Assessment of Glucocorticoid Lung Targeting by *ex-Vivo* Receptor Binding Studies in Rats

Guenther Hochhaus,[1,4] Ricardo J. Gonzalez-Rothi,[2] Anatoly Lukyanov,[2] Hartmut Derendorf,[1] Hans Schreier,[3] and Teresa Dalla Costa[1]

Received May 26, 1994; accepted August 15, 1994

Triamcinolone acetonide (TA, 22 μg) was given to rats by intravenous (IV) injection or intratracheal (IT) instillation. Free glucocorticoid receptors were monitored over time in liver and lung using an *ex-vivo* receptor binding technique. After IV administration of a TA solution, the reduction of free receptors over time was very similar in lung and liver (AUC_{Lung} = 280 ± 47 %*h; AUC_{Liver} = 320 ± 76 %*h). Intratracheal instillation of the same solution produced time profiles which mirrored those of IV injection (AUC_{Lung} = 260 ± 41 %*h; AUC_{Liver} = 330 ± 50 %*h). The lack of lung targeting was also reflected in the failure to show any significant difference in the pulmonary targeting factor T (AUC_{Lung}/AUC_{Liver}) between IV (T = 0.84 ± 0.18) and IT (T = 0.78 ± 0.03) administration. In contrast, a certain degree of lung specificity was observed after IT instillation of a glucocorticoid suspension (22 μg; AUC_{Lung} = 160 ± 135 %*h; AUC_{Liver} = 65 ± 91 %*h, T = 2.3 ± 0.5) as indicated by significant differences in T between IV injection and IT instillation (p = 0.038). The method presented provides a means of simultaneously assessing pulmonary and systemic effects after different forms and routes of administration and might be of value in further studying multiple aspects of inhalation glucocorticoid therapy.

KEY WORDS: triamcinolone acetonide; lung instillation; lung targeting; *ex-vivo* receptor binding; corticosteroids.

INTRODUCTION

Asthmatic disorders are commonly treated with inhaled glucocorticoids. Clinical studies have demonstrated antiasthmatic efficacy (e.g., prevention and control of symptoms) and reduced systemic side effects of glucocorticoid inhalation therapy. However, the extent to which these antiinflammatory effects are mediated via topical or systemic actions is not easily discernible from these studies. Indeed, some controversy has emerged in the literature whether the antiasthmatic effects of inhaled glucocorticoids might be primarily mediated by systemic absorption rather than through locally active drug effects (1-3).

While systemic pharmacological endpoints such as the effects on blood lymphocytes have been identified (4), sensitive pharmacological endpoints with high resolution are not available for the determination of glucocorticoid effects on pulmonary tissues. The extent of the pharmacological glucocorticoid response, however, is directly related to the fraction of occupied glucocorticoid receptors (5). Glucocorticoid receptors have been identified in liver (6) and lung (7) including a number of pulmonary cell types, which could be involved in both the pathogenesis and treatment of asthma (8). We describe here an animal model by which we simultaneously assess systemic spill-over and topical activity of pulmonary delivered drug by monitoring free glucocorticoid receptors over time in lung and liver after intratracheal instillation. This form of administration circumvents the oral absorption component generally observed after inhalation but reaches wide areas of the lung including alveoli (9). It further allows exact dosing and homogenous distribution throughout both lung sections (10). The model was used to compare triamcinolone acetonide after pulmonary instillation and intravenous injection and is suitable to assess lung specific activity of glucocorticoids.

MATERIALS AND METHODS

Materials. Unlabeled chemicals of analytical grade were obtained from Sigma Chemical Co. (St. Louis, MO). [1,2,4-^{3}H]triamcinolone acetonide (45 Ci/mmol) was purchased from New-England Nuclear (Boston, MA).

Animals. Specific-pathogen-free male F-344 rats, weighing approximately 250 g were housed in a 12-hr light dark cycle constant temperature environment. Animals were allowed free access to water and rat chow.

