Deterioration of epithelium mediated mechanisms in diabetic-antigen sensitized airways of guinea pigs

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Abstract

Background: The onset of diabetes causes disruption of respiratory epithelial mediators. The present study investigates whether diabetes modifies the epithelium mediated bronchial responses in hyper-reactive airway smooth muscle (ASM) primarily through nitric oxide (NO), cyclooxygenase (COX), and epithelium derived hyperpolarizing factor (EpDHF) pathways. **Methods:** Experimental model of guinea pigs having hyper-reactive airways with or without diabetes were developed. The responses of tracheal rings to cumulative concentrations of acetylcholine (ACh) and isoproterenol (IP) in the presence and absence of epithelium and before and after incubation with NO, K^+_{ATP} and COX inhibitors, N-(ω)-Nitro-L-arginine methyl ester (L-NAME; 100 µM), glybenclamide (10 µM) and indomethacin (100 µM) were assessed. **Results:** In diabetic guinea pigs with hyper-reactive airways, a decrease in ACh induced bronchoconstriction was observed after epithelium removal and after incubation with L-NAME/indomethacin, suggesting damage to NO/COX pathways. Hyper-reactivity did not alter the response of trachea to ACh but affected the response to IP which was further reduced in hyper-reactive animals with diabetes. The ASM response to IP after glybenclamide treatment did not alter in hyper-reactive guinea pigs and diabetic guinea pigs with hyper-reactive airways, suggesting damage to the EpDHF pathway. Treatment with indomethacin reduced IP response in the hyper-reactive model, and did not produce any change in diabetic model with hyper-reactive airways, indicating further disruption of the COX pathway. **Conclusion:** EpDHF pathway is damaged in hyper-reactive guinea pigs and in diabetic guinea pigs with hyper-reactive airways. Diabetes further aggravates the NO and COX mediated pathways in diabetic guinea pigs with hyper-reactive airways.

Key words: diabetes, asthma, epithelium, K^+_{ATP}, cycloxygenase pathway, NO

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Introduction

A number of population studies have indicated that the prevalence and severity of asthma in diabetic patients is less than in patients suffering only from asthma (1–3). The mechanism of negative association between these two diseases is unclear (4) and suggests a protective role for diabetes. Contrary to this, a recent study (5) has shown a strong association between the occurrence of type 1 diabetes and symptoms of asthma, suggesting that diabetes may damage the respiratory epithelium. Epidemiological studies have also shown that the incidence of asthma, COPD, pulmonary fibrosis, and pneumonia, is greater in people with diabetes than in those without diabetes and suggested a harmful role for diabetes (6–9).

A previous study from our group indicates that the onset of diabetes modulates the respiratory epithelium due to loss of NO, prostaglandins and K^+_{ATP} channels (10). The respiratory epithelium, similar to vascular endothelium, releases both relaxing and constrictive factors like nitric oxide (NO), prostaglandins (PG) and EpDHF, all of which are important epithelial mediators in airway physiology (11–14). PGs produced by constitutive enzyme isoforms have a beneficial role in physiological functions, while formations of PG via inducible isoforms are involved mainly in inflammatory and pathological pathways (15). Inhibition of K^+_{ATP} channels leads to depolarization and contraction of airway smooth muscle while activation of K^+_{ATP} channels via the EpDHF pathway leads to hyperpolarisation and relaxation of airway smooth muscle (16). Exaggerated or unbalanced PG production and reduction in the EpDHF pathway plays a key role in the pathophysiology of asthma (17).

Studies on experimental diabetes have shown a decrease in the responsiveness of the smooth muscle cells of the tracheal rings, independent of NO in the presence or absence of allergy (10, 18).

Therefore the aim of the present work was to determine whether diabetes augments or ameliorates the damage caused to the respiratory epithelium which is in a hyper-reactive state.

Materials and Methods

Animals

Dunkin-Hartley Guinea pigs (450–750 g) of either sex were maintained according to the recommendations given by the National Accreditation Board of Testing and Calibration Laboratories (NABL), and the study was approved by the VP Chest Institute's animal ethical committee. During treatment the guinea pigs were housed at constant room temperature, humidity, and light cycle (12:12 h light-dark), with free access to tap water and were fed with standard chow *ad libitum*. The guinea pigs were weighed prior to and on the last day of treatment, before conducting the experiments.

To study the effect of hyper-reactivity alone, and to study the effect of diabetes combined with airway hyper-reactivity on the trachea of the guinea pigs (*n*=10), hyper-reactivity was induced by an *ip* injection of ovalbumin (OA) (1 ml/kg (5%) on two consecutive days) followed by an inhaled ovalbumin challenge after 21 days.

