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Down-regulation of mast cell activation and airway reactivity in diabetic rats: role of insulin

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Down-regulation of mast cell activation and airway reactivity in diabetic rats: role of insulin. S.C. Cavalher-Machado, W. Tavares de Lima, A.S. Damazo, V. de Frias Carvalho, M.A. Martins, P.M.R. e Silva, P. Sannomiya. ©ERS Journals Ltd 2004.

ABSTRACT: Hormones play a modulating role in allergic inflammation. An inverse relationship between atopy and diabetes mellitus was reported. The mechanisms regulating this interaction are not completely understood. This study examined whether insulin influences mast cell activation following antigen challenge in rats.

The experimental design included alloxan-induced diabetic rats and matching controls. Experiments were performed 30 days after alloxan injection. The animals were sensitised by *s.c.* injection of ovalbumin (OA) and aluminium hydroxide. OA-induced airway contraction, morphometric analysis of airway mast cells and tissue histamine quantification were evaluated in the isolated main bronchus and intrapulmonary bronchus upon exposure to antigen *in vitro*.

Relative to controls, a reduced contraction to OA was observed in bronchial segments isolated from diabetic rats. This was accompanied by a 50% reduction in the number of degranulated mast cells and in histamine release. A complete recovery of the impaired responses was observed under the influence of insulin.

In conclusion, the data suggested that insulin might modulate the controlling of mast cell degranulation; therefore, the early-phase response to antigen provocation, which represents a new insight into a better understanding of the mechanisms, accounted for the decreased risk of asthma among type-1 diabetic patients.

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Hormones and other endocrine factors play a modulating role in allergic inflammation, with the effects of glucocorticoids or adrenergic agents being obvious examples. Experimental findings agree with clinical evidence that striking examples of this issue are premenstrual exacerbation of asthma [1, 2], worsening of asthma and chronic urticaria by hyperthyroidism [3, 4], and the reciprocal exclusion between atopic diseases and diabetes mellitus [5, 6]. Experimental evidence indicates that an overall reduced inflammatory reaction is observed whenever a relative lack of insulin occurs in an organism [7]. Altered vascular responsiveness to inflammatory mediators [8, 9], defective leukocyte-endothelial interactions and inflammatory cell dysfunctions [10, 11] are described in insulin-deficient states. It has been already demonstrated that diabetic rats present markedly reduced inflammatory reactions to allergen challenge in the airways and the pleural space, which are unrelated to changes in the number of total blood leukocytes or blood glucose levels [12, 13]. Reversal of the impaired responses is attained by treatment of the animals with insulin [12, 13]. However, the mechanisms responsible for the reduced allergic reaction in diabetic rats are still unknown. This study supports the hypothesis that insulin might modulate the level of mast cell degranulation, therefore, controlling the inflammatory component of asthmatic responses.

Materials and methods

Animals

Male Wistar rats that weighed 180–200 g at the beginning of experiments were used. The animals were allowed a standard pellet diet and tap water *ad libitum*, and they were maintained at 23°C under a cycle of 12 h light/darkness. All experiments were in accordance with ethical principles in animal research, as adopted by the Brazilian College of Animal Experimentation, and were approved by the Biomedical Sciences Institute/University of São Paulo-Ethical Committee for Animal Research.

Induction of diabetes mellitus

Diabetes mellitus was induced by an *i.v.* injection of 42 $mg \cdot kg^{-1}$ of alloxan monohydrate (Sigma Chemical Co., St. Louis, MO, USA) dissolved in physiological saline. Control rats were injected with physiological saline only. The presence of diabetes was verified by blood glucose concentrations >11.2 $mMol \cdot L^{-1}$, determined with a blood glucose monitor (Eli Lilly, São Paulo, Brazil) in samples obtained from the cut tip of the tail. Animals were rendered diabetic 10 days before

active sensitisation, and experiments were performed 30 days after alloxan injection. Blood glucose levels were estimated immediately before the experiments.

Active sensitisation

The animals were actively sensitised against ovalbumin (OA) by *s.c.* and a subsequent *i.p.* injection of 0.2 mL sterile physiological saline, containing 100 μg OA (grade III; Sigma Chemical Co.) and 8 mg aluminium hydroxide. A second sensitisation was repeated 14 days later, and experiments were performed 7 days thereafter.

