (ATII) cell β-AR signaling pathway (18, 19). Specifically, we have observed that ATII cells, freshly isolated from rats after 48 h of Iso infusion at a rate of 400 μg·kg⁻¹·h⁻¹, display β-AR downregulation (18), a decreased ability of forskolin to increase intracellular cAMP (19), and an inability of cAMP to stimulate the cAMP-dependent protein kinase (PKA) (19). The latter observation, coupled with a loss of ability of the stable cAMP analog and PKA activator 8-bromoadenosine-3',5'-cyclic monophosphothioate, Sp-isomer (Sp-8-Bromo-cAMPS) to increase ALC in rats infused with 400 μg·kg⁻¹·h⁻¹ Iso, suggests that defective PKA signaling may play a critical role in producing the impaired ability of β-AR agonists to increase ALC in Iso-infused rats (19).

In its inactive form, the PKA holoenzyme consists of two catalytic and two regulatory subunits. When stimulated by cAMP, the holoenzyme dissociates, and the catalytic subunits become free to phosphorylate their targets. In this study, we attempted to repair the PKA defect by using a newly developed protein delivery reagent (Chariot; Active Motif, Carlsbad, CA) to deliver either the active PKA catalytic subunit or the inactive PKA holoenzyme into the distal lung epithelial cells of Iso-infused rats. When mixed with a protein, Chariot forms a noncovalent complex that stabilizes the macromolecule and subsequently allows the complex to be internalized (20). After internalization, the complex dissociates, and the protein becomes free to exert its biological effect.

METHODS

Experimental model. Male Sprague-Dawley rats (n = 86) weighing 250–300 g (Harlan, Chicago, IL) were used in this study. The rats were housed in the Comparative Medicine Unit at the Northeastern Ohio Universities College of Medicine (NEOUCOM) for at least 1 wk before use under temperature-controlled conditions [20(2)°C] and at a relative humidity of 50% (SD 10). The rats were fed a standard rat chow and had water ad libitum. All experiments were approved by the NEOUCOM Institutional Animal Care and Use Committee.

Desensitization of the ALC response to β-AR agonists was produced by infusing (−)isoproterenol (+)-bitartrate (Iso; Sigma Chemical, St. Louis, MO) for 48 h at a rate of 400 μg·kg⁻¹·h⁻¹ using miniosmotic pumps (Alzet model 2001; Durect, Cupertino, CA) as previously described (18, 19). The pumps were filled under sterile conditions with Iso dissolved in 0.001 N HCl and primed in sterile saline at 37°C overnight. The next morning, the pumps were aseptically implanted subcutaneously under halothane anesthesia. No pumps containing the HCl vehicle were implanted in control rats in this study, because we have previously demonstrated that ALC in vehicle-infused rats did not differ from that of nonperfused rats (unpublished data).

Preparation of Chariot-PKA complex. Chariot was dissolved in phosphate-buffered saline (PBS) according to the manufacturer’s instructions. Chariot (6 μl) was mixed with 100 μl of PBS containing either the PKA catalytic subunit (500 units, purified from bovine heart; Promega, Madison, WI) or the holoenzyme (500 units, purified from bovine heart; Sigma Chemical). After a 2-h incubation (0.4%
CO₂, 37°C), the complex was added to the 5% albumin instillate (prepared by dissolving 50 mg/ml BSA in lactated Ringer solution) for use in the ALC experiments.

**Measurement of ALC.** ALC was measured in pentobarbital sodium (80 mg/kg ip)-anesthetized, tracheotomized, and ventilated rats as previously described (18, 19). The lungs were ventilated with FICO₂ = 1.0 at a respiratory rate of 40 breaths/min with an average tidal volume of 2.7 (SD 0.4) ml. Peak inspiratory pressure was 8.7 (SD 1.0) Torr under baseline conditions, and end-expiratory pressure was atmospheric. The rat was placed at a 45° angle (head elevated), and a polyethylene catheter (PE-50; Clay Adams, Becton Dickinson, Parsippany, NJ) was inserted through the tracheal cannula and into the lungs for the liquid instillation. The 5% BSA solution (3 ml/kg, with or without the Chariot-PKA catalytic subunit or holoenzyme complex) was then instilled into the distal air spaces of the left lung. The alveolar instillate was left in the lungs for 2 h. After this time, a thoracotomy was done, and a blood sample was obtained via aortic puncture for blood gas analysis. The rat was killed by exsanguination, the lungs were removed, and a sample of the remaining instillate liquid was aspirated for analysis of albumin concentration using either refractometry (18, 19) or the Lowry technique (15) modified for microtiter plates. Similar values for ALC were obtained with both techniques. ALC (expressed as % of instilled volume) was determined using the following mass balance equation: ALC = (1 – Alb/Alb) (100), where Alb and Alb are initial and final instillate albumin concentrations, respectively.