Induction of both diabetes and airway hyper-reactivity in guinea pigs (*n*=10) was accomplished first by induction of diabetes with a single *ip* injection of streptozotocin (180 mg/kg) and then following confirmation of the diabetic state, OA was injected as described above. The doses used were the same in all groups. The control (healthy) guinea pigs (*n*=10) were given normal balanced diet. Studies on hyper-reactive animals were performed after 4 weeks of treatment. In animals with diabetes along with airway hyper-reactivity, the studies were performed 8 weeks after treatment, which corresponds to 4 weeks post induction of hyper-reactivity.

Estimation of bronchial hyper-responsiveness to histamine

In order to assess bronchial hyper-responsiveness the measurement of specific airway conductance (SGaw) to inhaled histamine was carried out following the ovalbumin inhaled challenge by using a non-invasive body plethysmographic technique in all animals, four days before induction of hyper-reactivity and also prior to sacrificing the animals. A log dose response curve was plotted and the concentration of histamine producing 35% fall in SGaw was calculated (ED_{35} histamine) (19).

Oral Glucose Tolerance Test (OGTT)

All the groups were subjected to OGTT (20) before treatment as well as before sacrificing. Animals with impaired glucose tolerance were considered diabetic.

Broncho-reactivity studies

Tracheas from all the groups were carefully dissected out from the guinea pigs and cleaned of connective tissue and divided into ring segments that were 2–3 mm in length. For isometric tension recording, tracheal rings were mounted in an organ bath, between a stationary stainless steel hook and an isometric force transducer (Grass FT-03, USA). Changes in isometric tension were recorded by a PowerLab data acquisition system (8SP 20B, AD Instruments, Australia) with a computerized analysis programme (Chart 5.4.2, AD Instruments, Australia). Tracheal rings were maintained at 37°C in an organ bath containing 10 ml of modified Krebs-bicarbonate buffer solution of the following composition (in mM): NaCl 118; KCl 4.8; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 2.5; $CaCl_2$ 2.5; and glucose 11.0; pH, 7.4, bubbled with 95% O_2 and 5% CO_2. The tracheal rings before drug administration were subjected to a tension of 2 g that was readjusted every 15 min during a 120 min equilibration period. Tracheal rings were initially exposed to ACh (10 µM) to check their functional integrity.

To evaluate the bronchial reactivity, both dependent and independent of the respiratory epithelium, concentration-response curves were performed with a bronchoconstrictor (ACh 10^{-12} to 10^{-4} M) and also with a bronchodilator (IP 10^{-12} to 10^{-4} M) that was first contracted with ACh, in both epithelium intact and epithelium denuded tracheal rings. The epithelium was removed by rubbing the lumen with forceps. Denudation of the epithelium was confirmed by histology (data not shown).

The degree of activation of the smooth muscle prior to the addition of IP was assessed, since the potency and even direction (contraction or relaxation) of the effect of the mediators (drugs) may depend on the basal tone that is present when the effect of the mediator is evaluated.

After the end of treatments, the tracheal rings showed no significant difference in contraction or sensitivity to 10 µM ACh when compared with the control tracheal rings. Thus, the relaxation responses of tracheal rings previously contracted with 10 µM ACh to IP were studied at equal levels of pre-contraction in healthy and experimental tissues by means of concentration-response curves to IP.

To evaluate the effects of diabetes along with hyper-reactivity on the epithelial mediators NO, PGs and EpDHF