Measurement of ovalbumin-induced airway contraction in vitro

Under deep chloral hydrate anaesthesia (400 $mg \cdot kg^{-1}$ *i.p.*), the animals were exsanguinated by sectioning the abdominal aorta. The chest wall was opened, and the trachea and lungs were removed. As previously described [14], the main bronchus and the intrapulmonary bronchus were isolated and dissected out of the surrounding tissues. Ring segments of both bronchi were fixed with the aid of two steel hooks and suspended in 8 mL Krebs-Henseleit solution of the following composition: 115.0 mM NaCl, 4.6 mM KCl, 2.5 mM $CaCl_2.2H_2O$, 1.2 mM KH_2PO_4, 2.5 mM $MgSO_4.7H_2O$, 25.0 mM $NaHCO_3$ and 11.0 mM glucose. The solution was aerated with 5% CO_2 and 95% O_2 at 37°C. Tissues were allowed to equilibrate for 60 min under an initial tension of 0.5 g. During this period, the bathing solution was replaced every 20 min. The tension was then adjusted to 1.0 g, and the viability of the tissue was assessed by the addition of iso-osmolar Krebs-bicarbonate solution, containing 60 mM KCl. The bronchial segments, isolated from previously sensitised animals, were subsequently exposed to a solution of OA at a final concentration of 100 $\mu g \cdot mL^{-1}$, and the contractile responses were measured using an F-60 isometric transducer coupled to a recorder (Narco Biosystem Co., Houston, TX, USA). Results were presented as tension in g per 100 mg tissue weight.

Morphometric analysis of airway mast cells

The animals, previously sensitised against OA, were anaesthetised with chloral hydrate (400 $mg \cdot kg^{-1}$ *i.p.*), and the lungs were removed for isolation of bronchial segments, as described previously. Tissues were allowed to equilibrate in Krebs-Henseleit solution aerated with 5% CO_2 and 95% O_2 at 37°C for 60 min. During this period, the bathing solution was replaced every 20 min. The main bronchus and intrapulmonary bronchus segments were exposed to a solution of OA at a final concentration of 100 $\mu g \cdot mL^{-1}$ for 15 min. Control segments were maintained in Krebs-Henseleit solution only. The segments were fixed in 10% paraformaldehyde prepared in Sorensen phosphate buffer, pH 7.4 at 4°C for 24 h, then dehydrated in increasing concentrations of ethanol solution and washed in xylene. Samples were embedded in paraplast embedding media (Sigma Chemical Co.), sectioned at 5 μm and stained with 0.25% toluidine blue in 1% acetic acid (Sigma Chemical Co.). Airway sections were observed for the presence of intact and degranulated mast cells, using a ×40 light microscopic objective and a 100 μm^2 reticule in the focal plane. Determinations were made in three randomly selected fields of 10 serial airway 5-μm sections (at 15-μm intervals) obtained from both the main and intrapulmonary bronchi. Data were then averaged for each animal, with five animals in each group. Determinations were made by an unaware observer. Results were presented as the number of intact or degranulated mast cells per 100 μm^2.

Tissue histamine quantification

The main and intrapulmonary bronchi were isolated, as described previously. Both segments were placed in 800 μL of Hank's balanced salt solution (Sigma Chemical Co.) containing Ca^{2+} and Mg^{2+}, and then exposed, or not, to a solution of OA at a final concentration of 400 $\mu g \cdot mL^{-1}$ for 15 min at 37°C. The supernatant was then collected and stored at -20°C until needed. Histamine was measured by radioenzymatic assay [15, 16]. Briefly, 10 μL of standard histamine solution (Sigma Chemical Co.) or supernatant samples were added to 50 μL of a freshly prepared mixture containing histamine-N-methyl-transferase, partially purified from guinea pig brain [15], 0.04 μCi of (methyl-^{3}H) S-adenosyl methyonine (specific activity 15.0 $Ci \cdot mMol^{-1}$; Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) in Hank's balanced salt solution, and 0.05 M sodium phosphate buffer (pH 7.9). Measurements were made in duplicate. Blanks were prepared by replacing the samples with 0.05 M of sodium phosphate buffer (pH 7.9). After incubation at 4°C overnight, the enzymatic reaction was stopped by the addition of 0.5 mL of 1 N NaOH. The ^{3}H-methyl-histamine formed was then extracted into 3 mL of chloroform. After evaporation of the organic phase, the radioactivity was counted in a Scintilograph (Beckman Instruments Inc., Fullerton, CA, USA). Results were presented as histamine in ng per mg tissue weight. It is noteworthy that the recoverability of histamine from the main bronchi, following incubation with 20 $ng \cdot mL^{-1}$ of histamine, was comparable. Values in the absence and the presence of histamine were 11.6±1.25 and 42.4±8.4 $ng \cdot mL^{-1}$, and 6.2±0.6 and 38.4±10.6 $ng \cdot mL^{-1}$ (mean±SEM; n=3) for naïve and diabetic tissues, respectively.

Insulin treatment

The effect of insulin was observed in two situations: 1) after the treatment of diabetic animals with a single dose (4 IU *s.c.*) of neutral protamine Hagerdorn (NPH) insulin (Iolin®; Biobrás, São Paulo, Brazil), 4 h before isolation of the main bronchus and intrapulmonary bronchus; and 2) after the exposure of both segments to crystalline insulin (Biobrás, São Paulo, Brazil) at a final concentration of 1 $mU \cdot mL^{-1}$, 10 min before allergen challenge *in vitro*.

