P-Glycoprotein Transporter Expression on A549 Respiratory Epithelial Cells Is Positively Correlated With Intracellular Dexamethasone Levels

Joanna S. Cohen, MD,† Angela S. Benton, BA,‡ Fisayo Nwachukwu, BA,§ Tugba Ozedirne, BA,|| Stephen J. Teach, MD, MPH,*†¶ and Robert J. Freishtat, MD, MPH*†‡***

Background: Several mechanisms of glucocorticoid resistance in asthma have been proposed. P-glycoprotein (P-gp), a ubiquitous efflux transport protein, is associated with variability in the disposition of many drugs and interindividual variability in drug treatment response. This study was undertaken to determine the effect of P-gp expression on glucocorticoid efflux from airway epithelial cells.

Hypothesis: Decreasing respiratory epithelial P-gp expression in dexamethasone-exposed airway epithelial cells in vitro will increase intracellular dexamethasone concentration.

Methods: A549 lung epithelial cells, transfected with small interfering RNA (siRNA) targeted at messenger RNA for the gene encoding P-gp, were exposed to 100-nM dexamethasone for 15 minutes. Transfection efficiency of siRNA, P-gp expression, and intracellular dexamethasone were measured with flow cytometry.

Results: Cells transfected with both negative siRNA and siRNA targeted at P-gp exhibited a positive correlation of P-gp expression with intracellular dexamethasone. The mean ± SEM correlation coefficients were 0.78 ± 0.07 for cells transfected with negative siRNA and 0.79 ± 0.08 for cells transfected with siRNA targeted at P-gp.

Discussion: Contrary to our hypothesis, the positive correlation between P-gp expression and intracellular dexamethasone suggests that P-gp is not a primary transporter of glucocorticoids from airway epithelial cells. Increased P-gp expression is unlikely to be an important mechanism of glucocorticoid resistance in asthma.

Key Words: asthma, glucocorticoids, P-glycoprotein

(*J Investig Med* 2010;58: 991–994)

Glucocorticoids are a mainstay for acute and maintenance therapy for asthma, but 5% of patients with asthma have "difficult asthma," defined as a lack of response to long-term treatment with inhaled glucocorticoids and symptomatic treatment with a short-acting β-agonist.[1] Patients with asthma who show no clinical improvement after treatment with 40 mg of prednisolone or equivalent steroid dosages for 2 weeks are considered glucocorticoid resistant.[2] These patients with difficult asthma account for 50% of the total health care costs of asthma.[1,3] Understanding the mechanisms of glucocorticoid resistance in asthma may improve treatment for these patients with refractory symptoms and for patients with other glucocorticoid-resistant inflammatory diseases.[4]

Several mechanisms of glucocorticoid resistance in asthma have been described including glucocorticoid-resistant genes, defective glucocorticoid receptor binding and nuclear translocation, and environmentally altered nuclear factors.[2,4–6] Another proposed mechanism is the increased levels of efflux transporters such as P-glycoprotein (P-gp). P-glycoprotein, a member of the ATP-binding cassette transporter superfamily, is a 170-kd ATP-dependent efflux transporter initially identified as a surface glycoprotein that increases the efflux of chemotherapeutic drugs from cancer cells, a result known as multidrug resistance (MDR).[7–9] In addition to its role in the transport of chemotherapeutic agents, P-gp has been shown to efflux glucocorticoids in intestinal, brain, and adrenal epithelial cells in vitro.[10–12] High levels of P-gp expression have been identified on lymphocytes and intestinal epithelial cells of patients with glucocorticoid-resistant inflammatory bowel disease and on lymphocytes of patients with refractory rheumatoid arthritis.[13–15] However, despite the known expression of P-gp on airway epithelium, it remains unclear if the variable expression of P-gp contributes to glucocorticoid resistance in respiratory inflammatory diseases, particularly glucocorticoid-resistant asthma.[16–21]

The purpose of this study, therefore, was to examine the effect of P-gp expression on intracellular glucocorticoid concentrations in airway epithelial cells in vitro. We hypothesized that decreasing respiratory epithelial P-gp expression in dexamethasone-exposed airway epithelial cells in vitro will increase intracellular dexamethasone concentration. We decreased P-gp expression in lung epithelial cells with small interfering RNA (siRNA) targeted to *MDR1*, the gene encoding P-gp. After exposing these cells to fluorescein-labeled dexamethasone, we used flow cytometry to simultaneously measure intracellular dexamethasone and surface P-gp expression. Contrary to our hypothesis, our data showed a positive correlation between P-gp expression and intracellular dexamethasone levels.