Three different experimental studies were performed. Epithelium-intact tracheal rings were preincubated separately for 30 min with the NOS inhibitor, L-NAME (100 µM), the PG inhibitor, indomethacin (100 µM) and the K^+_{ATP} inhibitor glybenclamide (10 µM). The effects of these inhibitors on the airways were separately studied by comparing the amount of contraction or relaxation induced by ACh and IP in the absence or presence of these inhibitors.
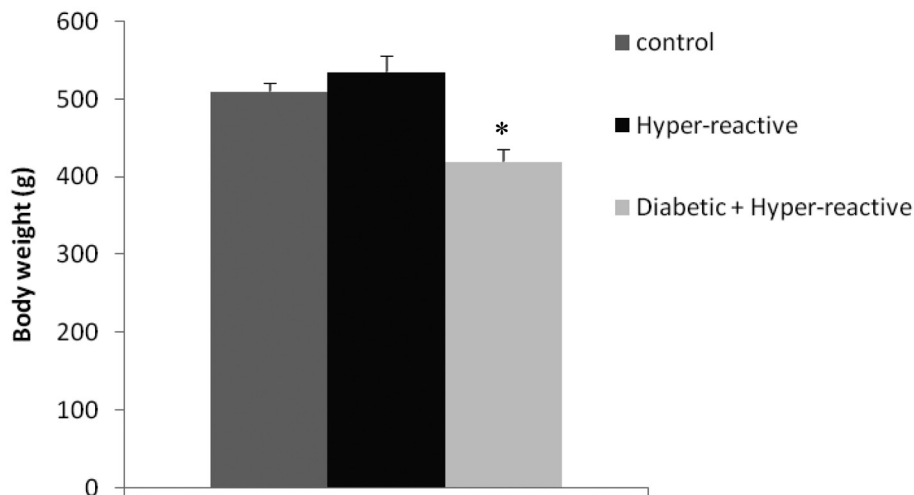
Fig. 1. Body weight in grams in healthy, hyper-reactive and diabetic hyper-reactive airways of guinea pigs. Data represents mean ± S.E.M. *P< 0.05 from control (*n*=10).

Statistical analysis

Numerical data is expressed as the mean ± S.E.M. of the number of animals used in each experiment. In the bronchial reactivity experiments, bronchodilator responses were expressed as the % change of the previous contraction to ACh and bronchoconstrictor responses were expressed as absolute values in gram tension. To compare the effect of L-NAME, glybenclamide, indomethacin on the response to ACh (10 µM) and IP (10 µM) in tracheal rings of normal, hyper-reactive alone and diabetic along with hyper-reactive guinea pigs, the results were expressed as % change in response to ACh (10 µM) and IP (10 µM) before and after incubating with of L-NAME, glybenclamide, indomethacin. The results were analyzed by one way analysis of variance (ANOVA) followed by post-hoc Tukey's test. Differences were considered to be statistically significant at *P*<0.05.

Results

In guinea pigs treated with streptozotocin for induction of diabetes the mean body weight significantly decreased (Fig. 1). A single intraperitoneal injection of streptozotocin (180 mg/kg) was sufficient enough to cause a significant increase in the postprandial blood glucose in guinea pigs from the baseline value (Fig. 2a), which was accompanied by a decreased blood glucose tolerance after glucose load challenge (Fig. 2b).

Effect of hyper-reactivity on SGaw

In guinea pigs sensitized with ovalbumin for induction of hyper-reactivity alone and in guinea pigs with both diabetes and airway hyper-reactivity, the specific airway conductance (SGaw) to histamine was significantly (*P*<0.05) decreased, indicating hyper-reactivity in both (Fig. 2c).

Epithelial dependence of the bronchial reactivity in diabetes along with airway hyper-reactivity