**Chariot control experiments.** In three rats, ALC was measured after instillation of Chariot alone to confirm that the Chariot vehicle did not alter baseline ALC. In three additional rats, terbutaline (10⁻⁴ M) was mixed with the instillate-Chariot mixture to determine if the Chariot vehicle affected the ability of β-AR agonists to stimulate ALC. The results from this group of rats were compared with a group of rats (n = 3) receiving terbutaline in the absence of Chariot.

**PKA catalytic subunit experiments.** This set of experiments was designed to determine whether delivery of the active PKA catalytic subunit to the distal lung epithelium of Iso-infused rats would increase ALC. The PKA catalytic subunit used in this study migrates as 35 kDa as determined by SDS-PAGE and Coomassie blue staining. Immunocytochemistry to confirm that the PKA catalytic subunit to the distal lung epithelium of Iso-infused rats would increase ALC. Sp-8-Bromo-cAMPS infusion or no infusion (Table 1). The basic control group for this set of experiments (no Iso infusion, no PKA catalytic subunit experiments). Ten animals were used for this group, because we wanted to ensure that baseline ALC remained stable during the interval between doing the PKA catalytic subunit and holoenzyme experiments.

**Terbutaline administration in Iso-infused rats receiving Chariot-PKA holoenzyme complex.** In six Iso-infused rats, we instilled fluid containing the Chariot-PKA holoenzyme complex and terbutaline (10⁻⁴ M). Although these animals were still receiving Iso during the period in which ALC was measured (because the implanted osmotic minipumps were still functioning), terbutaline was administered to determine if a greater degree of β-AR stimulation could increase ALC after delivery of the PKA holoenzyme in these desensitized rats.

**Evaluation of localization of Chariot-delivered PKA.** Sections of lung tissue from a lung instilled with Chariot-PKA catalytic subunit complexes were examined by immunocytochemistry to confirm that the Chariot-PKA complexes were transported into cells lining the respiratory tract. Lung tissue from a lung instilled with Chariot served as a control. After instillation of the Chariot-PKA complex, the lungs were rinsed with heparinized saline, infused with a solution containing 4% paraformaldehyde, and subsequently infused with aqueous mounting medium. The lungs were cut into 1-mm³ blocks and fixed at 4°C in 4% paraformaldehyde overnight. After fixation, lung tissues were cryoprotected in 20% sucrose for 72 h, after which they were mounted on wooden blocks and sectioned at 10 μm in a Hacker-Bright cryostat. Sections were mounted on coated slides, air-dried, and processed for the presence of PKA.

**Sections of Chariot-PKA-infused lungs were reacted with mouse monoclonal anti-PKA (Abcam, Cambridge, MA) diluted 1:100 in 0.1 M PBS. Binding of the primary antibody was localized using an indirect peroxidase technique (Vectastain kit). Sections were covered-slipped with Permount for microscopy. Chariot-instilled lungs reacted with anti-PKA served as a control for binding. Punctate deposits of dark granules were assumed to reflect binding of the antibody. Sections stained for PKA localization were photographed with a digital camera linked to an Olympus microscope. Images were not retouched except for alterations in brightness and contrast.

**Statistical analysis.** The ALC data were analyzed by a one-way analysis of variance (ANOVA). A Student-Newman-Keuls test was performed post hoc to determine differences between groups as indicated by ANOVA.

**RESULTS**

Rats infused with Iso for 48 h exhibited a 6.2% (SD 3.1) weight loss during the infusion period, a value similar to that observed in our previous studies using this infusion rate (18, 19). Average blood gas determinations for all experiments were: P0₂, 459 Torr (SD 167); PCO₂, 38.2 Torr (SD 10.7); pH, 7.43 (SD 0.10).

Chariot did not alter either baseline or β-AR-stimulated ALC. ALC in rats administered Chariot alone averaged 34.1% (SD 3.2), a value similar to that observed in control rats (Fig. 1). Similarly, Chariot did not affect the ability of terbutaline to increase ALC. Terbutaline increased ALC in Chariot-treated and control rats to, respectively, 59.9% (SD 5.1) and 63.2% (SD 1.2).