Data analysis

All data were presented as means±SEM. One-way ANOVA was performed, followed by the Tukey-Kramer multiple comparisons test. A p-value <0.05 was considered to be statistically significant.

Results

Characteristics of the study group

Animals that were rendered diabetic by the injection of alloxan exhibited a significant reduction in body weight gain during the experimental period, relative to controls (12±10 and 114±9, respectively; n=17; $p<0.0001$). Blood glucose

levels had significantly increased from control values of 5.26±0.05 to 21.78±0.50 mMol·L^{-1} ($p<0.001$).

Ovalbumin-induced airway contraction in vitro*: role of insulin*

Relative to controls, a reduced contraction to OA was observed in both segments, the main and intrapulmonary bronchi, which were isolated from diabetic rats. Treatment of the animals with a single dose of NPH insulin, 4 h before, completely restored airway contractions to OA. The values attained matched those observed in the control group (fig. 1). Blood glucose levels decreased from 23.52±1.23 mMol·L^{-1} before treatment to 12.32±1.29 mMol·L^{-1} after insulin treatment (n=6, $p<0.0001$). Similar results were obtained when insulin was added to the solution where airway segments from diabetic rats were suspended. OA-induced airway contractions matched those observed in the control group. Results are presented in figure 2. Addition of insulin alone to airway segments that were isolated from control animals was without effect (data not shown).

Effect of insulin on mast cell degranulation and histamine content in the airways

In another set of experiments, the presence of intact and degranulated mast cells was analysed in the main and intrapulmonary bronchi, isolated from animals previously sensitised to OA. Light microscopy confirmed the presence of mast cells with intact metachromatic granules in the connective tissue of bronchial segments, which were not exposed to the antigen. These mast cells were mainly localised near smooth muscle, vessels and nerves. The number of intact mast cells in segments that were not exposed to the antigen

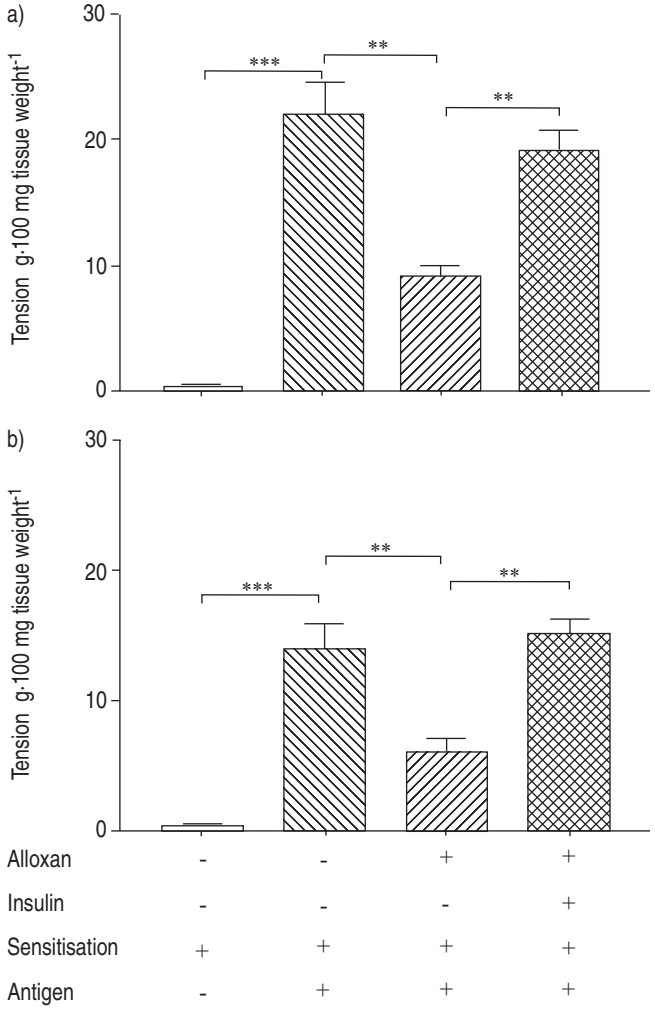

Fig. 1. – Ovalbumin (OA)-induced contraction (tension in g per 100 mg tissue weight) of the main bronchus (a) and intrapulmonary bronchus (b) isolated from control nondiabetic (n=5) and diabetic-sensitised rats (n=5). Some diabetic rats were treated with neutral protamine Hagerdorn insulin (4 IU *s.c.*) 4 h before the airway isolation procedure (n=6). Bronchial segments were exposed to OA (100 μg·mL^{-1}), and contractions were measured by means of an isometric transducer coupled to a recorder. Data are presented as mean±SEM. +: positive; -: negative. □: sensitised non-challenged control rats; ▧: sensitised challenged control rats; ▨: sensitised challenged diabetic rats; ■: sensitised challenged diabetic rats treated with insulin. **: $p<0.01$; ***: $p<0.001$.