ACh produced a concentration-dependent contraction in intact trachea of healthy, hyper-reactive alone, and diabetic guinea pigs with hyper-reactive airways (Table 1). Removal of the epithelium significantly increased the contraction produced by ACh in healthy and hyper-reactive airways. However, in guinea pigs with both diabetes and airway hyper-reactivity the contractions produced by ACh in tracheal rings after removal of the
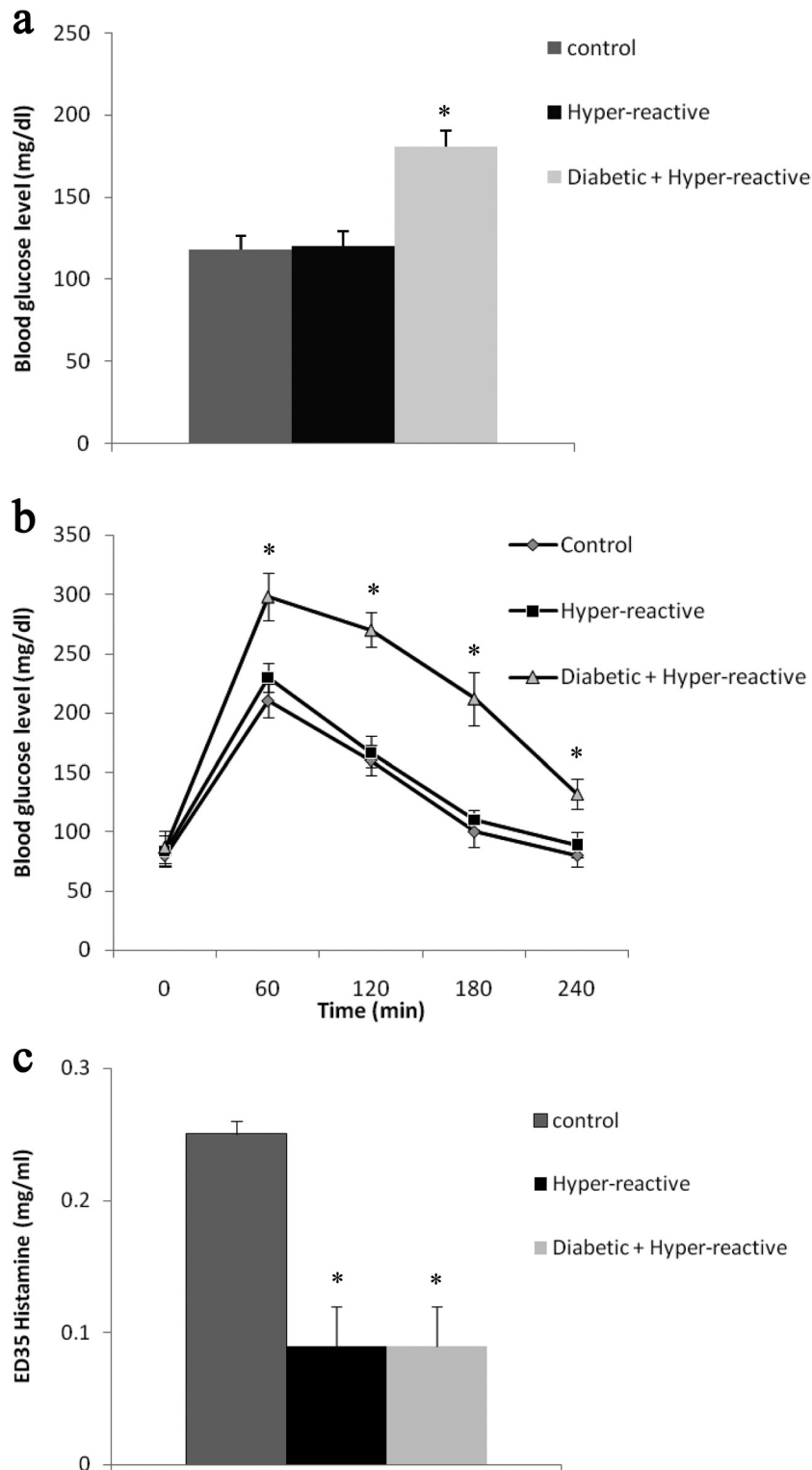
Fig. 2. Validation/Confirmation of experimental animal models.

a: Postprandial blood glucose level in healthy, hyper-reactive and diabetic hyper-reactive airway-guinea pigs. Data represents mean ± S.E.M. (n=10). *P<0.05 from control.

b: Blood glucose level after 0, 60, 120, 180 min by means of oral glucose tolerance test done in healthy, hyper-reactive and diabetic hyper-reactive airway guinea pigs. Data represents mean ± S.E.M. *P<0.05 from control (n=10).

c: Fall of ED$_{35}$ by histamine (mg/ml) in SGaw (sec $^{-1}$ cm H$_2$O $^{-1}$) in healthy, hyper-reactive and diabetic hyper-reactive airway guinea pigs. Data represents mean ± S.E.M. *P<0.05 from control (n=10).

Table 1. Effect of hyper-reactivity and diabetes +hyper-reactivity in guinea pigs on maximum response (Rmax) and sensitivity (pD$_2$) to acetylcholine (ACh), and isoproterenol (IP) in epithelium intact tracheal rings

	ACh		IP	
	Rmax (g)	pD$_2$	Rmax (%)	pD$_2$
Control	1.2 ± 0.02	5.74 ± 0.11	91.7 ± 0.26	7.63 ± 0.05
Hyper-reactive	1.1 ± 0.07	5.69 ± 0.06	84.9 ± 0.21*	7.52 ± 0.15*
Diabetes + Hyper-reactive	1.2 ± 0.09	5.54 ± 0.16	79.4 ± 0.23*#	7.46 ± 0.04*#

Results for IP are expressed as % of the previous contraction to ACh and pD$_2$ is expressed as −log one half; *$P<0.05$ from Control. #$P<0.05$ from Hyper-reactive (n=12).

