Impaired alveolar liquid clearance after 48-h isoproterenol infusion spontaneously recovers by 96 h of continuous infusion

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Submitted 17 January 2006; accepted in final form 14 February 2006

Maron, Michael B., Hans G. Folkesson, Sonya M. Stader, and Cheryl M. Hodnichak. Impaired alveolar liquid clearance after 48-h isoproterenol infusion spontaneously recovers by 96 h of continuous infusion. Am J Physiol Lung Cell Mol Physiol 291: L252–L256, 2006. First published February 17, 2006; doi:10.1152/ajplung.00022.2006.—We previously demonstrated that 48-h isoproterenol (Iso) infusion in rats impaired the ability of β-adrenoceptor (β-AR) agonists to increase alveolar liquid clearance (ALC). In this study, we determined whether this impairment persisted over longer time periods by infusing 400 µg·kg⁻¹·h⁻¹ Iso by osmotic minipump for 24–144 h (n = 6–7/group). ALC in control rats was 19.0 ± 2.4 (SD)% of instilled volume absorbed per hour. In Iso-infused rats, ALC was elevated at 24 h (34.9 ± 2.4%) and decreased at 48 h (15.2 ± 4.4%) and had recovered to 24 h values at 96 h (37.3 ± 3.8%) and 144 h (35.2 ± 3.3%). Plasma Iso concentrations remained elevated at all Iso infusion times. Peripheral lung β2-AR expression exhibited a parallel time course, with a reduction in expression observed at 48 h, followed by an increase to 24 h values at 96 and 144 h. Propranolol prevented the increase in ALC observed at 96 and 144 h, indicating that recovery in ALC was mediated by a recovery of the ability of the distal lung epithelium to respond to β-AR stimulation.

pulmonary edema; β-adrenergic receptor signaling pathway; receptor desensitization.

We showed previously (8, 10, 11) that 48-h isoproterenol (Iso) infusion in rats produces a dose-dependent desensitization of the alveolar epithelial β-adrenoceptor (β-AR) signaling pathway. This in turn impairs the normal ability of β-AR agonists to increase the rate at which excess fluid (e.g., pulmonary edema fluid) is removed from the alveolar air spaces (10). We subsequently found (8, 10, 11) that this desensitization occurs at multiple steps in the alveolar epithelial β-AR signaling pathway. In this regard, alveolar epithelial type II (ATII) cells isolated from rats infused with 400 µg·kg⁻¹·h⁻¹ Iso for 48 h exhibited a reduced β-AR density (10) and impaired abilities of forskolin and cAMP to stimulate, respectively, adenyl cyclase (AC) and cAMP-dependent protein kinase (PKA) (8, 11). In intact rats infused with this Iso dose for 48 h, these cellular changes were accompanied by a loss in the ability of forskolin and the PKA activator 8-bromoadenosine-3′,5′-cyclic monophosphorothioate, Sp isomer, as well as β-AR agonists, to increase alveolar liquid clearance (ALC) (8, 11). The impairment in β-AR-stimulated ALC could be reversed by the replacement of the desensitized PKA with either the catalytic subunit of PKA or the PKA holoenzyme followed by cAMP activation, thus suggesting that PKA may be a rate-limiting step (8).

These observations may have potential clinical significance in view of suggestions that β-AR agonists might be used for the treatment of severe pulmonary edema (1, 5, 15). In this regard, Perkins et al. (15) recently reported that extravascular lung water was significantly lower in patients with acute lung injury after 7 days of continuous intravenous albuterol infusion compared with those receiving a saline infusion. Our initial studies (8, 10, 11) were restricted, however, to a 48-h Iso infusion period. In preliminary studies, we extended the Iso infusion period up to 144 h and found that the impaired ability of β-AR agonists to increase ALC appeared to spontaneously recover by 96 h of continuous Iso infusion. This observation was surprising, because there does not appear to be evidence that agonist-promoted desensitization can recover in the continued presence of the desensitizing agonist. Accordingly, the objectives of this study were to document this phenomenon and to determine whether the recovery of β-AR agonist-stimulated ALC resulted from a recovery of β-AR function.