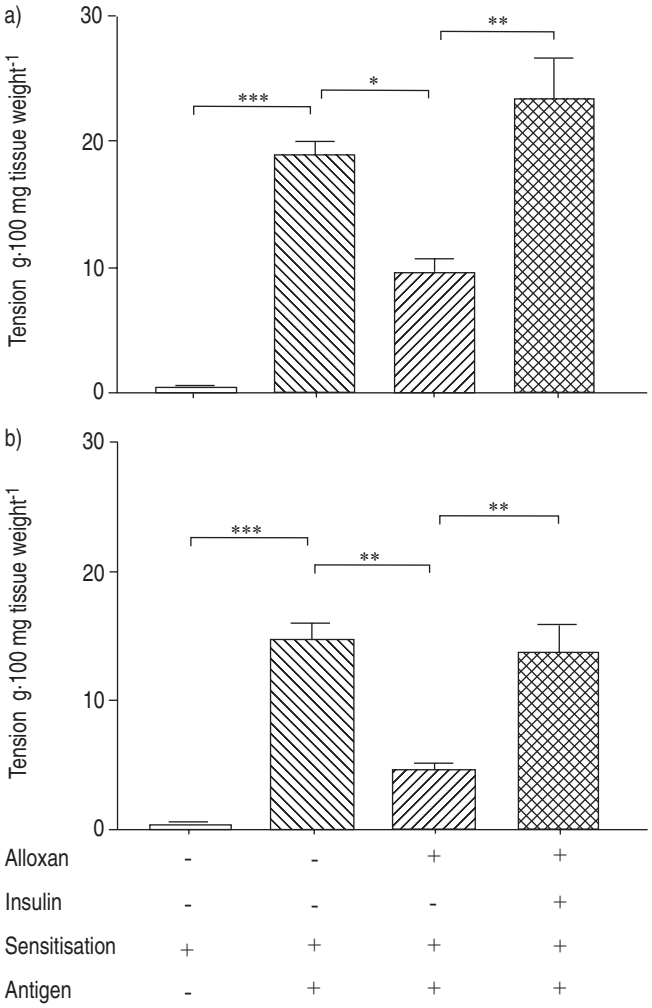

Fig. 2. – Ovalbumin (OA)-induced contraction (tension in g per 100 mg tissue weight) of main bronchus (a) and intrapulmonary bronchus (b) isolated from control nondiabetic (n=7) and diabetic-sensitised rats (n=7). In another set of experiments (n=9), insulin (1 mU·mL^{-1}) was added to the organ bath 10 min before the exposition of diabetic-derived bronchial segments to OA (100 μg·mL^{-1}). Contractions were measured by means of an isometric transducer coupled to a recorder. Data are presented as mean±SEM. +: positive; -: negative. □: sensitised non-challenged control rats; ▧: sensitised challenged control rats; ▨: sensitised challenged diabetic rats; ■: sensitised challenged diabetic rats treated with insulin. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

Table 1. – Number of intact and degranulated mast cells in ovalbumin-induced anaphylactic response in rat bronchial segments

Groups	Mast cells·100 μm^{-2}		
	Intact	Degranulated	%
Main Bronchus			
Control	0.1±0.1	3.8±0.3	97
Diabetic	2.4±0.2$^{\#}$	2.6±0.2$^{\#}$	52
Diabetic + insulin	0.5±0.2	4.5±0.3¶	90
Intrapulmonary Bronchus			
Control	0±0	3.2±0.4	100
Diabetic	1.5±0.2$^{\#}$	1.8±0.2$^{\#}$	55
Diabetic + insulin	0.3±0.2	3.2±0.2	91

Data are presented as mean±SEM and %. Determinations were made in three randomly selected fields of airway sections (10 samples per animal, five animals per group). $^{\#}$: $p<0.001$ *versus* other groups; ¶: $p<0.001$ *versus* controls.