Table 2. Effect of hyper-reactivity and diabetes + hyper-reactivity in guinea pigs on maximum response (Rmax) and sensitivity (pD$_2$) to acetylcholine, and isoproterenol in epithelium denuded tracheal rings

	ACh		IP	
	Rmax (g)	pD$_2$	Rmax (%)	pD$_2$
Control	1.6 ± 0.01	5.93 ± 0.07	86.7 ± 0.21	7.62 ± 0.04
Hyper-reactive	1.7 ± 0.02	5.92 ± 0.06	78.4 ± 0.13*	7.42 ± 0.05*
Diabetes + Hyper-reactive	1.2 ± 0.04	5.28 ± 0.27*#	76.8 ± 0.18*	7.48 ± 0.14*

Results for IP are expressed as % of the previous contraction to ACh and pD$_2$ is expressed as −log one half. *$P<0.05$ from Control, #$P<0.05$ from Hyper-reactive (n=12).

epithelium were significantly smaller in comparison to both healthy and hyper-reactive airways (Table 2).

In healthy guinea pigs, IP produced a concentration dependent relaxation in epithelium intact tracheal rings while epithelial denudation of tracheal rings significantly reduced the IP induced relaxation response, suggesting that the relaxant response to IP is mediated in part through the epithelium (Table 1 and 2). When the same experiment was repeated on the intact trachea of hyper-reactive guinea pigs, IP produced, a smaller response in hyper-reactive guinea pigs as compared to healthy guinea pigs. An even significantly smaller relaxation to IP was observed in diabetic guinea pigs with hyper-reactive airways (Table 1 and 2). Epithelial denudation partially decreased the response in the hyper-reactive condition but did not alter the IP induced relaxant response when diabetes occurred along with airway hyper-reactivity (Table 2).

Effect of epithelial mediators on the trachea of guinea pigs having both diabetes and hyper-reactive airways

To assess the potential contribution of the NO, EpDHF and COX pathways in the modulation of tracheal epithelium, after induction of airway hyper-reactivity alone, and diabetes combined with airway hyper-reactivity, L-NAME which blocks NOS, glybenclamide a K$^+_{ATP}$ channel inhibitor which blocks the EpDHF-mediated responses and indomethacin which is an inhibitor of COX pathway were used separately.

L-NAME enhanced the ACh response in hyper-reactive airways and in healthy airways. However this enhancement of ACh response in diabetes combined with hyper-reactive airways was significantly smaller in comparison to healthy and also hyper-reactive airways. Glybenclamide did not produce any change in the response to ACh in all the groups (data not shown). Indomethacin augmented the ACh response in all the three groups indicating, the involvement of the COX pathway. However the percent change produced by indomethacin was significantly lower in tracheal rings from diabetic animals with hyper-reactive airways as

compared to trachea from both hyper-reactive animals and healthy animals. These results suggest that there is only a fractional disruption of the NO and COX pathways in the ACh response of the tracheal rings of diabetic guinea pigs with hyper-reactive airways. These results are very similar to those obtained with epithelium denuded preparations, suggesting that ACh induced contraction in diabetes combined with hyper-reactive airway condition deteriorate due to disruption of both the NO and COX pathways (Fig. 3 and Fig. 4).

When indomethacin was added to intact tracheal rings to inhibit the activity of cyclooxygenase a $34.3 \pm 0.7\%$ change in the relaxation induced by IP in healthy guinea pigs was noted. This percent change in response to IP was significantly reduced in hyper-reactive tracheal rings and also in tracheal rings from diabetic guinea pigs with hyper-reactive airways ($P<0.05$). This percent change in response to IP in the tracheal rings from diabetic guinea pigs with hyper-reactive airways was also significantly less than that noted in hyper-reactive airways (Fig. 5). The percent change in the relaxant response to IP before and after incubation with glybenclamide was significantly reduced in tracheal rings from hyper-reactive animals and in those with both diabetes and airway hyper-reactive condition ($P<0.05$) in comparison to healthy tracheal rings (Fig. 6).

L-NAME did not produce any change in the response to IP in all the groups (data not shown).

Discussion

The present study revealed that diabetes worsens the epithelium dependent respiratory response in tracheal rings of diabetic guinea pigs having airway hyper-reactivity. Diabetes and hyper-reactivity synergistically augment the dysfunction of the respiratory epithelium and may be the cause of increased incidence of asthma, COPD, pulmonary fibrosis, and pneumonia in diabetic patients (21, 22). The damage to the epithelium dependent bronchial reactivity in diabetes combined with airway hyper-reactivity was specifically due to the increased disorder in the NO and COX pathways.