**METHODS**

**Experimental Model**

Male Sprague-Dawley rats (n = 103) 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 for at least 1 wk under temperature-controlled conditions [20 ± 2 (SD)°C] and at a relative humidity of 50 ± 10% before experimental use. The rats were fed a standard rat chow and had water ad libitum. All experiments were approved by the Northeastern Ohio Universities College of Medicine Institutional Animal Care and Use Committee.

Miniosmotic pumps (Alzet model 2001; Durect, Cupertino, CA) were filled under sterile conditions with the β-AR agonist (−)-isoproterenol (+)-bitartrate (Iso; Sigma Chemical, St. Louis, MO) dissolved in 0.001 N HCl and primed in sterile saline at 37°C overnight. This pump model is designed to release drugs continuously for 7 days. The next morning, the filled pumps were aseptically implanted subcutaneously under halothane anesthesia as previously described (8, 10, 11). An Iso concentration was selected to allow the pumps to deliver the drug at an infusion rate of 400 µg·Iso base·kg⁻¹·h⁻¹. This dose was selected because it produced the largest degree of desensitization in previous studies (10).

**Determination of ALC**

ALC was measured as previously described (3, 8, 10, 11). The rats were anesthetized with 80 mg/kg (ip) pentobarbital sodium (Abbott

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Laboratories, N. Chicago, IL), with the anesthetic being supplemented as needed. Body temperature was monitored with a rectal temperature probe and maintained with a water-perfused heating pad. A polyethylene tracheal cannula (PE-240; Clay Adams, Becton-Dickinson, Sparks, MD) was placed in the rat’s airway via a tracheotomy and connected to a mechanical ventilator (Harvard Apparatus, Nantucket, MA). The rat was ventilated with an inspired O_{2} fraction of 1.0 at a respiratory rate of 40 breaths/min with an average tidal volume of 2.7 ± 0.2 (SD) ml. Peak inspiratory pressure was 9.2 ± 1.8 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) was inserted through a port in the tracheal cannula and into the lungs for liquid instillation. The rats were allowed to stabilize for 10 min after surgery before the start of the experiment. At this time, 3 ml/kg of a 5% bovine serum albumin (Sigma) solution in Ringer lactate (Baxter Healthcare, Deerfield, IL) was instilled into the left lung at a rate of 0.20 ml/1.5 min with a 1-ml syringe. The solution had been adjusted previously with NaCl to an osmolality of 315 mosmol/kgH_{2}O. The alveolar instillate was left in the lungs for 1 h beginning at the completion of instillation. After 1 h, a thoracotomy was done, the rat was euthanized by exsanguination, the lungs were removed, and a sample of the remaining instilled liquid was aspirated for analysis of albumin concentration by refractometry. The refractometer (American Optical, Buffalo, NY) was calibrated with a series of albumin standards (Sigma). ALC was determined with the following mass balance equation: \[ \text{ALC} = \frac{1 - \frac{\text{Albi}}{\text{Albf}}}{(100)}, \] where ALC is expressed as the percentage of instilled liquid that left the air spaces during the 1-h observation period and Alb{B} are the initial and final instillate albumin concentrations, respectively.

Experimental Design

Effect of prolonged Iso infusion on Iso-stimulated ALC. In this set of experiments, rats (n = 6–7/group) were infused with Iso for 24, 48, 96, or 144 h and then ALC was measured as described above. Measurement of baseline ALC was made for comparison in rats receiving no Iso infusions (n = 7). We previously found (unpublished data) that baseline ALC is unaffected by implantation of pumps containing the Iso vehicle.

Analysis of plasma Iso concentration. Arterial blood samples were drawn at the end of the ALC experiment for analysis of plasma Iso (n = 2 rats each for 48-, 96-, and 144-h Iso infusions). Blood samples from two additional Iso-infused rats were obtained 4 h after pump implantation to determine how quickly plasma Iso concentration reached a steady-state level. Iso was extracted from plasma with acid-washed aluminum oxide and the catecholamine extraction protocol of BioAnalytical Systems (BAS). Iso was then resolved on a HPLC electrochemical detection system with a CoulArray analytical cell (ESA BioScience, Chelmsford, MA) using a BAS Phase II OAS 3 μm 100 × 3.2-mm column, and a mobile phase consisting of (mM) 27.4 citric acid, 50 sodium acetate, 10 sodium hydroxide, 0.1 EDTA, and 5% methanol at pH 4.5.