The effects of PKA catalytic subunit administration are shown in Fig. 1. Two hours after liquid instillation, ALC in control rats was 29.2% (SD 4.0) of the instilled volume.

| Table 1. Experimental groups for PKA holoenzyme replacement study |
| --- | --- | --- | --- |
| Condition | PKA Holoenzyme | Sp-8-Bromo-cAMPS Infusion | n |
| No infusion | PKA | Sp-8-Bromo-cAMPS | 10 |
| PKA | Sp-8-Bromo-cAMPS | 6 |
| Isoproterenol, 400 μg·kg⁻¹·h⁻¹ | Sp-8-Bromo-cAMPS | 5 |
| PKA | Sp-8-Bromo-cAMPS | 5 |
| PKA | Sp-8-Bromo-cAMPS | 5 |

PKA, protein kinase A; Sp-8-Bromo-cAMPS, 8-bromoadenosine-3’-5’-cyclic monophosphorothioate, Sp-isomer.
Administration of the PKA catalytic subunit increased ALC (P < 0.05) by 77.9%. After 48 h of Iso infusion, ALC [27.9% (SD 5.8)] was no different from that observed in control rats. PKA catalytic subunit administration to the Iso-infused rats increased ALC (P < 0.05) by 71.2%. The increased ALC produced by the PKA catalytic subunit in Iso-infused rats was not statistically different from that produced by the PKA catalytic subunit in control rats.

The effects of PKA holoenzyme administration are shown in Fig. 2. ALC in control rats (no Iso infusion, no PKA holoenzyme administration, and no Sp-8-Bromo-cAMPs infusion) was 29.2% (SD 4.0). The administration of Sp-8-Bromo-cAMPs increased ALC to 46.0% (SD 8.7) (P < 0.05) in rats receiving no Iso infusion or PKA holoenzyme. Infusion of Sp-8-bromo-cAMPs after PKA holoenzyme delivery did not further increase ALC [46.2% (SD 7.2)]. Administration of the inactive PKA holoenzyme did not increase ALC in control rats.

Baseline ALC in the Iso-infused rats was 27.9% (SD 2.8). Administration of either Sp-8-Bromo-cAMPs or the inactive PKA holoenzyme to Iso-infused rats did not increase ALC. Activation of the PKA holoenzyme by infusion of Sp-8-Bromo-cAMPs in Iso-infused rats, however, increased ALC to 41.7% (SD 8.8) (P < 0.05) a value that was 40.7% greater than that observed in Iso-infused rats receiving only the PKA holoenzyme (Fig. 2), but not significantly different from that observed in the control rats receiving only Sp-8-bromo-cAMPs (Fig. 2).

Terbutaline did not increase ALC in Iso-infused rats in which the Chariot-PKA holoenzyme complex had been delivered. ALC in these rats was 33.8% (SD 5.8).

Alveolar pneumocytes from Chariot-PKA-instilled lungs had punctate dark brown intracellular deposits after reaction of tissue with anti-PKA (Fig. 3, B–D). These deposits were absent from control Chariot-instilled samples (Fig. 3A), hence the deposits reflected the uptake of Chariot-PKA into alveolar cells. Additionally, the epithelial lining of the intrapulmonary bronchioles reacted more strongly to anti-PKA (Fig. 3, B and C) than did the airways of the Chariot instilled (control) lung tissues (Fig. 3A). Macrophages stained weakly or not at all in both PKA-infused and control preparations. No staining was observed in the endothelial cells lining the vasculature of the lung.

**DISCUSSION**

We previously found that the ability of β-AR agonists to increase ALC was eliminated in rats that had received a 48-h continuous Iso infusion at a dose of 400 μg·kg⁻¹·h⁻¹ (18). Although β-AR desensitization and downregulation are commonly considered to play an important role in the development of agonist-promoted tachyphylaxis of this signaling pathway, recent studies using a variety of cell and tissue types have indicated that impaired signaling can also develop at multiple points downstream of the receptor (8, 10, 12, 13, 17, 22–26). We have found this to be true for the alveolar epithelial β-AR signaling pathway as well (19). Of special interest were our previous observations showing that Iso infusion impaired the ability both of Sp-8-Bromo-cAMPs to increase ALC in the intact rat and of cAMP to increase PKA activity in ATII cells freshly isolated from rats infused with Iso (19). These observations suggested that prolonged Iso infusion resulted in the development of a rate-limiting postreceptor signaling defect at the level of PKA.