Drug Preparations. Triamcinolone acetonide (TA) was dissolved in ethanol to obtain a concentration of 15 mg/ml. Immediately before dosing, this stock solution was diluted with isotonic saline to obtain 22 μg/100 μl and administered before visible precipitation occurred. In one part of this study, a commercially available triamcinolone acetonide suspension (Kenalog-10®, Westwood-Squibb, Buffalo, NY.) was diluted with saline to achieve the same drug concentration of 22 μg/100 μl.

Drug Administration Procedure. All animal procedures were approved by the Animal Care Committee of the University of Florida. The animals were anesthetized via intraperitoneal injection with a combination of xylezine 20 mg/ml, ketamine 100 mg/ml, and acepromazine 10 mg/ml. The anesthetic mixture was prepared daily and used within two hours after mixing. The dose of the mixture ranged from 0.2-0.5 ml per animal.

For the IV injection, a 1 cm midline incision was made in the area of the femoral vein. Using a tuberculin syringe with a 27 gauge needle, 0.1 ml of saline or TA solution were slowly injected into the vein. The incision was then sutured with 4.0 silk. For IT instillation, the neck of the completely anesthetized, but spontaneously breathing animal was shaved and a 1 cm midline vertical incision was made originating above the sternal notch in a cephalad direction. The neck muscles were carefully dissected midline, until the trachea was exposed. Using a tuberculin syringe with a 27 gauge needle, 0.1 ml of saline, triamcinolone acetonide solution or suspension, vortexed immediately before use, were slowly injected into the trachea just above the carina. Immediately after injection, the animal was rotated slowly in all

[1] Department of Pharmaceutics, College of Pharmacy, University of Florida, Gainesville, Florida, 32610.
[2] College of Medicine, Department of Medicine, Division of Pulmonary Medicine, University of Florida, Gainesville, Florida, 32610.
[3] Center for Lung Research, Vanderbilt University, School of Medicine, Nashville, Tennessee, 37232-2650.
[4] To whom correspondence should be addressed.

0724-8741/95/0100-0134$07.50/0

Fig. 1. Photomicrograph of whole rat lung saggital sections fixed after *in-vivo* intratracheal instillation of india ink. The thin arrows identify india ink deposition along the tracheo-bronchial tree, bold arrows show deep alveolar deposition in both lungs.

positions with the head up, to allow gravity to evenly distribute the injected material. The incision was then sutured with 4.0 silk, and the animal placed on its abdomen in a cage with rodent bedding, and allowed to recover while observed. On a given day, animals (one animal per time point) were decapitated 15 min, 1 hr, 3 hr and 6 hr after drug administration (IT instillation or IV injection) and 4 hours after saline administration (sham). Their lungs and livers were immediately processed for receptor binding studies.

Three separate experiments were performed on different days for every form of administration (IV or IT administration of TA solution, IT instillation of TA suspension).

To visually document intrapulmonary deposition via the intratracheal instillation technique, 0.1 ml of undiluted india ink was instilled into the lungs of untreated animals. The lung were excised en bloc, fixed in formalin and whole lung saggital sections were imbedded in paraffin, stained with hematoxylin and eosin and photographed.