MATERIALS AND METHODS

Cell Culture

CCL-185 A549 cells (ATCC Cell Biology Collection, Manassas, VA) were maintained and grown in medium containing Dulbecco modified Eagle medium (Gibco/Invitrogen, Carlsbad, CA), 10% fetal bovine serum (Invitrogen), and 1% penicillin/streptomycin (Sigma-Aldrich, St Louis, MO), at 37°C in a humidified incubator. We maintained cells in a monolayer and passaged them at 70% confluence using phosphate-buffered saline (Fisher, Houston, TX) and trypsin (Sigma-Aldrich).

From the *Division of Emergency Medicine, Children's National Medical Center; †Department of Pediatrics, School of Medicine and Health Sciences, George Washington University; ‡Centers for Genetic Medicine, Children's National Medical Center, Washington, DC; §Temple University, Philadelphia, PA; ||University of California – Davis, Davis, CA; ¶Center for Clinical and Community Research, Children's National Medical Center; and **Department of Integrative Systems Biology, School of Medicine and Health Sciences, George Washington University, Washington, DC.

Received June 29, 2010, and in revised form August 18, 2010.
Accepted for publication August 18, 2010.

Reprints: Joanna S. Cohen, MD, 111 Michigan Ave NW, Washington, DC 20010. E-mail: jcohen@cnmc.org.

Supported by The Board of Visitors, Children's National Medical Center, Washington, DC (to J.S.C.).

Copyright © 2010 by The American Federation for Medical Research

ISSN: 1081-5589
DOI: 10.231/JIM.0b013e3181f88503

Copyright © 2010 American Federation for Medical Research. Unauthorized reproduction of this article is prohibited.

J Investig Med: first published as 10.2310/JIM.0b013e3181f88503 on 15 December 2015. Downloaded from file:/ on April 24, 2024 by guest. Protected by copyright.

J Investig Med: first published as 10.2310/JIM.0b013e3181f88503 on 15 December 2015. Downloaded from file:/ on April 24, 2024 by guest. Protected by copyright.

TABLE 1. siRNA Sequences and Knockdown Efficacy

siRNA No.	Sequence	P-gp Knockdown, Mean ± SEM, %*
1	r(UGA AGU UAU UGG UGA CAA A) dTdT	19.7 ± 1.0
2	r(GGU CGG GAU GGA UCU UGA A) dTdT	64.8 ± 11.8
3	r(GGA GUA UCU UCU UCC AGG A) dTdT	56.0 ± 13.6
4	r(GAU GAU GUC UCU AAG AUU A) dTdT	18.2 ± 3.6

*Normalized to P-gp expression in negative control siRNA-transfected cells.

Transfection

The design and synthesis of 3′-Alexa Fluor 647–modified *MDR1* siRNA and AllStars Negative siRNA were completed by Qiagen (Valencia, CA). See Table 1 for the nucleotide sequences of sense *MDR1* siRNAs. An RNAi Human/Mouse Starter kit containing HiPerFect Transfection Reagent, siRNA Suspension Buffer, AllStars Negative Control siRNA (Alexa Fluor 488–labeled), and Hs/Mm_MAPK1 Control siRNA was also purchased from Qiagen. We diluted the siRNA with a negative control suspension buffer per the manufacturer's recommendations and stored it at −20°C. We incubated the cells for 5 to 10 minutes in 3 μL of HiPerFect reagent to allow for formation of transfection complexes before incubating with 3 μL of siRNA suspended in 100 μL of serum-free medium. We incubated the cells for 24 hours.

Dexamethasone Exposure

After 24 hours of incubation, we changed the cell culture medium and added 100 nM dexamethasone fluorescein (catalog no. D-1383; Invitrogen) to all wells. After preliminary studies indicated no significant difference in dexamethasone uptake at 60, 30, and 15 minutes, cells were incubated with dexamethasone fluorescein for 15 minutes. The secretions were discarded. We applied trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Sigma-Aldrich) to the cells for 5 minutes to achieve a single cell suspension. Cells were subsequently fixed with 1% paraformaldehyde.