In this study, we report additional evidence that supports this hypothesis. We initially found that administration of the PKA catalytic subunit increased ALC in Iso-infused rats to a level produced either by the catalytic subunit in control rats (Fig. 1) or by β-AR agonists in normal rats (18). This result indicates that the impaired ability of Sp-8-Bromo-cAMPs to increase ALC that occurs in Iso-infused rats (19). Figure 2 represents defective PKA signaling rather than desensitization at points downstream of PKA. Importantly, the ability of the PKA...
Fig. 3. Sections of lung tissue stained for PKA activity. A: Chariot-instilled lung tissue reacted with anti-PKA as a control for staining. Note that the preparation, including the bronchiolar wall (br), is unstained. B: Chariot-PKA instilled lung tissue reacted with anti-PKA. Note that the bronchiolar walls exhibit strong PKA expression. C and D: lung tissue instilled with Chariot-PKA immunoreacted to show PKA activity. Note the intense expression of PKA in the bronchiolar wall in C. Arrowheads: punctate staining of PKA within the cells forming the alveolar lining in C and D. Collectively these data show that Chariot carries PKA into the cells lining the bronchioles and into cells lining the alveoli of the lungs. Scale bar = 50 μm in A and B, 30 μm in C and D.

catalytic subunit to increase ALC in Iso-infused rats indicates that prolonged Iso infusion does not significantly impair the ability of the alveolar epithelial Na⁺ transport proteins (apical Na⁺ channels and basolateral Na⁺-K⁺-ATPases), when appropriately stimulated, to develop a transepithelial osmotic pressure gradient capable of producing a maximal ALC. These data do not rule out the possibility, however, that Iso infusion produced some degree of desensitization of the alveolar epithelial Na⁺ transport machinery. If significant desensitization developed at these effector sites, however, administration of the PKA catalytic subunit would not have significantly increased ALC. Rather, the data indicate that the rate-limiting impairment resides primarily within the alveolar epithelial β-AR signaling pathway.

In a subsequent set of experiments, we found it possible to deliver the inactive PKA holoenzyme to the distal lung epithelium of control rats without increasing ALC (Fig. 2). Holoenzyme delivery to Iso-infused rats also did not increase ALC, but activation by Sp-8-Bromo-cAMPS increased ALC significantly (Fig. 2). On one level, these data indicate that the PKA holoenzyme can be delivered to the distal lung epithelium in its inactive state and retain its ability to become activated by cAMP. On another level, the failure of the holoenzyme alone to increase ALC in the Iso-infused rats provided additional insight into the relative significance of the other Iso-induced alveolar epithelial β-AR pathway signaling defects that we have identified (18, 19). Because we did not remove the miniosmotic pumps containing Iso during the measurement of ALC, the rats continued to receive Iso during the period in which ALC was measured. If impaired PKA function was the only β-AR pathway signaling defect, the holoenzyme should have been activated by an Iso-induced increase in endogenous cAMP, and ALC should have increased. Similarly, our attempt to increase the amount of β-AR stimulation by administering terbutaline to Iso-infused rats after PKA holoenzyme delivery did not increase ALC. These data thus suggest that an additional signaling defect(s) in the β-AR signaling pathway located upstream of PKA prevented both Iso and terbutaline from increasing ALC, even after the PKA defect had been repaired. Previous studies in our laboratory (18, 19) have shown the β-AR and adenylyl cyclase to be potential sites of the upstream signaling impairment. In this regard, we have previously found that ATII cells, freshly isolated from rats infused with Iso at a rate of 400 μg·kg⁻¹·h⁻¹ for 48 h, exhibited both β-AR downregulation (18) and an impaired ability of forskolin to increase cAMP concentration (19). There are other potential sites of upstream desensitization that might also play a role. These include the G proteins, G protein-coupled receptor kinases (GRKs), and cAMP phosphodiesterase. Finney et al. (8) evaluated the possibility of desensitization of these signaling sites and regulatory mechanisms in rats infused with the β-AR agonist albuterol for 7 days. They observed decreases in β-AR density and Gsα expression and increases in cAMP phosphodiesterase activity and GRK-2 expression in lung tissue from these animals. Downregulation of Gsα was considered to be, however, the primary functional impairment responsible for the desensitized ability of albuterol to protect against acetylcholine-induced bronchoconstriction observed in these rats.