Effect of Iso infusion on peripheral lung β_{2}-AR expression. These experiments were done to determine whether changes in peripheral lung tissue β_{2}-AR protein expression were correlated with the recovery of Iso-stimulated ALC. Lung tissue from five rats at each time point was homogenized on ice in T-Per reagent (Pierce, Rockford, IL) containing protease inhibitors [aprotinin (30 μg/ml; Sigma) and leupeptin (1 μg/ml; Sigma)] with a homogenizer (Tissue Tearer). The tissue homogenate was centrifuged at 13,000 g for 5 min at +4°C. The supernatant (membrane and cytosol fraction) was collected, aliquotted in multiple vials per sample, and snap-frozen in liquid nitrogen. One vial was used to determine total protein concentration of the sample to ensure equal loading of the electrophoresis gel. Aliquots were stored at −80°C until analysis.

Polycarboxylamide gel electrophoresis and transfer to nitrocellulose membranes (Pierce) were carried out with standard protocols. After electrophoresis and transfer, the nitrocellulose membranes were blocked [SuperBlock Dry Block blocking buffer (Tris-buffered saline; TBS; Pierce)] for 1 h. Primary antibody incubations were then carried out overnight at +4°C on an orbital shaker. Anti-β_{2}-AR antibody was purchased from Santa Cruz Biotechnology (H-20; sc-569; Santa Cruz, CA) and directed against the human β_{2}-AR COOH terminus. This antibody specifically recognizes a membrane protein of 68 kDa in rats. After the incubation, the membranes were washed 5 × 10 min (pH = 7.5; TBS with 0.1% Tween 20). The membranes were then incubated with the enzyme-conjugated secondary antibody (goat-anti-rabbit IgG) for 1 h at room temperature. After incubation, membranes were washed again. The substrate solution (SuperSignal West Femto; Pierce) was then added and incubated for 5 min. The luminescence signal was detected with a Kodak image analyzer and analyzed densitometrically with TotalLab software (Nonlinear Dynamics, Newcastle upon Tyne, UK).

Effect of terbutaline on ALC after prolonged Iso infusion. Terbutaline [at a dose known to maximally stimulate ALC (10^{-4} M, dissolved in the instillate)], was used in this set of experiments for two reasons. First, terbutaline-stimulated ALC in the absence of Iso infusion was used as a reference point to compare the extent of the recovery in ALC occurring by 96 h of Iso infusion. Second, terbutaline was administered at all Iso infusion time points to determine the maximal β-AR-stimulated ALC. Rats (n = 6/group) were infused with Iso for 24, 48, 96, or 144 h and then anesthetized for ALC measurements. Terbutaline instilled into the lungs of normal rats (n = 6) served as the control group for these experiments.

Effect of propranolol on ALC after prolonged Iso infusion. This set of experiments was done to determine whether the recovery of ALC observed by 96 h was mediated by a recovery of β-AR function. Rats (n = 6–7/group) were infused with Iso for 24, 48, 96, or 144 h and then anesthetized for ALC measurements. For these experiments, β_{1}-propranolol (2.5 × 10^{-4} M; Sigma) was dissolved in the instillate. Propranolol instilled into the lungs of normal rats (n = 7) served as a control group for the Iso infusion studies.

Statistical analysis. Multigroup comparisons of data were done by ANOVA followed by Student-Newman-Keuls post hoc test. Comparisons between two groups were made by unpaired Student’s t-test.