Flow Cytometry

We purchased phycoerythrin (PE)-labeled monoclonal mouse antihuman P-gp (clone 17F9) and appropriate isotype control antibodies from BD Pharmingen (Franklin Lakes, NJ). We centrifuged the cells for 5 minutes at 2200 rpm at 4°C and then aspirated the supernatant. The cells were resuspended in 100 μL of fixation solution and incubated in the dark for 20 minutes. We washed the cells 3 times with 1 mL of permeabilization buffer before the addition of 20 μL of pretitrated intracellular antibodies or appropriate isotype controls. The cells were incubated in the dark for 20 minutes and then washed again with 1 mL of permeabilization buffer. We resuspended the cells in 500 μL of flow cytometry staining buffer (eBioscience, San Diego, CA) and stored them on ice in the dark before analysis on a FACS Calibur System (BD Biosciences, San Jose, CA).

Statistical Analysis

We tested a total of 9 experimental replicates for each of the 2 conditions, negative control siRNA, and MDR1 siRNA–treated cells. Using FlowJo 7.5 Software (Tree Star, Inc, Ashland, OR), we used correlation analysis to determine the correlation between intracellular dexamethasone positivity and P-gp positivity

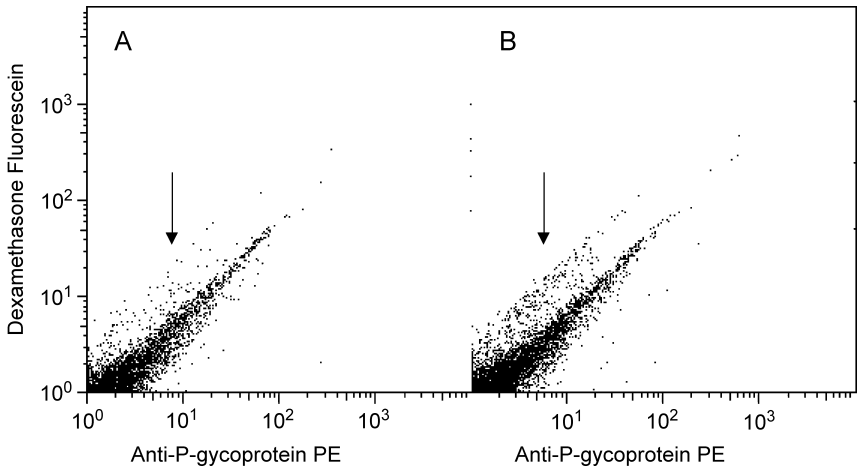

FIGURE 1. Flow cytometric measurements of anti–P-gp PE and intracellular dexamethasone fluorescein in A549 cells transfected with negative control siRNA (A) and MDR1 siRNA (B) demonstrate a consistent positive correlation between P-gp expression and intracellular dexamethasone. Transfected cells were incubated with 100 nM dexamethasone fluorescein for 15 minutes and then fixed and labeled with anti–P-gp PE. Shown are the flow cytometric results from negative control siRNA-transfected cells (A) compared with MDR1 siRNA-transfected cells (B). Cells transfected with both negative siRNA and siRNA targeted at P-gp exhibited a positive correlation of P-gp expression with intracellular dexamethasone. The arrows in both A and B indicate the population of cells with lower P-gp expression. Of note, cells transfected with MDR1 siRNA have a larger population with lower P-gp expression (B) when compared with cells transfected with negative control siRNA (A).

© 2010 The American Federation for Medical Research

Copyright © 2010 American Federation for Medical Research. Unauthorized reproduction of this article is prohibited.

for each condition. We calculated the mean correlation coefficient and SEM.

RESULTS

We tested 4 MDR1 siRNA duplexes targeted to the *MDR1* gene on chromosome 7q21.12 and modified with sense 3′-Alexa Fluor 488 for gene-silencing efficacy. Transfections of all 4 *MDR1* siRNAs were successful, with varying degrees of P-gp protein expression knockdown ranging from 20% to 65% as measured with flow cytometry (Table 1). Because of its high knockdown efficiency in this initial studies, siRNA #2 was chosen for further analysis. A subsequent FACS analysis determined that transfection with MDR1 siRNA #2 yielded some knockdown of P-gp in 95% of cells and a 20-U overall decrease in the geometric mean expression of P-gp (baseline P-gp: geometric mean ± SEM = 389 ± 64). As a result, MDR1 siRNA #2 was selected for further experimentation.