In an earlier study, Aoshiba et al. (2) used the same protein delivery agent, Chariot, to deliver caspase-3 to the lungs of mice to produce alveolar wall apoptosis and emphysematous changes. To our knowledge, the current study represents the first attempt to use a protein delivery reagent to repair a defective step in a cellular signaling pathway in vivo. Recent experimental studies have focused on the possibility of using viral vectors to deliver DNA into the lung followed by transcription and translation to produce a desired protein. This approach, however, requires time for transcription and translation to occur, and some viral vectors that have been used appear to elicit an inflammatory response (21). For experimental studies, the ability to use a nontoxic protein delivery reagent to administer a biologically active protein to the distal lung may have a major advantage in producing a biological effect.
with a faster onset. In this regard, we observed a PKA-induced increase in ALC within the 2-h experimental time frame of our experiments. It is possible that this approach may also eventually have clinical applicability. Although β-AR agonist therapy has been suggested as a treatment for severe pulmonary edema (3, 5, 9, 16), the development of desensitization when prolonged administration is needed could be an issue (18, 19). If so, it might be possible to circumvent this problem by using a protein delivery reagent to deliver replacements for defective signaling molecules to the alveolar epithelium. With respect to the potential for intratracheal administration in the clinical condition of pulmonary edema, issues such as mixing of the protein delivery reagent-drug complex with any preexisting alveolar edema would need to be addressed.

Although β-AR agonists and cAMP analogs are well known to increase ALC in intact animals and isolated lungs, we are unaware of any reports showing that more distal signaling molecules of the β-AR signaling pathway (e.g., PKA) can independently increase ALC. The ability of the PKA catalytic subunit to stimulate ALC thus indicates that the administration of β-AR signaling molecules downstream of adenylyl cyclase can increase ALC. Previous supporting evidence for a role of PKA signaling in β-AR-stimulated ALC are observations by Yue et al. (27), who reported that PKA increased the open time and open probability of low-amiloride-affinity Na+ channels in isolated rat ATII cells.

To determine the intrapulmonary localization of the Chariot-PKA delivery, we used immunocytochemistry to detect PKA and observed staining in both the alveolar and distal airway epithelium (Fig. 3). Although we were not able to distinguish between alveolar epithelial type I (ATI) and ATII cell uptake of the Chariot-PKA complex, it appeared that both cell types were stained. These data indicate that the Chariot-PKA complex was delivered to multiple cell types of the distal lung epithelium, including the alveolar epithelial cells. We previously reported that PKA desensitization appeared to play a major role in the impaired ability of β-AR agonists to increase ALC in rats infused with Iso for 48 h and that freshly isolated ATII cells obtained from Iso-infused rats exhibited a reduced ability of cAMP to stimulate PKA activity (19). Although historically, the ATII cell has been generally considered to be responsible for alveolar epithelial vectorial Na+ transport, recent studies have shown that the ATII cell expresses Na+ transport proteins and engages in Na+ transport (6, 11). More recently, Liebler et al. (14) have shown that ATII cells also express β-ARs and GRK-2 and have suggested that β-ARs of both ATI and ATII cells may become desensitized after prolonged β-AR agonist exposure. Additionally, a number of studies have shown that distal airway epithelial cells have the ability to transport Na+, suggesting that some portion of the lung’s ability to clear fluid may reside at the distal airway epithelium (1, 4). Airway epithelial cells also express β-ARs (7). Although we have shown that ATII cells exhibit PKA desensitization (19), it is not clear from our data if repair of the PKA signaling defect in the ATII cell was uniquely responsible for the observed recovery of a stimulated ALC after the delivery of PKA.

In conclusion, we found that the administration of either the active PKA catalytic subunit or the inactive PKA holoenzyme followed by activation with Sp-8-Bromo-cAMPS increased ALC in Iso-infused rats. These data are consistent with our previous observations suggesting that prolonged Iso infusion results in the development of a rate-limiting postreceptor signaling defect in the alveolar epithelial β-AR signaling pathway at the level of PKA (19). Moreover, the results of this study also support the concept that protein delivery reagents might be used to repair signaling pathway defects in the distal lung epithelium.

Grants

This study was supported by American Heart Association, Ohio Valley Affiliate Grant 0355364B, and a grant from the Ohio Board of Regents Research Challenge program.

References

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