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Insulin induces a hypercontractile airway smooth muscle phenotype

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Abstract

This study aims to investigate the effects of insulin on bovine tracheal smooth muscle phenotype in vitro. Contractility of muscle strips and DNA-synthesis ([^{3}H]thymidine incorporation) of isolated cells were used as parameters for smooth muscle phenotyping. Insulin (1 μM) was mitogenic for bovine tracheal smooth muscle and potentiated DNA-synthesis induced by other growth factors. In contrast, after pretreatment of unpassaged bovine tracheal smooth muscle cells in culture, the mitogenic response induced by growth factors was strongly diminished, with no difference in the basal incorporation. Pretreatment of bovine tracheal smooth muscle strips in organ culture with insulin increased maximal contraction to methacholine and KCl. These results show that insulin acutely augments DNA-synthesis in the presence of other growth factors. In contrast, insulin pretreatment induces a hypercontractile phenotype with a decreased mitogenic capacity. This mechanism may be involved in the putative negative association between asthma and type I diabetes. In addition, these findings may have implications for the use of aerosolized insulin in diabetes mellitus.

Keywords: Insulin; Airway smooth muscle; Phenotype; Asthma; Diabetes

1. Introduction

Cultured airway smooth muscle cells are known to develop a less contractile phenotype when exposed to serum-rich culture media and growth factors, characterized by a decreased shortening capacity and contractile protein expression, while the proliferative and synthetic capabilities of these cells are enhanced (Halayko et al., 1996; Halayko and Solway, 2001; Ma et al., 1998). Phenotype switching is known to be regulated by extracellular matrix proteins that either promote (e.g. collagen type I, fibronectin) or inhibit (e.g. laminin) progression toward the less contractile and more proliferative state (Hirst et al., 2000). Recently, we have demonstrated that intact airway smooth muscle, embedded in its own extracellular matrix, is also sensitive to phenotype changes induced by exogenously applied growth factors (Gosens et al., 2002). Progression to the less contractile state can be induced by serum, platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1) and epidermal growth factor (EGF), which is linearly related to the mitogenic response of the growth factor applied (Gosens et al., 2002).

It is unknown whether the observed relationship between proliferation and phenotypic modulation is shared by all growth factors that stimulate receptors with intrinsic tyrosine kinase activity, including insulin. Insulin is known to be mitogenic for cultured human airway smooth muscle cells and to potentiate airway smooth muscle mitogenesis induced by other receptor tyrosine kinase agonists, such as EGF, and by G-protein coupled receptor agonists, such as thrombin (Ediger and Toews, 2000). Consequently, one would expect insulin to promote progression toward the less contractile state. However, induction of functionally hypercontractile myocytes has been reported after treatment with serum-free media containing insulin (Ma et al., 1998). Hence, it is of great interest to solve this discrepancy. Moreover, insight in the long-term effects of insulin on airway smooth muscle phenotype is warranted in view of recent publications on the application of aerosolized insulin in diabetes mellitus (Henry et al., 2003; Skyler et al., 2001; Steiner et al., 2002). Inducing a phenotype

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switch by this mode of administration could limit its use, especially in patients suffering from airway diseases. Moreover, long-term effects of insulin could also explain the repeatedly reported negative association between type I diabetes and asthma (Abrahamson, 1941; Douek et al., 1999).

Therefore, we investigated the effects of insulin on bovine tracheal smooth muscle phenotype in vitro, using both intact tissue and isolated cells, in which we measured contractility and proliferative responsiveness, respectively, as parameters for smooth muscle phenotype. Insulin was acutely mitogenic for bovine tracheal smooth muscle cells and synergistically potentiated mitogenesis induced by PDGF, IGF-1 and EGF. However, pretreatment with insulin induced a hypercontractile and hypoproliferative phenotype of these cells.

