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Author response to letter to editor: Hyperinsulinemia adversely affects lung structure and function

Suchita Singh,¹ Manish Bodas,¹ Naveen K. Bhatraju,¹ Bijay Pattnaik,¹ Atish Gheware,¹ Praveen Kolumam Parameswaran,⁵ Michael Thompson,⁵ Michelle Freeman,⁵ Ulaganathan Mabalirajan,¹ Reinoud Gosens,⁶ Balaram Ghosh,¹ Christina Pabelick,⁵ Allan Linneberg,^{2,3,4} Y. S. Prakash,⁵ and Anurag Agrawal¹

¹Center of Excellence for Translational Research in Asthma and Lung Disease, CSIR-Institute of Genomics and Integrative Biology, New Delhi, India; ²Research Centre for Prevention and Health, the Capital Region of Denmark, Copenhagen, Denmark; ³Department of Clinical Experimental Research, Glostrup University Hospital, Glostrup, Denmark; ⁴Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark; ⁵Departments of Anesthesiology and Physiology and Biomedical Engineering, Mayo Clinic, Rochester, Minnesota; and ⁶Department of Molecular Pharmacology, Groningen Research Institute for Asthma and COPD (GRIAC), University of Groningen, Groningen, Netherlands

TO THE EDITOR: We appreciate the interest shown by Wolff et al. (17) regarding our recent publication in the *American Journal of Physiology Lung Cellular and Molecular Physiology* (11). We acknowledge the convenience of an inhaled insulin formulation, the extensive safety data required by the FDA prior to approval, and also the inherently unknown nature of long-term side effects of any drug despite appropriate safety studies and extensive financial investment by the parent company, academia and various organizations. Accordingly, given clinical introduction of inhaled insulin formulations, the intent of our study was to explore the effects of inhaled insulin in a mouse model and to dissect out some of the mechanisms by which insulin influences human airway epithelial and smooth muscle cells in vitro. In this regard, the polemic arguments notwithstanding, via this letter in response to that by Wolff et al. (17), we look forward to stimulating a healthy debate on the subject of long-term risks vs. benefits of inhaled insulin.

The primary objection raised by Wolff et al. (17) seems to be to a statement in our article that “Our work has important general implications that should hopefully lead to questioning of many current trends or practices including use of inhaled insulin formulations in diabetes” and of the data behind the statement (11). After a careful review of the critique by Wolff et al. (17), we stand by our statement.

A major focus of the criticism is the mode and quantity of insulin delivered, sometimes with contradictory elements, i.e., too little is delivered to the lungs by intranasal (i.n.) delivery and in subsequent sections, that the dose of insulin was too high. We recognize that i.n. administration of small volumes in mice results in a substantial fraction being delivered to the GI tract rather than the lung. While the fraction delivered varies between protocols, ~10% delivery to the lung can be expected with the 30 μ l volumes that we used. We should have specified that 3 μ l of the 0.01 N HCl vehicle in which insulin is freshly dissolved (as recommended by the manufacturer; Sigma), is made up to a final i.n. injectate of 30 μ l with a weakly acidic pH estimated between 3 and 4, which is well tolerated with no distress or weight loss. In fact, when comparing to the initial

weight, at the end of the 12-day protocol, if anything, there was a small but significant weight gain of about 0.4 g (paired *t*-test, $P < 0.05$), which was not different between vehicle- and insulin-treated groups. While we have the capacity for direct intratracheal (i.t.) delivery of drugs, the simple i.n. protocol was devised for considerations that included propensity of insulin to precipitate or form amyloid-like fibrillar aggregates at higher pH, with agitation, or on hydrophobic surfaces (5, 13) as well as the potential for substantial animal discomfort or injury during repeated i.t. access. We accept that we did not try to precisely quantify the amount of insulin delivered; instead we selected the appropriate i.n. dose from multiple pilot doses, by physiological end points such as elevation of insulin in blood and absence of hypoglycemia. This design was well suited for the specific purpose of understanding the molecular and cell physiological signaling activated by inhaled insulin in lung tissue and how that would influence overall lung physiology.

Before we provide specific responses to the other concerns raised by Wolff et al. (17), it is important to clarify that it is somewhat misleading to portray our findings as isolated among a sea of contrary evidence. Similarly, we posit that it is scientifically incorrect to take the implicit stance that FDA approval confers a basis to ignore (or not publish) contrary data. The potential for bias in reporting of sponsored research is very well known and a conflict of interest in postmarket surveillance is extensively documented elsewhere (7, 8). Thus independent studies such as ours are of value to researchers, clinicians, and their patients, and to industry itself, contrary to the assertion by Wolff et al.