was equivalent in control and diabetic rats. Values were 4.6±0.3 and 4.2±0.2 cells·100 μm^{-2} in the main bronchus and 2.9±0.2 and 3.0±0.2 cells·100 μm^{-2} in the intrapulmonary bronchus, both segments isolated from control and diabetic rats, respectively (seven animals per group). The addition of OA to the preparations determined a reduction in the number of intact cells and a corresponding increase in the number of degranulated mast cells (table 1). However, whereas the average rate of degranulation was 97% in the main bronchus and 100% in the intrapulmonary bronchus isolated from the control animals, the level of degranulation was only 52% in the main bronchus and 55% in the intrapulmonary bronchus in diabetic rats. The addition of insulin, prior to exposition of the preparations to OA, completely restored the level of mast cell degranulation. Values were 90% in the main bronchus and 91% in the intrapulmonary bronchus (table 1). Representative sections of these preparations are illustrated in figures 3 and 4. Similar results were obtained when both segments were evaluated for the release of histamine upon exposure of the preparations to OA *in vitro*. Release of histamine was very low (main bronchus) or undetectable (intrapulmonary bronchus) in segments derived from sensitised control rats not stimulated with OA. Release of histamine was remarkably increased after contact of sensitised preparations with OA. Results are illustrated in figure 5. In contrast, the OA-induced release of histamine in segments derived from diabetic rats was significantly reduced, particularly in the main bronchus. Values of histamine release were restored after the addition of

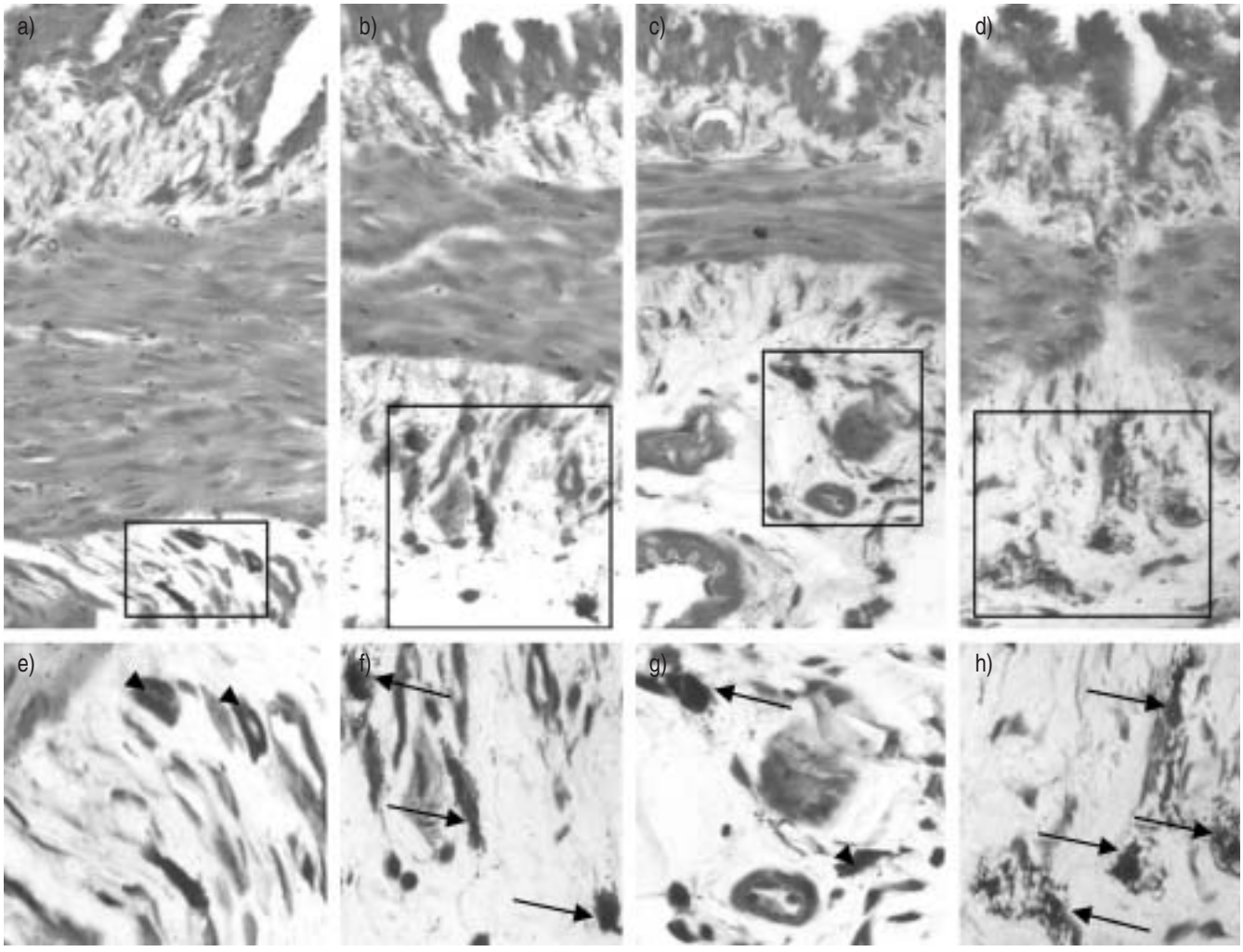

Fig. 3. – Photomicrographs of airway sections after ovalbumin challenge *in vitro*. Main bronchus (a–d, with the corresponding e–h) sections (5 μm) were isolated from sensitised rats before challenge (a and e), sensitised rats after challenge (b and f), diabetic-sensitised rats after challenge (c and g), and insulin-treated diabetic-sensitised rats after challenge (d and h). Intact (arrowhead) and degranulated (arrow) mast cells were observed surrounding the airway tissue (Toluidine blue stain; a–h).