2. Methods

2.1. Tissue preparation and organ culture procedure

Bovine tracheae were obtained from local slaughterhouses and rapidly transported to the laboratory in Krebs-Henseleit (KH) buffer of the following composition (mM): NaCl, 117.5; KCl, 5.60; $MgSO_4$, 1.18; $CaCl_2$, 2.50; NaH_2PO_4, 1.28; $NaHCO_3$, 25.00; and glucose 5.50, pregassed with 5% CO_2 and 95% O_2; pH 7.4. After dissection of the smooth muscle layer and careful removal of mucosa and connective tissue, tracheal smooth muscle strips were prepared while incubated in gassed KH-buffer at room temperature. Care was taken to cut tissue strips with macroscopically identical length (1 cm) and width (2 mm). Tissue strips were washed once in sterile Dulbecco's modification of Eagle's medium (DMEM), supplemented with $NaHCO_3$ (7 mM), HEPES (10 mM), sodium pyruvate (1 mM), nonessential amino acid mixture (1:100), gentamicin (45 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (1.5 μg/ml). Next, tissue strips were transferred into suspension culture flasks and a volume of 7.5 ml medium was added per tissue strip. Strips were maintained in culture in an incubator shaker (37 °C, 55 rpm) for 8 days, refreshing the medium on day 4. Either fetal bovine serum or insulin were present during the entire incubation period, when applied.

2.2. Isometric tension measurements

Tissue strips, collected from suspension culture flasks, were washed with several volumes of KH-buffer pregassed with 5% CO_2 and 95% O_2, pH 7.4 at 37 °C. Subsequently, strips were mounted for isometric recording (Grass force–displacement transducer FT03) in 20 ml water-jacked organ baths, containing KH-buffer at 37 °C, continuously gassed with 5% CO_2 and 95% O_2, pH 7.4. During a 90-min equilibration period, with washouts every 30 min, resting tension was gradually adjusted to 3 g. In separate experiments, it was established that strips stretched to 3 g passive tension responded optimally. Subsequently, muscle strips were precontracted with 20 and 30 mM isotonic KCl solutions. Following two washouts, basal smooth muscle tone was established by the addition of 0.1 μM (−)-isoprenaline and tension was re-adjusted to 3 g, immediately followed by two changes of fresh KH-buffer. Following another equilibration period of 30 min, cumulative concentration response curves were constructed to stepwise increasing concentrations of isotonic KCl (5.6–50 mM) or methacholine (1 nM–100 μM). The increase in KCl concentration was compensated for by substitution with NaCl to maintain iso-osmolarity. When maximal KCl or methacholine-induced tension was obtained, the strips were washed several times and basal tone was re-established using (−)-isoprenaline (10 μM).

2.3. Isolation of bovine tracheal smooth muscle cells

Tracheal smooth muscle was chopped using a McIlwain tissue chopper, three times at a setting of 500 μm and three times at a setting of 100 μm. Tissue particles were washed two times with the medium mentioned above, supplemented with 0.5% fetal bovine serum. Enzymatic digestion was performed in the same medium, supplemented with collagenase P (0.75 mg/ml), papain (1 mg/ml) and soybean trypsin inhibitor (1 mg/ml). During digestion, the suspension was incubated in an incubator shaker (Innova 4000) at 37 °C, 55 rpm for 20 min, followed by a 10-min period of shaking at 70 rpm. After filtration of the obtained suspension over 50-μm gauze, cells were washed three times in medium supplemented with 10% fetal bovine serum.

2.4. [^{3}H]thymidine-incorporation

Bovine tracheal smooth muscle cells were plated in 24-well cluster plates at a density of 30,000 cells per well in 10% fetal bovine serum containing medium at 37 °C in a humidified 5% CO_2-incubator. After attachment overnight, cells were washed two times with sterile phosphate buffered saline (PBS, composition (mM): NaCl, 140.0; KCl, 2.6; KH_2PO_4, 1.4; $Na_2HPO_4.2H_2O$, 8.1; pH 7.4). Subsequently cells were made quiescent by incubation for 72 h in serum-free medium supplemented with 0.1% FCS, apotransferrin (5 μg/ml) and ascorbate (100 μM). When pretreatment effects of insulin were studied, 0.1% fetal bovine serum was replaced for insulin (1 μM).

