

## The allergic inflammatory reaction in neonatal streptozotocin-induced diabetic rats: evidence of insulin resistance and microvascular dysfunction

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Received 18 October 2007; returned for revision 20 December 2007; received from final revision 4 March 2008;  
accepted by I. Ahnfelt-Rønne 24 March 2008

Published Online First 13 November 2008

**Abstract.** *Objective:* To investigate the allergic reaction in neonatal streptozotocin (nSTZ)-induced diabetes mellitus.

*Material:* Male newborn Wistar rats were made diabetic by the injection of streptozotocin (160 mg/kg, i.p.) and used 8 weeks thereafter.

*Treatment:* Animals were sensitized against ovalbumin (OA, 50 µg and Al(OH)<sub>3</sub>, 5 mg, s.c.) and challenged 14 or 21 days thereafter.

*Methods:* OA-induced airway inflammation and OA-induced pleurisy models were used to investigate leukocyte migration (total and differential leukocyte counts) and lung vascular permeability (Evans blue dye extravasation).

*Results:* nSTZ-diabetic rats presented glucose intolerance and insulin resistance. Relative to controls, nSTZ rats exhibited a 30% to 50% reduction in lung vascular permeability. Leukocyte infiltration in both models of allergen-induced inflammation, and number of pleural mast cells did not differ between groups.

*Conclusions:* Data suggest that the reduction of allergic inflammatory reactions in nSTZ rats is restricted to microvascular dysfunctions and associated, probably, with insulin resistance in lung microvascular endothelium.

**Key words:** Type 2 diabetes – Allergic inflammatory reaction – Lung vascular permeability

### Introduction

Hormones and other endocrine factors play a modulating role in inflammation [1]. Experimental evidence indicates that an overall reduced inflammatory reaction is observed whenever a relative lack of insulin occurs in an organism [1, 2]. Abnormalities observed in experimental models of Type 1 diabetes include decreased microvascular responses to inflammatory mediators such as histamine and bradykinin [3, 4]; leading to decreased protein leakage and edema formation [5–7]. The early local exudative cellular reaction to inflammatory stimuli is reduced in alloxan-diabetic rats [8–10]. Decreased leukocyte-endothelial cell interactions [11–13], reduced mast cell degranulation [14, 15], and increased inducible nitric oxide synthase expression and activity in neutrophils [16] are, at least in part, responsible for the impaired leukocyte infiltration in inflammatory lesions of diabetic animals. In addition, increased lymphocyte apoptosis [17], and a reduction in lymph node retention capacity [18] have been described.

Alloxan-induced diabetic rats when sensitized against ovalbumin (OA) present markedly decreased cell yields from bronchoalveolar lavage after antigen challenge, which can be reversed by insulin treatment [19]. Insulin is required for the development of airway inflammation, loss of neuronal M<sub>2</sub>-muscarinic receptor function, and subsequent hyperresponsiveness in antigen-challenged rats [20]. These experimental findings agree with clinical evidence that the relationship between Type 1 diabetes and asthma is to some extent reciprocal, as children with diabetes [21, 22], and their siblings [21] are partially protected against asthma. That insulin plays an essential role in regulating the course of allergic

inflammatory reactions was clearly demonstrated in studies carried out in type 1 models of diabetes. The present study was aimed at investigating the relationship between asthma and Type 2 diabetes. When STZ is administered to neonatal (nSTZ) 2 days old rats it produces a type 2 diabetes model in adulthood, characterized by glucose intolerance and insulin resistance [23, 24]. In a previously published study, Cuman and coworkers [25] demonstrated that nSTZ-induced type 2 diabetic rats exhibit reduced inflammatory responses to carageenan, which are partially corrected by adrenalectomy.

The present study was designed to investigate protein leakage and leukocyte migration in ovalbumin (OA)-sensitized and challenged nSTZ-induced type 2 diabetic rats.

## Materials and methods

### Drugs and reagents

Streptozotocin sodium citrate, glucose, ovalbumin, phosphate-buffered saline, and ethylenediaminetetracetic sodium salt were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Aluminum hydroxide was obtained from EMS Sigma Pharma (São Paulo, SP, Brazil). Heparin (Liquemine®) was obtained from Roche (Rotkreuz, Switzerland). Evans blue and formamide were purchased from Merck (Darmstadt, Germany).

### Animals

The animals were allowed a standard pellet diet and tap water *ad libitum*, and were maintained at  $23 \pm 2^\circ\text{C}$  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 approved by the Biomedical Sciences Institute/University of São Paulo-Ethical Committee for Animal Research.

### Induction of diabetes mellitus

Type 2 diabetes was induced in male newborn (2 days old) Wistar rats by an intraperitoneal injection of 160 mg/kg STZ dissolved in citrate buffer (10 mM, pH 4.5) as previously described [25]. Control rats were injected with citrate buffer only. Eight weeks later, animals were placed in a metabolic cage during 24 h to evaluate food and water consumption and urine volume. Glycosuria was qualitatively assessed in urine with the aid of reagent strips (Diastix®, SP, Brazil). The presence of diabetes was verified by blood and urine glucose levels, 24-h urinary volume, food and water intake, and body weight changes.