RNA was isolated from cells transfected with MDR1 siRNA #2, and MDR1 messenger RNA (mRNA) amplification was measured with reverse transcription–polymerase chain reaction using 3 primers targeted at different regions of the *MDR1* gene. There was no amplification of *MDR1* mRNA for any of these primers; however, there was no alteration in the amplification of the control housekeeping gene *β_2-microglobulin* mRNA.

Cells transfected with MDR1 siRNA #2 and cells transfected with negative control siRNA were exposed to 100-nM dexamethasone for 15 minutes and subjected to flow cytometry (Fig. 1). Cells transfected with both negative siRNA and siRNA targeted at P-gp exhibited a positive correlation of P-gp expression with intracellular dexamethasone. The mean correlation coefficients were 0.78 ± 0.07 for cells transfected with negative siRNA and 0.79 ± 0.08 for cells transfected with siRNA targeted at P-gp.

DISCUSSION

This study was undertaken to determine the effect of altered P-gp expression on glucocorticoid efflux in respiratory epithelial cells. We hypothesized that knocking down P-gp expression in respiratory epithelial cells would result in lower dexamethasone efflux in vitro. However, our results demonstrate the contrary: a positive correlation between P-gp expression and intracellular dexamethasone. This positive correlation is constant with varying levels of P-gp expression.

Glucocorticoids freely diffuse across cell membranes and exert their effect by binding to a cytoplasmic glucocorticoid receptor, which translocates to the nucleus where it functions as a transcription factor.[22,23] Patients with glucocorticoid-resistant asthma are still susceptible to the Cushingoid adverse effects of glucocorticoids, suggesting that the mechanism of resistance is different from that seen in cases of primary cortisol resistance, which has been attributed to a variety of disorders of glucocorticoid receptor number, translocation, and function.[24,25]

P-glycoprotein has been identified, and it seems to be functional in both bronchial and alveolar epithelial cells.[16,26–28] Mutations in similar transmembrane transporters in the lung are found in disease states such as cystic fibrosis, Tangier disease, and chronic obstructive pulmonary disease.[21,29–31] Most likely, in the respiratory system, P-gp functions to rid the lungs of pathogens, irritants, pollutants, and other agents that produce oxidative stress.[27]

The relationship between P-gp and glucocorticoids may be complicated by the fact that P-gp expression is upregulated by cellular stress, cytokine release, and inflammation.[26,29,32] In addition, the significance of P-gp expression and function as a determinant of inhaled glucocorticoid delivery is debatable. Multiple studies attempting to characterize P-gp expression and function in the respiratory epithelium in vitro have generated contradictory results. Some studies suggest that P-gp activity in respiratory epithelium has an insignificant effect on drug absorption in vitro, particularly compared with intestinal models.[18,19,33–35] Further, inhaled glucocorticoids have been shown to decrease P-gp expression by reducing *MDR1* gene activity in lymphocytes of children with asthma.[36] In our experiments, a brief exposure to dexamethasone is unlikely to induce de novo P-gp synthesis. However, it seems plausible that glucocorticoids could increase surface P-gp expression by inducing its translocation from the cytosol to the cell surface.

Our study is limited by the epithelial cell model used. Although A-549 cells have many properties in common with human respiratory epithelial cells, they may have different drug permeability. In addition, they lack the polarization of human respiratory epithelial cells. The population of A549 cells may contain various cell sizes that could be correlated with different amounts of cell surface P-gp expression and different amounts of dexamethasone uptake. This could contribute to the direct relationship observed between P-gp expression and intracellular dexamethasone. In addition, the dose of dexamethasone used in our experiment may not reflect physiologic dosing and the 15-minute exposure may be too long or too short to accurately reflect the duration of cellular drug exposure in vivo. Our study may also not reflect physiologic conditions in that, because of its higher molecular weight, dexamethasone fluorescein is incorporated into cells at a slightly slower rate than dexamethasone.