RESULTS

Systemic Effects of Iso Infusion

Plasma Iso concentrations for each of the Iso infusion periods are shown in Fig. 1 and remained elevated at a steady-state level during the entire period. By 4 h of Iso infusion, plasma Iso concentration had achieved a steady-state level (44.9 ± 8.2 ng/ml). After 24 h of Iso infusion, the rats exhibited an average weight loss of 5.0%. Body weight remained stable at 48 h [5.4% loss, a value similar to that previously observed at this Iso infusion rate at 48 h (8, 10, 11)]. At 96 and 144 h, body weight was increased by 4.8% and 13.3%, respectively, over baseline values.

Effect of Iso Infusion on ALC and Peripheral Lung β_{2}-AR Expression

Figure 2 compares the effect of Iso on ALC and peripheral lung β_{2}-AR expression. Twenty-four-hour Iso infusion increased ALC to 34.9 ± 2.4% of instilled volume absorbed per hour (baseline ALC in control rats was 19.0 ± 2.4%). By 48 h, Iso-stimulated ALC was reduced to a degree (56%) similar to that observed in our previous studies (8, 10, 11). By 96 h, ALC had recovered to values observed at 24 h, with no further
change observed at 144 h, β2-AR expression was reduced to 80 ± 12% of its baseline value after 24 h of Iso infusion and to 34 ± 4% of baseline by 48 h of Iso infusion (Fig. 2). By 96 h of Iso infusion, β2-AR expression had recovered to values observed at 24 h and remained stable thereafter.

**Effect of Terbutaline on ALC After Prolonged Iso Infusion**

Terbutaline increased baseline ALC by 107% (Fig. 3). At 24 h of Iso infusion, ALC was equivalent to that observed in control rats administered terbutaline and was not further increased by terbutaline. By 48 h of Iso infusion, both Iso- and terbutaline-stimulated ALC were significantly reduced to values observed in our previous studies (8, 10, 11). Terbutaline administration at either 96 or 144 h did not further increase ALC over that produced by Iso alone.

**Effect of Propranolol on ALC After Prolonged Iso Infusion**

Propranolol did not affect baseline ALC (Fig. 4). Propranolol administration decreased the elevated ALCs observed at 24, 96, and 144 h of Iso infusion but had no effect on the depressed ALC observed at 48 h (Fig. 4).

**DISCUSSION**

We previously found (10) that 48-h continuous Iso infusion in rats resulted in a dose-dependent impairment in the ability of β-AR agonists to increase ALC. At the highest Iso infusion rate (400 μg·kg⁻¹·h⁻¹ over 48 h), the distal lung epithelial liquid clearance mechanisms became refractory to β-AR stimulation (8, 10, 11). The major finding of the present study was that this impairment spontaneously recovered in the continued presence of Iso when the infusion period was extended to 96 and 144 h (Fig. 2). The basis for this conclusion is the observation that ALC at 96 h of Iso infusion was equivalent to that observed at 24 h of Iso infusion (Fig. 2) and that produced by terbutaline (at a dose generally considered to maximally stimulate ALC in this model) in control rats (Fig. 3). To further test our conclusion that the increased ALC rates observed at 96 and 144 h of Iso infusion represented maximum β-AR-stimulated rates, we measured ALC in additional rats at these time points in the presence of terbutaline. ALC was not further increased over that produced by Iso alone (Fig. 3).