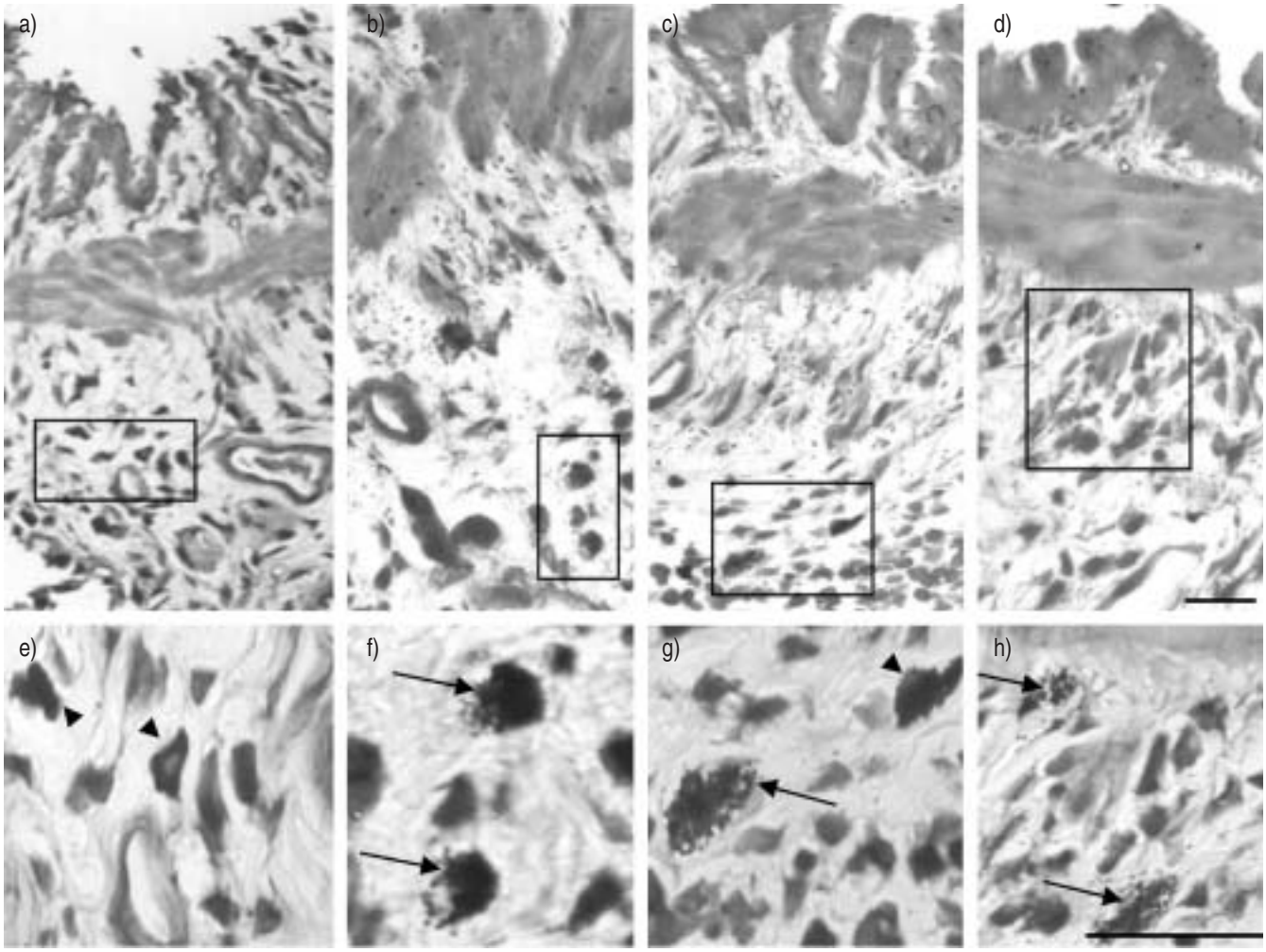

Fig. 4. – Photomicrographs of airway sections after ovalbumin challenge *in vitro*. Intrapulmonary bronchus (a–d, with the corresponding e–h) sections (5 μm) were isolated from sensitised rats before challenge (a and e), sensitised rats after challenge (b and f), diabetic-sensitised rats after challenge (c and g), and insulin-treated diabetic-sensitised rats after challenge (d and h). Intact (arrowhead) and degranulated (arrow) mast cells were observed surrounding the airway tissue (Toluidine blue stain; a–h). Scale bars=15 μm.

insulin, prior to exposure of preparations to the antigen (fig. 5).