After quiescence, cells were washed with PBS and stimulated with mitogens in serum-free medium for 28 h, the last 24 h in the presence of [^{3}H]thymidine (0.25 μCi/ml), followed by two washes with PBS at room temperature and one with ice-cold 5% trichloroacetic acid.

Cells were treated with this trichloroacetic acid-solution on ice for 30 min; subsequently, the acid-insoluble fraction was dissolved in 1 ml NaOH (1 M). Incorporated [^{3}H]thymidine was quantified by liquid-scintillation counting.

2.5. Data analysis

All data represent means ± S.E.M. from n separate experiments and EC_{50} was expressed as the concentration required to induce half the maximal effect (E_{max}). pD_2 values were calculated as $-\log EC_{50}$. The statistical significance of differences between data was determined by the Student's t-test for paired observations (two-tailed). Differences were considered to be statistically significant when $P<0.05$.

2.6. Materials

DMEM and methacholine chloride were obtained from ICN Biomedicals (Costa Mesa, CA, USA). Fetal bovine serum, $NaHCO_3$ solution (7.5%), HEPES solution (1 M), sodium pyruvate solution (100 mM), nonessential amino acid mixture, gentamycin solution (10 mg/ml), penicillin/streptomycin solution (5000 U/ml/5000 μg/ml), amphotericin B solution (250 μg/ml) (Fungizone) and trypsin were obtained from Gibco Life Technologies (Paisley, UK). EGF (human recombinant), IGF-1 (human recombinant), PDGF (human recombinant), insulin (from bovine pancreas), apotransferrin (human) and soybean trypsin inhibitor were obtained from Sigma (St. Louis, MO, U.S.A.). [*Methyl*-^{3}H]thymidine (specific activity 25 Ci/mmol) was obtained from Amersham (Buckinghamshire, UK). Papain and collagenase P were from Roche Diagnostics (Mannheim, Germany). All other chemicals were of analytical grade.

3. Results

3.1. Acute effects of insulin on bovine tracheal smooth muscle DNA-synthesis

Acute effects of insulin on DNA-synthesis were assessed, using cells that were made quiescent in serum-free medium for a period of 3 days. Insulin (1 μM) increased [^{3}H]thymidine incorporation to 159 ± 11% of basal (Fig. 1A, $P<0.01$). In combination with other growth factors (PDGF, IGF-1, EGF), insulin induced a synergistic enhancement of the responses. Synergism was expressed as the difference between the sum of individual responses and the measured combined response. Interestingly, as compared to IGF-1 (10 ng/ml), PDGF (10 ng/ml)-induced and EGF (10 ng/ml)-induced incorporation were potentiated to a larger extent (Fig. 1A,B).

3.2. Effects of pretreatment with insulin on bovine tracheal smooth muscle DNA-synthesis

In order to investigate the effect of pretreatment with insulin on bovine tracheal smooth muscle DNA-synthesis, cells were made quiescent in media with and without insulin (1 μM) for a period of 3 days. After this period, cells were washed and stimulated with growth factors. No difference in basal [^{3}H]thymidine incorporation could be observed for pretreatment without and with insulin that averaged 3661 ± 803 and 3459 ± 740 dpm/well ($n=15$), respectively. However, the mitogenic effect induced by PDGF (10 ng/ml) was significantly reduced after pretreatment with insulin. Similarly, a reduction in incorporated [^{3}H]thymidine was observed for IGF-1 (10 ng/ml), whereas the response to EGF (10 ng/ml) was suppressed completely (Fig. 2). Analysis of the concentration–response relationship for PDGF showed that the observed decrease manifested itself both as a