Our results suggest that, although it is likely that there are multiple molecular mechanisms of glucocorticoid insensitivity, glucocorticoid efflux by P-gp is unlikely to be a significant mechanism of glucocorticoid resistance in subjects with asthma. Understanding these mechanisms may ultimately help guide the management of subgroups of patients with glucocorticoid-resistant asthma.

REFERENCES

1. Barnes PJ, Woolcock AJ. Difficult asthma. *Eur Respir J*. 1998;12(5):1209.
2. Barnes PJ, Adcock IM. Glucocorticoid resistance in inflammatory diseases. *Lancet*. 2009;373(9678):1905.
3. Chung KF, Godard P, Adelroth E, et al. Difficult/therapy-resistant asthma: the need for an integrated approach to define clinical phenotypes, evaluate risk factors, understand pathophysiology and find novel therapies. ERS Task Force on Difficult/Therapy-Resistant Asthma. European Respiratory Society. *Eur Respir J*. 1999;13(5):1198.
4. Sousa AR, Lane SJ, Cidlowski JA, et al. Glucocorticoid resistance in asthma is associated with elevated in vivo expression of the glucocorticoid receptor beta-isoform. *J Allergy Clin Immunol*. 2000;105(5):943.
5. Goleva E, Hauk PJ, Hall CF, et al. Corticosteroid-resistant asthma is associated with classical antimicrobial activation of airway macrophages. *J Allergy Clin Immunol*. 2008;122(3):550.
6. Ito K, Chung KF, Adcock IM. Update on glucocorticoid action and resistance. *J Allergy Clin Immunol*. 2006;117(3):522.
7. Juliano RL, Ling V. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim Biophys Acta*. 1976;455(1):152.
8. Gros P, Croop J, Housman D. Mammalian multidrug resistance gene: complete cDNA sequence indicates strong homology to bacterial transport proteins. *Cell*. 1986;47(3):371.
9. Higgins CF. ABC transporters: from microorganisms to man. *Annu Rev Cell Biol*. 1992;8:67.
10. Mason BL, Pariante CM, Thomas SA. A revised role for

© 2010 The American Federation for Medical Research

J Investig Med: first published as 10.2310/JIM.0b013e3181f88503 on 15 December 2015. Downloaded from file:/ on April 24, 2024 by guest. Protected by copyright.

Copyright © 2010 American Federation for Medical Research. Unauthorized reproduction of this article is prohibited.

P-glycoprotein in the brain distribution of dexamethasone, cortisol, and corticosterone in wild-type and ABCB1A/B–deficient mice. *Endocrinology*. 2008;149(10):5244.