Discussion

The results presented suggest that mast cell degranulation is modulated by insulin. The suggestion is supported by the following observations. First, relative to controls, a reduced contraction to OA was observed in bronchial segments isolated from previously sensitised diabetic rats. Secondly, this was accompanied by marked reduction in the number of degranulated mast cells and in histamine release upon exposure of bronchial segments to the antigen. Thirdly, a complete recovery of the impaired responses was observed under the influence of insulin.

Allergen challenge *in vitro* induces the contraction of bronchial segments that were isolated from previously sensitised rats [14], as a result of an immunoglobulin (Ig)E-dependent mediator release by activated mast cells. Relative to controls, contraction of both segments, the main and the intrapulmonary bronchi, was markedly reduced when isolated from diabetic-sensitised rats. The impaired response was apparently not related to antibody production. In fact, the relationship between the refractoriness of diabetics to atopic diseases and the prevention of IgE production is still controversial. It has already been demonstrated that the formation of IgE antibodies to sensitising antigens appeared drastically reduced in alloxan-diabetic mice [17] and rats, providing the animals were induced to be diabetic with alloxan and then sensitised to OA [18]. However, in another study, it has been shown that alloxan-induced diabetic rats exhibited enhanced IgE antibody titres of the same magnitude as matching controls, with negligible IgG antibody production in both groups of animals [12]. These discrepancies can be attributed to the different sensitisation protocols and animal strains used. The mechanism underlying the impaired allergic contractile responses in diabetic rats cannot be ascribed to a reduction in the number of mast cells within the lungs, as demonstrated in the pleural [13] and peritoneal cavities [19] of alloxan-treated rats. In the present study, the number of intact mast cells was equivalent in both bronchial segments, isolated either from control or diabetic rats. Similar findings have been reported by BELMONTE *et al.* [20] in the case of streptozotocin-induced diabetes. There were also no differences in the number of mast cells in the airway wall and in the association with nerves between diabetic rats and their controls [20]. Differentiation and maturation of mast cells typically occurs in peripheral tissues, where they are associated with nerves and distributed in the proximity of

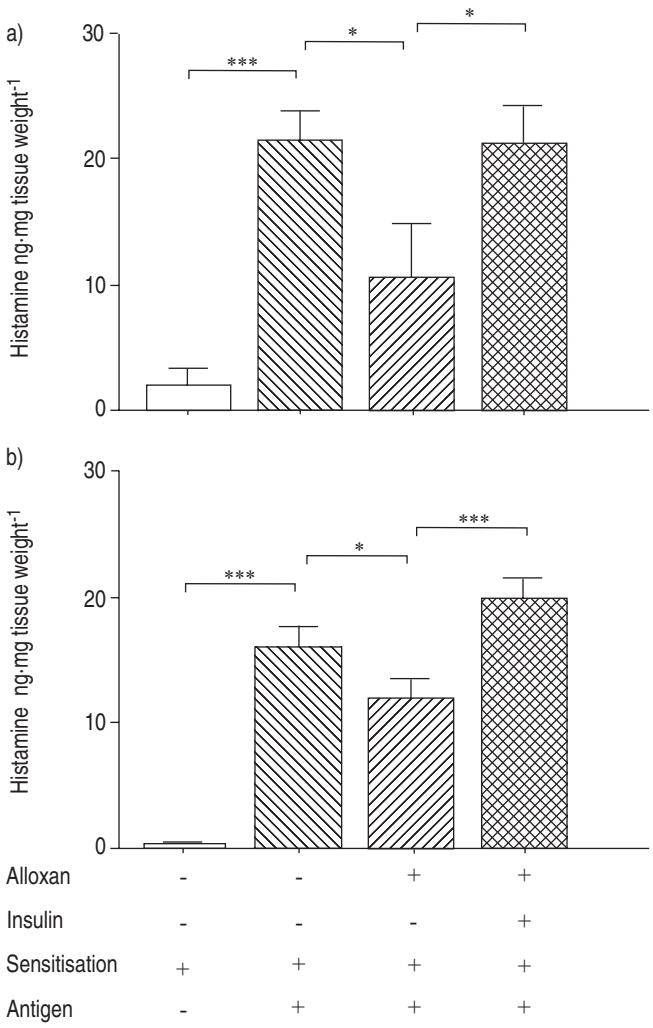

Fig. 5. – Histamine release (ng per mg tissue weight) from the main bronchus (a) and intrapulmonary bronchus (b) isolated from control nondiabetic (n=5) and diabetic (n=5) sensitised rats. In another set of experiments (n=7), insulin (1 mU·mL^{-1}) was added to the organ bath 10 min before the exposition of diabetic-derived bronchial segments to ovalbumin (400 μg·mL^{-1}). Histamine levels were determined by a radioenzymatic assay. Data are presented as mean±SEM. +: positive; -: negative. □: sensitised non-challenged control rats; ▨: sensitised challenged control rats; ▨: sensitised challenged diabetic rats; ■: sensitised challenged diabetic rats treated with insulin. *: $p<0.05$; ***: $p<0.001$.