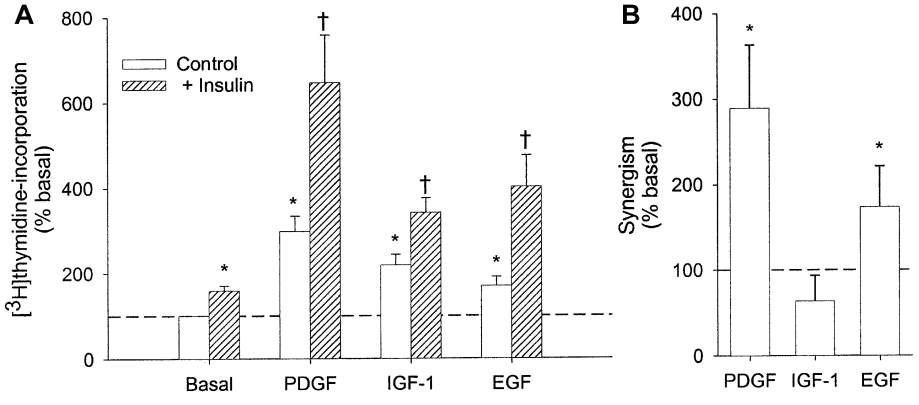

Fig. 1. (A) [^{3}H]Thymidine incorporation of unpassaged bovine tracheal smooth muscle cells. Basal responses and those in response to EGF (10 ng/ml), IGF-1 (10 ng/ml) and PDGF (10 ng/ml) were measured, both in the absence and presence of insulin (1 μM). (B) Calculated synergism of DNA-synthesis of the applied growth factors due to the presence of insulin. Data represent means ± S.E.M. of four to five experiments each performed in triplicate. *$P<0.05$ compared to control basal; †$P<0.05$ compared to absence of insulin.

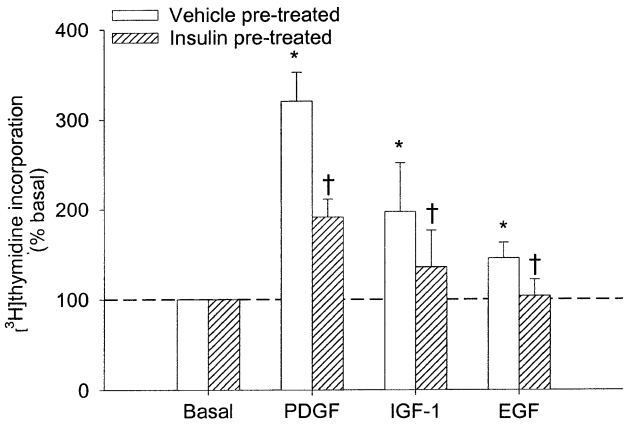

Fig. 2. [^{3}H]Thymidine incorporation of unpassaged bovine tracheal smooth muscle cells, pretreated with serum-free medium with or without insulin (1 μM) for a period of 3 days. Basal responses and those in response to EGF (10 ng/ml), IGF-1 (10 ng/ml) and PDGF (10 ng/ml) were measured. Data represent means ± S.E.M. of six experiments each performed in triplicate. *$P<0.05$ compared to basal; †$P<0.05$ compared to serum-free pretreatment.

decrease in maximal effect and as a rightward shift, indicating a decreased sensitivity (E_{max} = 338 ± 26 and 207 ± 20% of basal ($P<0.001$) and EC_{50} = 2.1 ± 0.7 and 4.0 ± 1.4 ng/ml ($P<0.05$) for pretreatment in medium without and with insulin, respectively, Fig. 3).