In guinea pigs with both diabetes and airway hyper-reactivity, a decrease in airway conductivity, decreased tolerance to glucose and a significant increase in post prandial blood glucose levels accompanied with loss in body weight was observed, suggesting the establishment of type I diabetes and airway hyper-reactivity. Previous studies have reported weight loss along with increase in blood glucose levels in streptozotocin treated guinea pigs (10). In hyper-reactive guinea pigs alone normal blood glucose levels and decrease in airway conductivity was observed. Previous study from our group has demonstrated that onset of diabetes *i.e.* four weeks after streptozotocin treatment, does not alter the airway conductivity but modulates the respiratory epithelium (10). The decrease in airway conductivity in guinea pigs having both diabetes and airway hyper-reactivity may be attributed to ovalbumin treatment and eight weeks post streptozotocin treatment. The current study was done four weeks post ovalbumin treatment in order to study whether diabetes modulates/deteriorates the respiratory epithelium function in hyper-reactive airways. For many years, the respiratory epithelium, which is a continuous layer of epithelial cells which covers the lumen of the conductive airway tract was thought to be relatively inert. It is now recognized however, that the respiratory epithelial cells have important paracrine, endocrine and autocrine functions, in addition to acting as a physical barrier to irritants/allergens and providing muco-ciliary clearance, hydration, host defence and gas exchange (13, 14). The respiratory epithelium releases various bronchio-active substances such as NO, EpDHF, and prostaglandin E_2 (PGE_2) which help in protecting the airway from excessive bronchoconstriction (13, 14, 23).

Response to ACh in epithelium intact trachea of guinea pigs with hyper-reactivity alone, diabetes along with hyper-reactivity and control guinea pigs were similar. In healthy and in hyper-reactive tracheal rings, the bronchoconstriction induced by ACh was augmented by removal of the epithelium indicating that epithelium
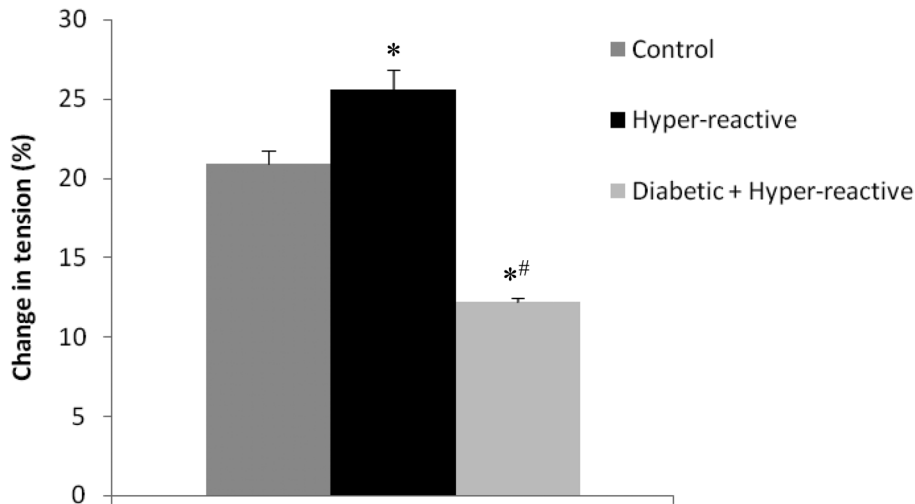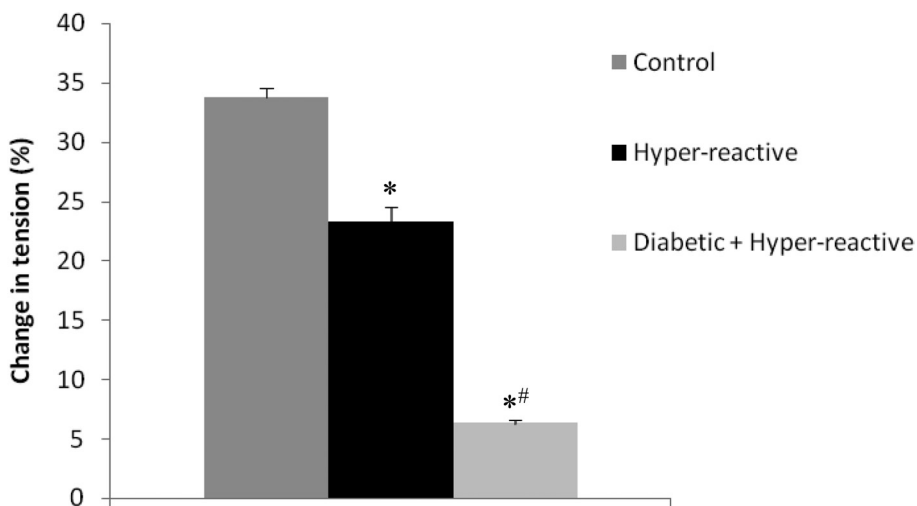
Fig. 3. Effect of diabetes and hyper-reactivity on NO modulation of the bronchoconstric-
tor response of ACh. % change in ACh (10 µM)-induced contraction of tracheal
rings with intact epithelium in the presence of L-NAME (100 µM). *$P<0.05$ from
Control, #$P<0.05$ from Hyper-reactive ($n=12$).