Receptor Binding Assays. Immediately after decapitation, the lung without trachea and a liver lobe were resected, and placed on ice. The weighed tissue was added to 10 volumes of ice-cooled incubation buffer (10 mM Tris/HCl, 10 mM sodium molybdate, 2 mM 1,4-dithiothreitol) and homogenized in a Virtis 45 homogenizer at 40 % of full speed, for 3 periods of 5 seconds with a 30 sec cooling period between each step. 1/10 vol of 5% charcoal (prepared in incubation buffer) was added to the homogenate and mixed. After 5 minutes, the suspension was centrifuged at 4 °C and 50,000g for 10 min in a Beckman centrifuge equipped with a JA-21 rotor to obtain a clear supernatant. This procedure gave identical results (B_{max} and K_d) to a previously published *ex-vivo* procedure (8), but was more convenient to perform. Aliquots of the supernatant (150 µl) were transferred into microcentrifugation tubes that contained 25 µl of ^{3}H-triamcinolone acetonide in incubation buffer (final concentration: 0.25, 0.5, 2, 4, 10 nM) and 25 µl of incubation buffer or 20 µl of unlabeled TA (10 µM) to determine non-specific binding. After a 16-24 hours incubation period at 4 °C (the variability in incubation time was shown to not affect the B_{max} of a preparation with 50% receptor occupancy), 20 µl of the mixture was removed to determine the amount of total radioactivity. The unbound glucocorticoid was removed by addition of a 2% suspension of activated charcoal in buffer (20 µl). The mixture was incubated for 5 min on ice and then was centrifuged at 10,000g for 5 min in a Fisher Microcentrifuge. The radioactivity (DPM) in 300 µl of supernatant was determined by liquid scintillation counting. All determinations were performed in duplicate.

Estimates for total bound ^{3}H-TA (TA_T), nonspecifically bound ^{3}H-TA (TA_{ns}) and free ^{3}H-TA (TA_F) were used to determine the number of available binding sites (B_{max}) and

Table I. Cumulative Receptor Occupancy (AUC) and Lung Targeting Factor T After Different Forms of Administration

	Occupied glucocorticoid receptors[a], AUC (% * h)		
	Intravenous solution	Intratracheal solution	Intratracheal suspension
Lung	280 ± 47	260 ± 41	160 ± 135
Liver	320 ± 76	330 ± 50	65 ± 91
Targeting factor[b] (AUC_{Lung}/AUC_{Liver})	0.84 ± 0.18	0.78 ± 0.03	2.3 ± 0.5

[a] Receptor occupancy time profiles from Fig. 2 were converted as % of occupied receptors and the AUC was calculated by the trapezoidal rule.

[b] Average and standard deviations calculated from paired samples.