3.3. Effects of pretreatment with insulin on bovine tracheal smooth muscle contractility

The effects of insulin (1 μM) on bovine tracheal smooth muscle phenotype were investigated using intact organ-cultured smooth muscle strips as described previously (Gosens et al., 2002). In view of the time course of the phenotypic switch in intact tissue ($t1/2$ = 2.8 days) (Gosens et al., 2002), strips were pretreated with insulin for a period of 8 days. As positive controls, some preparations

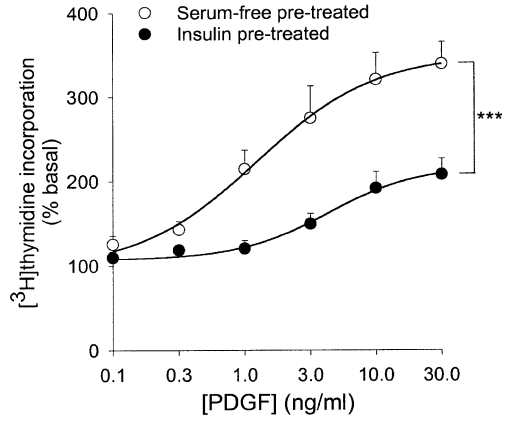

Fig. 3. PDGF-induced [^{3}H]thymidine incorporation of unpassaged bovine tracheal smooth muscle cells, pretreated with serum-free medium with or without insulin (1 μM) for a period of 3 days. Data represent means ± S.E.M. of six experiments each performed in triplicate. ***$P<0.001$.

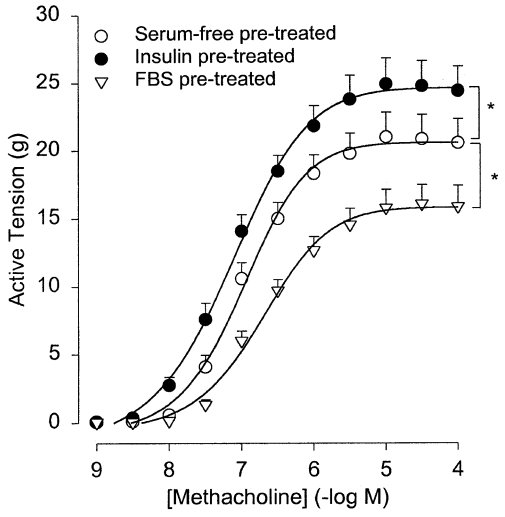

Fig. 4. Methacholine-induced contraction of bovine tracheal smooth muscle strips, pretreated with serum-free medium, medium containing 10% fetal bovine serum or medium containing insulin (1 μM) for a period of 8 days. Data represent means ± S.E.M. of eight experiments each performed in duplicate. *$P<0.05$.

were treated with 10% fetal bovine serum, known to switch to a less contractile phenotype. As expected, strips treated with 10% fetal bovine serum responded with a decrease in E_{max} for methacholine. No change in sensitivity (pD_2) was observed after treatment with 10% fetal bovine serum (Fig. 4). In contrast, strips treated with insulin responded with an increase in maximal contraction for methacholine when compared to serum-free medium pretreated strips. This increase was quantitatively similar to the decrease in E_{max} induced by 10% fetal bovine serum. In addition, a small but significant leftward shift could be

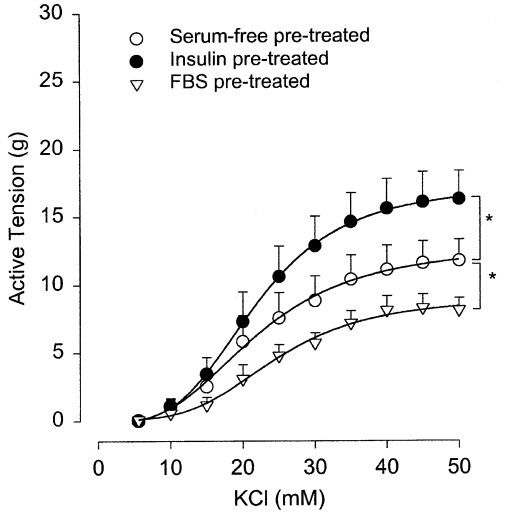

Fig. 5. KCl-induced contraction of bovine tracheal smooth muscle strips, pretreated with serum-free medium, medium containing 10% fetal bovine serum or medium containing insulin (1 μM) for a period of 8 days. Data represent means ± S.E.M. of eight experiments each performed in duplicate. *$P<0.05$.