Fig. 4. Effect of diabetes and hyper-reactivity on COX modulation of the bronchocon-
strictor response of ACh. % change in ACh (10 µM)-induced contraction of
tracheal rings with intact epithelium in the presence of indomethacin (10 µM).
*$P<0.05$ from Control, #$P<0.05$ from Hyper-reactive ($n=12$).

limits ACh induced bronchoconstriction. This effect was not observed in tracheal rings of guinea pigs having
both diabetes and hyper-reactivity, suggesting dysfunction of respiratory epithelium which may be attributed
to diabetes.

To confirm whether diabetes modulates/deteriorates the relaxation of the respiratory epithelium, responses
of the tracheal rings to β_2 adrenergic agonist IP was studied. IP a β_2 adrenergic agonist relaxes the airway
smooth muscle. Airways including the trachea have both anti allergic properties as well as bronchodilatory
activity and abundantly express β_2 adrenoceptors. Previous studies from our group and others have shown that
in healthy guinea pigs, removal of the epithelium from trachea caused a statistically significant decrease to IP
induced relaxation (10). In the present study a significant decrease in relaxation response to IP in the epithelium
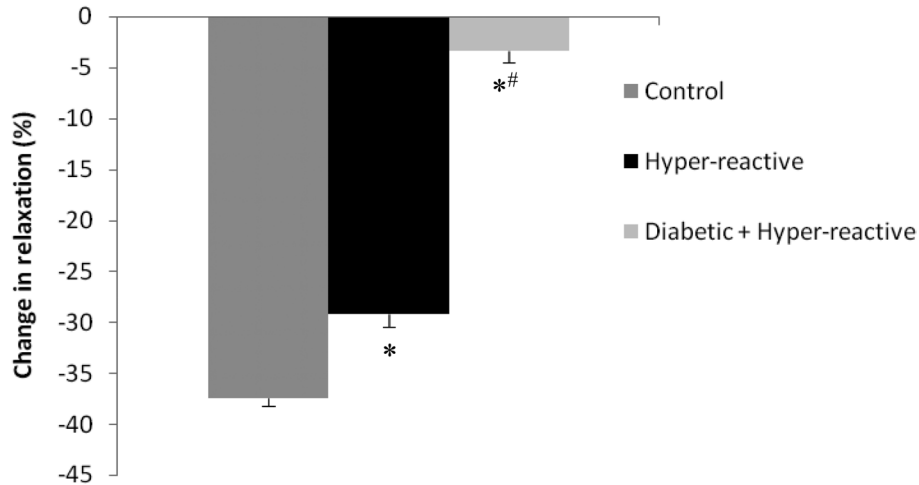
Fig. 5. Effect of diabetes and hyper-reactivity on COX pathway modulation of the bronchodilator response to IP. % change in IP (10 μM)-induced relaxation of tracheal rings with intact epithelium precontracted by ACh (10 μM) in the presence of indomethacin (100 μM). *$P<0.05$ from Control; #$P<0.05$ from Hyper-reactive (n=12).
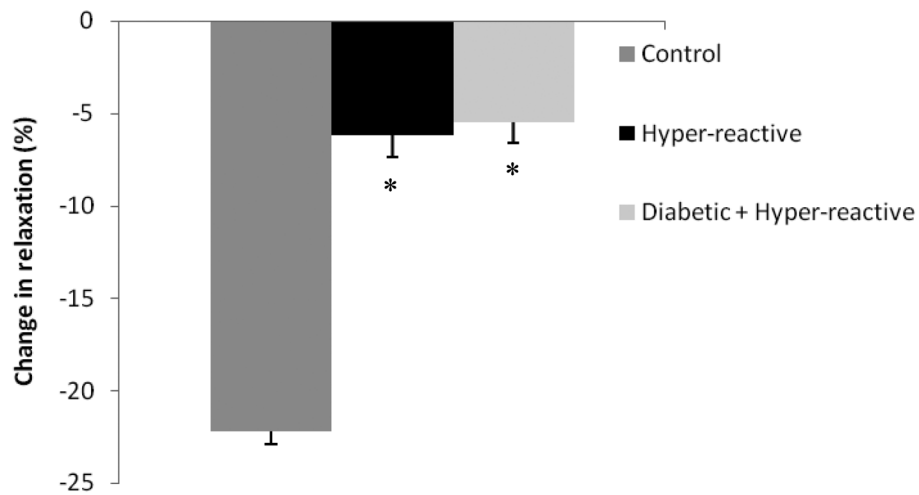