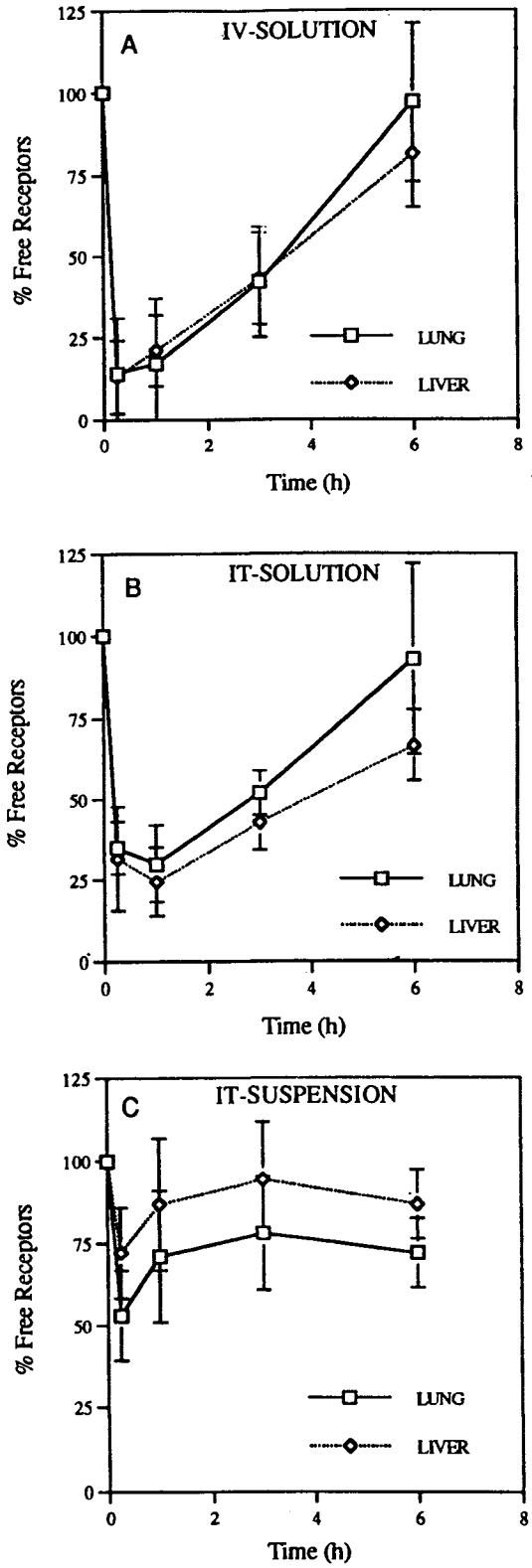

Fig. 2. Time course of free glucocorticoid receptors in lung and liver. Rats were treated with 22 μg triamcinolone acetonide (TA) and free receptors were determined by an *ex-vivo* receptor binding assay. Mean and standard deviations of three animals per time point are shown for intravenous administration of TA (A), intratracheal instillation of a TA solution (B) and intratracheal instillation of a TA suspension (C).

the equilibrium binding constant (K_d). Therefore, the data were fitted to equation 1 using the non-linear curve fitting procedure MINSQ (Micromath, Salt Lake City).

$$TA_T = (B_{max} * TA_F / (TA_F + K_d)) + TA_{NS} \quad (1)$$

In some cases, B_{max} could not be determined by this procedure, as the high *in-vivo* receptor occupancy did not allow a reliable estimate of free receptor sites. In this case, B_{max} was estimated from TA_T values after incubation with 10 nM ^{3}H-TA and the corresponding non-specific binding term. B_{max} values expressed as pmol/g liver were consequently transformed in % of control. The lung weight increased slightly (<50%) during the time of the experiment, even after intravenous administration of saline. This increase, likely to be related to the anesthesia, resulted in a decline of receptors over time when B_{max} values were expressed as pmol/g tissue, fmol/mg protein, but not as pmol/lung (results not shown). For better comparison, pulmonary and hepatic binding data were expressed as percent of B_{max} of baseline (sham rats). The cumulative change from baseline (AUC) was calculated for the 6 hour investigation period by the trapezoidal rule from percent free receptor-time profiles. The pulmonary targeting factor (T) was defined as AUC_{Lung}/AUC_{Liver}. Assuming a one compartment body model, the elimination rate k_e was determined from the slope of the linear portion of the effect-time profiles after intravenous administration, with the slope being equal to $B_{max} * k_e/4$ (11).

RESULTS

The intratracheally instilled india ink was visualized throughout the tracheo-bronchial tree and deep into the alveolar regions of both lung segments (Fig. 1).

In sham rats, glucocorticoid receptor concentrations were 15.7 ± 3.6 pmol/g (n = 9) in liver cytosol and 6.9 ± 2.6 pmol/lung in pulmonary tissue (n = 9, average lung weight: 1.2 ± 0.3 g). K_d values were 0.26 ± 0.05 nM in liver and 0.21 ± 0.04 nM in lung cytosol. After intravenous administration of 22 μg of triamcinolone acetonide, free receptor concentrations in liver and lung cytosol declined rapidly with the lowest free receptor concentration seen 15 min after dosing (Fig. 2a). Receptor levels returned to baseline within 6 hours. Receptor occupancy-time profile paralleled each other in liver and lung. There was, however, a small but significant difference between the AUC of hepatic and pulmonary receptor occupancy, which was also reflected in the lung targeting factor being slightly smaller than unity (0.84, Table I). From the slope of the decline of free receptors over time, an elimination half-life of 1.2 hours was calculated for TA. Fig 2b illustrates the time profile of receptor occupancy in liver and lung after intratracheal instillation of an aqueous solution of TA. The receptor occupancy-time profile was similar to the one after IV injection. Cumulative receptor occupancy-time profiles (AUCs) were not significantly different after IV injection and IT instillation of a TA solution ($p > 0.05$ for lung and liver data, respectively). As a result, the targeting factors of Table I (AUC_{lung}/AUC_{Liver}) did not differ significantly between the two routes of administration.