11. Oka A, Oda M, Saitoh H, et al. Secretory transport of methylprednisolone possibly mediated by P-glycoprotein in Caco-2 cells. *Biol Pharm Bull*. 2002;25(3):393.
12. Ueda K, Okamura N, Hirai M, et al. Human P-glycoprotein transports cortisol, aldosterone, and dexamethasone, but not progesterone. *J Biol Chem*. 1992;267(34):24248.
13. Farrell RJ, Kelleher D. Glucocorticoid resistance in inflammatory bowel disease. *J Endocrinol*. 2003;178(3):339.
14. Farrell RJ, Murphy A, Long A, et al. High multidrug resistance (P-glycoprotein 170) expression in inflammatory bowel disease patients who fail medical therapy. *Gastroenterology*. 2000;118(2):279.
15. Tsujimura S, Saito K, Nawata M, et al. Overcoming drug resistance induced by P-glycoprotein on lymphocytes in patients with refractory rheumatoid arthritis. *Ann Rheum Dis*. 2008;67(3):380.
16. Lechapt-Zalcman E, Hurbain I, Lacave R, et al. MDR1-Pgp 170 expression in human bronchus. *Eur Respir J*. 1997;10(8):1837.
17. Montano E, Schmitz M, Blaser K, et al. P-glycoprotein expression in circulating blood leukocytes of patients with steroid-resistant asthma. *J Investig Allergol Clin Immunol*. 1996;6(1):14.
18. Manford F, Riffo-Vasquez Y, Spina D, et al. Lack of difference in pulmonary absorption of digoxin, a P-glycoprotein substrate, in mdr1a-deficient and mdr1a-competent mice. *J Pharm Pharmacol*. 2008;60(10):1305.
19. Madlova M, Bosquillon C, Asker D, et al. In-vitro respiratory drug absorption models possess nominal functional P-glycoprotein activity. *J Pharm Pharmacol*. 2009;61(3):293.
20. van der Deen M, de Vries EGE, Timens W, et al. ATP-binding cassette (ABC) transporters in normal and pathological lung. *Respir Res*. 2005;6:59.
21. van der Deen M, Timens W, Timmer-Bosscha H, et al. Reduced inflammatory response in cigarette smoke exposed Mrp1/Mdr1a/1b deficient mice. *Respir Res*. 2007;8:49.
22. Barnes PJ, Adcock IM. How do corticosteroids work in asthma? *Ann Intern Med*. 2003;139(5 Pt 1):359.
23. Rhen T, Cidlowski JA. Antiinflammatory action of glucocorticoids—new mechanisms for old drugs. *N Engl J Med*. 2005;353(16):1711.
24. Lane SJ, Lee TH. Mononuclear cells in corticosteroid-resistant asthma. *Am J Respir Crit Care Med*. 1996;154(2 Pt 2):S49.
25. Malchoff DM, Brufsky A, Reardon G, et al. A mutation of the glucocorticoid receptor in primary cortisol resistance. *J Clin Invest*. 1993;91(5):1918.
26. Campbell L, Abulrob A-NG, Kandalaft LE, et al. Constitutive expression of P-glycoprotein in normal lung alveolar epithelium and functionality in primary alveolar epithelial cultures. *J Pharmacol Exp Ther*. 2003;304(1):441.
27. Scheffer GL, Pijnenborg ACLM, Smit EF, et al. Multidrug resistance related molecules in human and murine lung. *J Clin Pathol*. 2002;55(5):332.
28. van der Valk P, van Kalken CK, Ketelaars H, et al. Distribution of multi-drug resistance-associated P-glycoprotein in normal and neoplastic human tissues. Analysis with 3 monoclonal antibodies recognizing different epitopes of the P-glycoprotein molecule. *Ann Oncol*. 1990;1(1):56.
29. Lehmann T, Kohler C, Weidauer E, et al. Expression of MRP1 and related transporters in human lung cells in culture. *Toxicology*. 2001;167(1):59.
30. Brooks-Wilson A, Marcil M, Clee SM, et al. Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. *Nat Genet*. 1999;22(4):336.
31. Shulenin S, Nogee LM, Annilo T, et al. *ABCA3* gene mutations in newborns with fatal surfactant deficiency. *N Engl J Med*. 2004;350(13):1296.
32. Litman T, Druley TE, Stein WD, et al. From MDR to MXR: new understanding of multidrug resistance systems, their properties and clinical significance. *Cell Mol Life Sci*. 2001;58(7):931.
33. Florea BI, van der Sandt IC, Schrier SM, et al. Evidence of P-glycoprotein mediated apical to basolateral transport of flunisolide in human broncho-tracheal epithelial cells (Calu-3). *Br J Pharmacol*. 2001;134(7):1555.
34. Hamilton KO, Backstrom G, Yazdanian MA, et al. P-glycoprotein efflux pump expression and activity in Calu-3 cells. *J Pharm Sci*. 2001;90(5):647.
35. Patel J, Pal D, Vangal V, et al. Transport of HIV-protease inhibitors across 1 alpha,25di-hydroxy vitamin D_3-treated Calu-3 cell monolayers: modulation of P-glycoprotein activity. *Pharm Res*. 2002;19(11):1696.
36. Kopriva F, Dzubak P, Potesil J, et al. The anti-inflammatory effects of inhaled corticosteroids versus anti-leukotrienes on the lymphocyte P-glycoprotein (PGP) expression in asthmatic children. *J Asthma*. 2009;46(4):366.

J Investig Med: first published as 10.2310/JIM.0b013e3181f88503 on 15 December 2015. Downloaded from file:/ on April 24, 2024 by guest. Protected by copyright.

© 2010 The American Federation for Medical Research

Copyright © 2010 American Federation for Medical Research. Unauthorized reproduction of this article is prohibited.