observed in the dose–response relationship for methacholine after pretreatment with insulin ($pD_2 = 7.0 \pm 0.1$ and 7.2 ± 0.1 for pretreatment with and without insulin, $P < 0.01$). Almost similar results were found for KCl-induced contraction, both quantitatively and qualitatively. However, no shift in sensitivity (EC_{50}) after pretreatment with insulin was observed for KCl (Fig. 5).

4. Discussion

As shown in this study, the acute effects of insulin on bovine tracheal smooth muscle cells are dependent on the presence of other growth factors. Insulin, applied in a concentration generally used in airway smooth muscle cell culture media was mitogenic by itself and augmented the proliferative effects induced by submaximally effective concentrations of PDGF, IGF-1 and EGF (Kelleher et al., 1995). This augmentation was more profound for PDGF and EGF when compared to IGF-1, which is in line with results obtained by others using human airway smooth muscle cells (Ediger and Toews, 2000). Probably structural similarities between insulin and IGF-1 cause these two growth factors to act to some extent through the same receptors (Bayes-Genis et al., 2000).

In contrast to the acute effects of insulin, pretreatment with insulin induced a decrease in proliferative responsiveness. The presence of insulin during the quiescence period may have stimulated the cells to proliferate to a small extent, resulting in fewer cells that are available for stimulation by other growth factors. However, if this were the explanation for the decreased proliferative responses seen after pretreatment with insulin, basal thymidine incorporation should have been decreased as well. Moreover, proliferative responses to all growth factors should have been equally decreased. However, basal incorporated activity was similar for control and insulin pretreated cells, demonstrating that insulin-induced differences occurred selectively at the level of growth factor-induced thymidine incorporation. Furthermore, the decrease in proliferative responsiveness was dependent on the growth factor applied: the PDGF response was diminished by approximately 50%, whereas the EGF response was abolished. Since insulin pretreatment also increased contractility, the results indicate that insulin pretreatment induced a phenotypic shift towards a hypercontractile and less proliferative phenotype.

One could argue that the smooth muscle cells in strip preparations maintained in insulin are simply more viable due to the very presence of insulin and therefore respond more efficiently after 8 days in organ culture. However, serum-free maintained strips exhibit increased rather than decreased contractile responses as compared to freshly isolated strips. Moreover, growth factors which would stimulate rather than inhibit the number of viable cells decrease contractility of bovine tracheal smooth muscle strips (Gosens et al., 2002).

It is important to note that the increase in contractility after pretreatment with insulin and the decreased contractility after treatment with fetal bovine serum and other growth factors are observed for both methacholine and KCl-induced contraction. Methacholine requires receptor-induced stimulation of phosphoinositide turnover to induce calcium release, whereas KCl uses voltage-dependent calcium channels to induce calcium influx (Meurs et al., 2001). Therefore, qualitative and quantitative similarities between KCl and methacholine-induced contraction can be achieved only by modulating contraction downstream of intracellular Ca^{2+}-increases. Considering the long-term nature of the change in contractility (Gosens et al., 2002), changes at the level of the contractile machinery are the most likely explanation for the observed effects.

The hypercontractile phenotype is somewhat unexpected, since a previous report by our laboratory shows that regulation of contractility by growth factors, including IGF-1, is reciprocally related to their mitogenic responses (Gosens et al., 2002). Differences in the balance of activation of distinct kinase-isoforms may underlie this discrepancy: e.g. Akt1 and Akt2 are known to have opposite effects on skeletal muscle differentiation induced by insulin (Sumitani et al., 2002). These kinases both act downstream of phosphoinositide 3-kinase (PI 3-kinase). It should be noted that PI 3-kinase is involved, at least in part, in the growth factor-induced phenotype shift (Gosens et al., 2002).