After intratracheal administration of a TA suspension, however, the overall reduction in free receptors was less

pronounced (Fig. 2c), but free receptor levels did not return to baseline during the 6 hour observation period. In addition, the AUCs of occupied lung and liver receptors (Table I) differed significantly from those after IV-administration ($p < 0.05$ for lung and liver, Table I) for the 6 hour observation period. More importantly, the resulting targeting factor T (Table I) differed significantly from the other treatments (IV/IT-Suspension: $p = 0.038$, IT-Solution/IT-Suspension: $p = 0.034$).

DISCUSSION

The presented *ex-vivo* model is suitable to monitor systemic and local effects of glucocorticoids simultaneously with resolution and reproducibility acceptable for an *ex-vivo* receptor binding techniques.

Receptor occupancy time profiles after intratracheal administration of a TA solution were very similar to those observed after IV injection. Hence, lung targeting was not achieved after pulmonary instillation of a TA solution. Previous studies revealed an extremely fast pulmonary absorption of lipophilic substances including steroids (12-15). Theoretical calculations showed that for pulmonary targeting to occur the pulmonary absorption needs to be significantly slower than the systemic elimination (11). We estimated an elimination half life of 1.2 hours for TA and a fast pulmonary absorption as indicated by a fast onset of receptor occupancy in the liver. Thus, the lack of pulmonary targeting is linked to a fast pulmonary absorption of the lipophilic TA, in contrast to more hydrophilic drugs for which pulmonary targeting has been demonstrated (16).

A different profile was seen for the instillation of a TA suspension. A slow dissolution of these particles seems to be responsible for the smaller peak effects but also for the prolonged duration of action (i.e. sustained receptor occupancy). More importantly, a pulmonary targeting was observed. This suggests that the slow dissolution of particles indeed achieves a drug concentration at the site of pulmonary receptors, which exceeds that of the liver. However, the observation period of 6 hours was not sufficient to monitor the complete return to baseline and the resulting targeting factor for $AUC_{0\text{-}6h}$ might differ from the true AUC_{∞} based estimate. Thus, the observation period for slow release dosage forms should be prolonged in future studies. In addition, it needs to be stressed that the TA suspension used in these experiments is recommended for intra-articular use. Although the size of these particles is comparable with those used in aerosols (17) further studies should be performed with aerosol formulations.

In summary, a method to assess the pulmonary targeting of inhaled glucocorticoids has been presented with triamcinolone acetonide as an example. The method uses a molecular-pharmacological endpoint that allows a simultaneous assessment of topical and systemic activity after pulmonary delivery. The model might be useful in further evaluating pulmonary vs systemic delivery of glucocorticoids, and the effects of formulation parameters (e.g. sustained release, etc.) of pulmonary delivery systems.

ACKNOWLEDGMENTS

The assistance of Mr. Dave Soucy is acknowledged. We would also like to thank Dr. Dani Zander, University of Florida, Department of Pathology, for her assistance in preparing the lung sections. This work was funded by the NIH-08-RIGM46922A-01.