blood vessels. These processes are tightly regulated by cytokines and, perhaps, other microenvironmental factors, which results in the generation of heterogeneous populations of mast cells [21]. As a result of the vast heterogeneity of cell type, distribution, anatomic association and function, survival and proliferation of mast cells may be dissimilar in different tissues even under the same stimulus [21]. However, striking differences were observed in the number of degranulated mast cells after allergen challenge *in vitro*. The level of mast cell degranulation in diabetic rats was only 50–55% when compared to matching control values, either in the main or intrapulmonary bronchus, suggesting some kind of refractoriness to the action of OA. This finding was correlated with a marked reduction in the content of histamine that was released upon exposure of airway segments to the antigen. Accordingly, the reduced bronchoconstriction to allergen challenge *in vitro*, observed in both segments isolated from diabetic rats, could be ascribed to an impairment in mast cell degranulation, therefore, reducing the release of airway smooth muscle constrictor agents, including histamine and other rodent mast cell-specific spasmogens, such as serotonin.

Mast cells that were present in the bronchoalveolar fluid of patients with asthma exhibited increased spontaneous and IgE-dependent mediator release [22], including histamine, prostaglandin-D_2 and cysteinyl leukotrienes, which are potent spasmogens of airway smooth muscle [23]. Strong correlations have been observed between the severity of bronchial hyperresponsiveness and mast cell number, histamine concentration and spontaneous histamine release in the bronchoalveolar fluid of patients with asthma [24, 25]. Furthermore, mast cells are significantly more numerous in asthmatics than nonasthmatics [26, 27], even in patients with newly diagnosed asthma [28]. Interestingly, there is a remarkable difference between the number of mast cells in the airway smooth muscle in patients with asthma and the number in both normal subjects and patients with eosinophilic bronchitis, a condition with many of the features of asthma, but with normal airway function [29].

It is now recognised that mast cells play a significant role in the pathophysiology of asthma through their immunomediator secretory activities, as a response to their activation by both immunological and nonimmunological stimuli [23]. The current data suggest that type-1 diabetes might be associated to a reduced state of mast cell activation in the early-phase response of an organism to the allergen. In fact, it has been reported that mast cells from diabetic rats were shown to be refractory to antigen stimulation, both *in vivo* [13] and *in vitro* [30].

The following observations support the hypothesis that insulin might be required for the development of the initial events of the asthmatic reaction. First, full recovery of OA-induced bronchoconstriction was observed after treatment of diabetic rats with a single dose of NPH insulin, administered 4 h before airways isolation for challenge *in vitro*. The current data matched those observed in bronchial segments isolated from control nondiabetic rats. Secondly, similar results were obtained when insulin was added to the bathing solution 10 min before antigen challenge *in vitro*. Insulin *per se* had no effect on airway reactivity, but allowed the recovery of airway responses to OA. Thirdly, the finding was accompanied by an increase in the number of degranulated mast cells in diabetic rats, matching the number observed in controls. Finally, OA-induced release of histamine returned to normal levels under the influence of insulin.

Insulin given as a single dose was not sufficient to reduce blood glucose levels to normal values. Therefore, the impaired responses observed in diabetic rats may be primarily linked to a continuing deficiency of insulin, rather than to hyperglycaemia. The suggestion is further supported by the observation that reversal of the impaired responses was attained by direct contact of airway segments with the hormone *in vitro*.

Experimental evidence indicates that insulin appears to play a pivotal role in the development of airway inflammation. Alloxan-induced diabetic rats that were sensitised against OA present markedly decreased cell yields from bronchoalveolar lavage after antigen challenge, which can be reversed by insulin treatment [12]. Insulin is required for the development of airway inflammation, loss of neuronal M_2-muscarinic receptor function and subsequent hyperresponsiveness in antigen-challenged rats [20].

Data from recent epidemiological studies in children with type-1 diabetes mellitus [31, 32] are consistent with data from previously published studies [33–35], reporting an inverse relationship between atopy and diabetes mellitus. The suggestion is that the presence of a T-helper 1 cell-mediated disease may lower the prevalence of atopic diseases. However,

if insulin is required for the development of allergic airway inflammation [12], some children may have both diseases, implying that asthma may be suppressed in diabetic individuals because there is a relative lack of insulin, which, in turn, would allow asthma to manifest itself clinically.

In conclusion, the current data suggest that insulin might modulate mast cell degranulation controlling, therefore, the early-phase response to antigen provocation, which represents a new insight for a better understanding of the mechanisms, accounted for the decreased risk of asthma among type-1 diabetic patients.

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