Previous studies concerning a role for insulin in airway smooth muscle phenotype switching are not available. However, insulin is often used as a substituent in serum-free media, in which others have succeeded in inducing a hypercontractile canine airway smooth muscle phenotype (Ma et al., 1998). However, this was attributed to serum deprivation rather than to the presence of insulin (Halayko et al., 1999). Indeed, in chick gizzard smooth muscle cells, insulin has been shown to be involved in phenotypic switching to a hypercontractile phenotype (Hayashi et al., 1998). In addition, prolonged treatment of PAC1 cells with insulin induces a switch from a vascular smooth muscle phenotype to a skeletal muscle phenotype as demonstrated by the expression of skeletal muscle specific proteins. Interestingly, RT-PCR analysis in these cells showed that this smooth muscle to skeletal muscle differentiation is accompanied by increases in smooth muscle specific protein expression, such as myosin-light chain kinase (sm-MLCK), smooth muscle heavy chain (sm-MHC) and sm-calponin (Graves and Yablonka-Reuveni, 2000). These findings suggest that insulin-induced changes toward a (hyper)contractile phenotype may not be confined to smooth muscle of bovine tracheal origin.

A lower prevalence of asthma and atopy symptoms in patients with type I diabetes mellitus has been reported in a number of epidemiological studies (Abrahamson, 1941; Douek et al., 1999; Meerwaldt et al., 2002), although this is also debated (Stromberg et al., 1995). The mechanism of this putative association is still unclear. Based on the

present study, low plasma levels of insulin might be protective towards symptoms of asthma, since insulin may extend the range of airway smooth muscle phenotypic shifting either toward a proliferative or a hypercontractile phenotype, conditional on the presence of other growth factors. This could also contribute to the controversy with respect to the negative association of asthma and diabetes mellitus, since diabetics that are under well-controlled insulin treatment would be equally subjective to asthma as non-diabetic individuals. In line with this hypothesis, an increased function of inhibitory prejunctional muscarinic M_2-receptors and a decreased antigen challenge-induced influx of inflammatory cells in the airways have been demonstrated in rat model of streptozotocin-induced type I diabetes which could be reversed by the administration of insulin (Belmonte et al., 1998; Coulson et al., 2002). Using the same model, a diminished tracheal contractility was observed in long-term (8 week) diabetic rats (Cros et al., 1992), but not in 1-week diabetic rats (Belmonte et al., 1997; Coulson et al., 2002). A similar time-dependency has been observed for calmodulin expression (Ozturk et al., 1994). Phenotype switching in vivo may be a slower process than in vitro, since it is still continuing 35 days after the last challenge in repeatedly allergen-challenged rats (Moir et al., 2003), whereas growth factor induced phenotype switching in intact bovine tracheal smooth muscle in vitro is characterized by a $t1/2$ of 2.8 days (Gosens et al., 2002).

The long-term effects of insulin on airway smooth muscle phenotype switching may also be important in view of recent human studies on the effectiveness of aerosolized insulin in diabetes management (Henry et al., 2003; Skyler et al., 2001; Steiner et al., 2002). If used for diabetes treatment, lung concentrations of insulin will be chronically elevated as compared to other ways of administration. In diabetics suffering from airway diseases such as asthma as well, such treatment may worsen airway smooth muscle hyperplasia and contractility by extending the phenotype switching capacity.

In conclusion, insulin is mitogenic and potentiates mitogenesis induced by other growth factors. In contrast, pretreatment with insulin induces a hypercontractile and hypoproliferative bovine tracheal smooth muscle phenotype. Therefore, insulin may enhance either contractility or proliferation of airway smooth muscle, dependent on the duration of exposure to insulin. This may provide an explanation for the putative negative association between asthma and type I diabetes. In addition, this shows that aerosolized administration of insulin may result in adverse effects on airway smooth muscle mass and function.

Acknowledgements

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