REFERENCES

1. R. Brattsand, L. Kallstrom, U. Johansson, and M. Dahlback, *Route of administration and rapid inactivation as determinants of the lung-specific action of glucocorticoids. In* J.C. Hogg, R. Ellul-Micallef, and R. Brattsand (Eds.), *Glucocorticoids, inflammation, and bronchial hyperreactivity*. Excerpta Medica, Amsterdam, 1985, pp. 145-153
2. J.H. Toogood, C.W. Frankish, B.H. Jennings, J.C. Bakerville, O. Borga, N.H. Lefcoe, and S.-A. Johansson. A study on the mechanism of the anti-asthmatic action of inhaled budesonide. *J. Allergy Clin. Immunol.* 85: 872-880 (1990).
3. J.M. Henriksen and A. Wenzel. Effect of an intranasally administered corticosteroid (budesonide) on nasal obstruction, mouth breathing, and asthma. *Am. J. Resp. Dis.* 130: 1014-1018 (1984).
4. H. Derendorf, G. Hochhaus, H. Moellmann, J. Barth, M. Krieg, S. Tunn, and C. Moellmann. Receptor-based pharmacokinetic-pharmacodynamic analysis of corticosteroids. *J. Clin. Pharmcol.*, 33, 115-123 (1993).
5. F.D. Boudinot, R. D'Ambrosio, and W.J. Jusko. Receptor-mediated pharmacodynamics of prednisolone in the rat. *J. Pharmacokinet. Biopharm.* 14: 460-493 (1986).
6. H. Bojar, W. Westerkamp, and C.H. Broelsch. Identification and partial characterization of glucocorticoid receptors in human liver. *Acta Hepatogastroenterol. (Stuttg.)* 27: 176-182 (1979).
7. G. Hochhaus, P. Rhodewald, and H. Möllmann. Identification of glucocorticoid receptors in normal and adult human lung. *Res. Exp. Med.* 182: 71-78 (1983).
8. D.G. Beer, G.R. Cunha, and M. Malkinson. Autoradiographic demonstration of the specific binding and nuclear localization of ^{3}H-dexamethasone in adult mouse lung. *Lab. Investig.* 49: 725-734 (1983).
9. E.M. Effros and G.R. Mason. Measurement of pulmonary epithelial permeability in vivo. *Am. Rev. Resp. Dis.* 127: S59 (1983).
10. D.W. Baxter and C.D. Port. Large volume intratracheal instillation of particulate suspensions to hamsters. In Experimental Lung Cancer: Carcinogenesis and Bioassay. E. Karbe and J.F. Paik (Eds.). New York, Springer Verlag, 1974, pp. 86-91.
11. G. Hochhaus and H. Derendorf. *Dose Optimization based on pharmacokinetic/pharmacodynamic modeling*. In H. Derendorf and G. Hochhaus (eds.), Handbook of pharmacokinetic/pharmacodynamic correlations, CRC in press, New York.
12. J.A. Burton and L.S. Schanker. Absorption of corticosteroids from the rat lung. *Steroids* 23: 617-624 (1974).
13. A. Ryrfeldt, G. Persson, and E. Nilsson. Pulmonary disposition of the potent glucocorticoid budesonide, evaluated in an isolated perfused rat lung model. *Biochem. Pharmcol.* 38: 17-22 (1989).
14. M.D. Chaplin, W.C. Cooper, E.J. Segre, J. Oren, R.E. Jones, and C. Nerenberg. Correlation of flunisolide plasma levels to eosinopenic response in humans. *J. Allergy Clin. Immunol.* 65: 445-453 (1980).
15. S.J. Enna and L.S. Schanker. Absorption of drugs from the rat lung. *Am. J. Physiol.* 223: 1227-1231 (1972).
16. G. Hochhaus and H. Moellmann. Pharmacokinetic/pharmacodynamic characteristics of the beta-2-agonists terbutaline, salbutamol and fenoterol. *Int. J. Clin.Pharmacol. Ther. Toxicol.* 30: 342-362 (1992).
17. H.W. Moellmann, B. Armbruster, J. Barth, H. Derendorf, O.W. Floerke, G. Hochhaus, C.R. Moellmann, P. Rohdewald, E.W. Schmidt. Analyse von Form, Korngroessenverteilung und Aggregation der Kristalle in Glukokortikoid-Depotpraeparaten. Akt. Rheumatol. 15: 101-124 (1990).