Fig. 6. Effect of diabetes and hyper-reactivity on K^+_{ATP} channel modulation of the bronchodilator response to IP. % change in IP (10 μM)-induced relaxation of tracheal rings with intact epithelium pre-contracted by ACh (10 μM) in the presence of glybenclamide (10 μM). *$P<0.05$ from Control (n=12).

intact tracheal rings was observed in hyper-reactive guinea pigs and an even significantly smaller relaxation was noted in guinea pigs having both diabetes and airway hyper-reactivity in comparison to healthy trachea. Epithelial denudation was seen to further decrease the relaxant response in the hyper-reactive condition but did not alter the IP induced relaxant response in diabetes combined with hyper-reactive airways, further supporting that there is additional impairment of the respiratory epithelium due to diabetes.

Impairment of the epithelium in trachea may lead to alteration in ASM responses due to change in the synthesis and release of a number of biologically active contractile and relaxant substances such as NO, EpDHF and PGE_2 (11, 13, 14, 24). ACh induced bronchoconstriction is depressed by both L-NAME and indomethacin in the intact tracheal tissues of healthy guinea pigs, suggesting that NO and COX pathways have a reductive

role. Other studies have also found that high concentrations of L-NAME (10^{-4} M) partially increased the contractile effect of ACh (30). NO and PGE_2 are released from both airway epithelium and smooth muscle cells and are thought to be predominately broncho-protective (12, 25, 26). The percent change in response to IP/ACh in L-NAME incubated tracheal rings was significantly more in hyper-reactive airways in comparison to healthy airways, suggesting an increased production of NO in hyper-reactive airways. Accumulating evidence have also indicated that inflammatory diseases of the respiratory tract, especially asthma, are commonly associated with elevated production of NO (27). Contrary to the response in hyper-reactive guinea pigs, the percentage change in tracheal rings from animals with both diabetic and hyper-reactive airways in response to IP/ACh due to L-NAME was reduced, indicating that diabetes decreases the production of NO. A reduction in the percentage change in response to IP/ACh due to indomethacin was observed in epithelium-intact tracheal rings from both hyper-reactive guinea pigs and in guinea pigs with both diabetes and hyper-reactivity implying that the contractile/relaxant response due ACh/IP by the COX-mediated component is impaired. However, this impairment was found to be more in guinea pigs with both diabetes and hyper-reactivity suggesting that diabetes worsens respiratory epithelial dysfunction.

In epithelium intact trachea of healthy animals, IP-induced relaxation was attenuated by indomethacin and also by glybenclamide specifying that PGE_2 and K^+_{ATP} channels play a significant role in modulating the airways which is in agreement with the earlier findings in guinea pigs (14, 16). In contrast to its effect on healthy trachea, glybenclamide did not affect the broncho-relaxation response to IP in hyper-reactive guinea pigs, implying that the response mediated by K^+_{ATP} channel component of was already impaired. The remaining response appeared to be mediated by the COX pathway as the broncho-relaxation by IP was significantly attenuated in the presence of indomethacin. This suggests that the COX pathway is relatively resistant to hyper-reactivity. However, in the case of epithelium intact tracheal rings from guinea pigs having diabetic as well as hyper-reactive condition, neither glybenclamide nor indomethacin altered the IP induced bronchorelaxation, signifying that diabetes further deteriorates the already distressed/disturbed hyper-reactive respiratory epithelium condition by impairing the COX pathway and also the EpDHF pathway.

Diabetes is coupled with increased glucose in the airway surface liquid (ASL) which distresses the respiratory epithelium or vice versa (28). Damage to the respiratory epithelium may contribute to abnormal responses of the airway smooth muscle, resulting in respiratory disorders. Some studies as mentioned earlier have shown a strong positive association between the occurrence of type 1 diabetes and the symptoms of asthma, signaling that diabetes worsens the distress of respiratory epithelium (10, 18, 29, 30).

In conclusion, the data indicates that diabetes deteriorates epithelial function in hyper-reactive trachea as a consequence of the impairment of NO pathways, COX pathways and K^+_{ATP} channels, all of which mediate the relaxation and contraction responses of the trachea. Therefore, epithelium mediated mechanisms are more likely to be important in the development of the respiratory disorders as seen in diabetic individuals.

Conflict of interest

The authors declare that they have no conflict of interest.

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