The observation that plasma Iso concentration remained significantly elevated during the entire 144-h Iso infusion period importantly indicated that the recovery was not due to a decrease in agonist exposure during this period (Fig. 1). Rather, two lines of evidence suggested that the recovery of β-AR-stimulated ALC involved a recovery of distal lung β-AR signaling function. First, peripheral lung β2-AR expression underwent a pattern of change parallel to that observed for ALC, demonstrating a significant reduction in expression at 48 h of Iso infusion followed by an increased β2-AR expression at 96 h that remained stable at 144 h (Fig. 2). The reduction in peripheral lung β2-AR expression, as well as the impaired β-AR-stimulated ALC (8, 10, 11) observed at 48 h, was consistent with our previous observations of a reduction in...
reduced the Iso-induced elevations in ALC at 96 and 144 h, suggesting a regulatory process. Second, the days exhibited a reduction in peripheral lung tissue from the same animal. Although the parallel patterns of recovery in ISO-stimulated ALC, propranolol sensitivity, and peripheral lung β2-AR expression observed in this study are internally consistent, we have not been able to find precedent for such a response from a survey of studies in which ISO has been infused in rats to produce lung β-AR downregulation. For example, Nishikawa and colleagues (13), using a dosing regimen identical to that used in our study (400 μg·kg⁻¹·h⁻¹ delivered by osmotic minipump) for periods of up to 7 days, found persisting significant reductions in peripheral lung tissue β-AR density (as determined by radioligand binding) that did not recover between days 1 and 7. Both β1- and β2-subtype numbers remained decreased during the entire study period. Our results differed in that we observed minimal β2-AR downregulation at 24 h (Fig. 2), as well as recovery of β2-AR expression and function. The reason for these apparent differences is unknown but could relate to differences in rat strains studied (17), variation in possible extracellular regulatory (e.g., endocrine) responses that might intervene in the desensitization process (2, 9, 12), or differences in sample preparation and assay of β-AR numbers (13).

The inclusion of a 24-h evaluation time in this study allowed us to further refine the time course of ISO-induced desensitization of the ALC response. Previously, we observed no impairment in the ability of epinephrine to stimulate ALC after 4 h exposure (3) but have consistently observed an impaired...
continuous Iso exposure and becomes manifest by 48 h. At 24 h there was no apparent decrease in the ability of Iso or terbutaline to increase ALC, and $\beta_2$-AR expression remained at $\sim 80\%$ baseline levels. These data indicate that Iso-induced desensitization of the ALC response develops after 24 h of continuous Iso exposure and becomes manifest by 48 h.

Sartori et al. (16) also evaluated the possibility that prolonged $\beta$-AR agonist administration desensitizes the ability of $\beta$-AR agonists to increase ALC in a study in which they infused albuterol in mice for periods of up to 144 h. Consistent with our previous (10) and present results, they observed a reduction in distal lung tissue $\beta$-AR density and an impaired ability of the infused albuterol to increase ALC (the latter developing by 72 h of infusion). There were notable differences in response, however. As discussed previously (11), there was no evidence for the development of PKA desensitization in mice as observed in Iso-infused rats (8, 11). With respect to our current study, Sartori et al. (16) found that the ability of terbutaline to increase ALC was preserved when measured at 24, 72, and 144 h after albuterol infusion. No ALC measurements were made at 48 h, so it is unknown whether the terbutaline-stimulated ALC response underwent a similar pattern of desensitization and recovery in the mouse. In contrast to our observations that both $\beta_2$-AR protein expression (Fig. 2) and the ability of the infused Iso to increase ALC (Fig. 2) recovered by 96 h, Sartori et al. (16) found that the reduced $\beta$-AR density and the impaired ability of the infused albuterol to increase ALC did not recover. The reasons for these different patterns of response are unknown but could relate to species differences, characteristics of the agonist (e.g., the use of full vs. partial agonists), or drug doses.

In conclusion, we found that by 96 h of continuous Iso infusion, the impairment in Iso-induced and propranolol-sensitive ALC and reductions in peripheral lung $\beta_2$-AR expression observed at 48 h of Iso infusion had recovered to values observed at 24 h. These observations appear to be novel with respect to those of previous studies examining the process of $\beta$-AR desensitization and down-regulation in intact animals. The maintenance of elevated plasma Iso concentrations during the entire Iso infusion period coupled with the consistency in direction and time course of our ALC and $\beta_2$-AR expression results lend strength to the conclusion that, under some conditions, the desensitized ability of $\beta$-AR agonists to increase ALC can recover in the presence of the agonist responsible for desensitization. Finally, the maintained ability of $\beta$-AR agonists to stimulate ALC observed during the first 24 h of Iso exposure in this study suggests that, even at high doses, $\beta$-AR-agonist therapy might effectively help patients with severe pulmonary edema to clear fluid from their air spaces during this time frame.

**GRANTS**

This study was supported by American Heart Association, Ohio Valley Affiliate Grants 0051029B and 0355364B, and a grant from the Ohio Board of Regents Research Challenge program.

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