In terms of insulin effects on the lung, prior work has clearly suggested that insulin induces airway smooth muscle contractility and proliferation (2, 9, 10, 12) while airflow reduction has been reported in mice exposed to FDA-approved inhaled insulin formulations (5). Teeter and Riese (14), whose work is extensively cited by Wolff et al., found that both subcutaneous and inhaled insulin led to a decline in FEV₁, more so for inhaled insulin. Such a decline in FEV₁ has been consistently replicated in other studies (1). It is critical to note that spirometry is a poor test for distal airway disease and even small declines in FEV₁ may be associated with significant distal airway changes. More than 80% of small airways may be lost before spirometric diagnosis of chronic obstructive pulmonary

Address for reprint requests and other correspondence: A. Agrawal, Center of Excellence for Translational Research in Asthma and Lung Disease, CSIR-Institute of Genomics and Integrative Biology, Mall Rd., New Delhi, India (e-mail: a.agrawal@igib.in).

disease is made, and absence of spirometric abnormality does not exclude clinically significant airway disease (4, 6, 18). These facts invalidate the crux of the argument by Wolff et al. that current safety data with small declines in FEV₁ exclude significant adverse effects of inhaled insulin, in effect supporting our conclusions.

In terms of mechanisms, we have presented multiple lines of evidence using experiments on freshly dissected human lung tissue as well as mice, which lead us to conclude that high levels of insulin activate β -catenin signaling in the lung that further leads to remodeling (11). Regarding these, Wolff et al. have raised concerns only about the mouse data and therefore they likely agree that insulin induces β -catenin signaling in human lung tissue and drives airway smooth muscle proliferation, contractility, and collagen deposition in vitro. In that case, our mouse data only corroborates the same in vivo.

One of their main concerns is that the insulin dose was higher than the highest doses relevant to humans. It is self-evident based on the absence of hypoglycemia and the calculations provided by Wolff et al. that this is not the case, especially after considering the fractional lung delivery by the i.n. route. Their next concern is that we did not report data for naive untreated mice. The most appropriate control for a drug (here insulin) remains that using the vehicle, which is what we chose to show. Any differences between these groups are then attributable to the drug. We hope that the additional details of the injectate and the i.n. protocol clarify their concerns about acid aspiration injuries and the need for a third group. The physiology and lung histology of naive Balb/c mice is well studied. The data from the vehicle group is not significantly different from the range observed in naive Balb/c mice in our lab. This is also evident from normal values of respiratory system resistance and normal histology without evidence of inflammation or mucous metaplasia in vehicle-treated mice. Although not shown, these parameters were quantitated in multiple sections and found normal in both vehicle and insulin-treated mice. Masson's trichrome staining was increased in insulin-treated mice, and subsequently to eliminate known limitations of indirect imaging based quantification of collagen, we focused on direct measurement of collagen measured in total lung lysate by an independent method: Sircol assay. This was found to be increased, confirming the gross histological appearance and was shown as an independent panel (Fig. 1F of the main article). The statement by Wolff et al. that "appearance of true collagen in 12 days seems unrealistic" is incorrect and somewhat naive given the many acute models of lung fibrosis and reports of lung collagen deposition in less than 2 wk by low-dose infusion of asymmetric dimethyl-arginine in mice (15) and after repeated bronchoconstriction challenge in humans (3). We are well aware of the potential of nonspecific immune responses induced by siRNA and a scrambled siRNA was used as control, as indicated throughout the article.

To conclude, our work should be seen in the broader context of evidence supporting a potentially detrimental effect of high doses of insulin on lung function. Convenient delivery of insulin is not a panacea for type 2 diabetes either, given that the value of tight glycemic control is itself being debated (16). Thus there is a general need to more strictly look at risk-benefit profiles of diabetes therapy. In this context, further exploring the effects of high levels of insulin in the lungs and actively

questioning the risk-benefit profile of inhaled insulin formulations is not just desirable but necessary.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

S.S., M.B., N.K.B., Y.S.P., and A.A. edited and revised manuscript; S.S., M.B., N.K.B., B.P., A.G., P.K.P., M.A.T., M.F., U.M., R.G., B.G., C.M.P., A.L., Y.S.P., and A.A. approved final version of manuscript; A.A. drafted manuscript.

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