### Oral glucose tolerance test (oGTT)

Rats fasted for 6 h were given glucose (1 g/kg) by the oral route. Glycemia was estimated before and 30 min after glucose challenge in blood samples obtained from the cut tip of the tail with the aid of a glucose monitor (Advantage®, Roche Diagnostics Co., IN, USA).

### Intravenous glucose tolerance test (ivGTT)

Rats were fasted for 6 h and then anesthetized under ether atmosphere to i. v. glucose challenge (1 g/kg.). The animals were sacrificed under CO<sub>2</sub> atmosphere and blood samples were collected from the abdominal aorta immediately before and 5, 20 and 60 min after glucose challenge. After centrifugation at 4 °C for 10 min, 20 µl of plasma samples were immediately used for glucose determination by the glucose-oxidase test (Labtest

Diagnostica S.A., Lagoa Santa, MG, Brazil). The remaining plasma was kept at  $-20^\circ\text{C}$  until assayed using a commercial insulin radioimmunoassay kit (ICN Pharmaceuticals Inc., Orangeburg, NY, USA).

### OA-induced pleurisy

Active sensitization of the animals was achieved by a subcutaneous injection of 0.2 ml sterile physiologic saline containing 50 µg OA and 5 mg Al(OH)<sub>3</sub>. Fourteen days thereafter, 0.1 ml sterile physiologic saline containing 12 µg OA was injected into the pleural cavity of the animals, using a 27.5-gauge needle adjusted to 3 mm in length. Control rats received physiologic saline only by the same route. The animals were killed under CO<sub>2</sub> atmosphere 4 and 24 h after OA challenge. The chest wall was opened, and the inflammatory exudate was withdrawn after washing the pleural cavity with 3 ml of phosphate-buffered saline (PBS) containing ethylenediamine tetracetic acid (EDTA, 100 mM). Total cell counts were determined in a hemocytometer. The differential analysis was performed under oil immersion objective in cytocentrifuged smears coloured with May-Grunwald-Giemsa stain. A total of 100 cells were counted and classified as neutrophils, eosinophils or mononuclear cells on the basis of normal morphological criteria. Mast cell counts were determined in a hemocytometer after staining the cells with toluidine blue dye in PBS (1:10).

### OA-induced airway inflammation

The animals were actively sensitized against OA by a subcutaneous injection of 0.2 ml sterile physiologic saline containing 50 µg OA and 5 mg Al(OH)<sub>3</sub>. A second sensitization was repeated 14 days later and experiments performed 7 days thereafter. Under ether anesthesia the trachea was exposed through a midline ventral incision of approximately 0.5 cm length in the neck. With the aid of a 27.5-gauge needle, 0.2 ml physiologic saline containing 700 µg of OA was instilled into the airways for antigen provocation. Control rats received physiologic saline alone by the same route. Bronchoalveolar lavage (BAL) was performed 48 h after OA challenge. The animals were anesthetized as described above and the abdominal cavity was opened for exsanguination from the abdominal aorta. The lungs were then lavaged by instillation of 5 ml PBS containing EDTA (100 mM), at room temperature, using a polyethylene tubing, 1 mm in diameter, inserted into the trachea. The procedure was repeated 3 times. The BAL fluid was not used if the retrieved volume was less than 85 % of the 15 ml instilled. Total cell counts were determined in a hemocytometer. Differential leukocyte counts were carried out on stained films under oil immersion microscopy. A total of 100 cells were counted based on morphological criteria.

### Lung vascular permeability

Animals were actively sensitized to OA as described above. Lung vascular permeability was assessed by Evans blue dye extravasation as described by Sirois et al [26]. In brief, animals were given Evans blue dye (20 mg/kg, i. v.) immediately before OA challenge. OA (10 mg) dissolved in 1 ml physiologic saline was administered by an ultrasonic nebulizer (Pulmoclear II, Soniclear, São Paulo, Brazil) connected to a closed box in which the animal was kept for 15 min. The animals were sacrificed under CO<sub>2</sub> atmosphere, and exsanguinated from the abdominal aorta. The trachea, main and intrapulmonary bronchi, and lung parenchyma were removed, and divided in order to obtain 2 samples. Samples were weighed, one sample was placed in formamide (4 ml/g tissue wet weight) at 20 °C for 24 h, and the other one was put to dry in stove (37 °C) for 7 days. The concentration of Evans blue dye extracted by formamide was measured by spectrophotometry at 620 nm using standard dilutions of Evans blue in formamide (0.3–100 µg/ml). The dry/wet weight ratio of each sample was determined (index of edema) and used in the final calculation of dye extravasation, which was expressed as µg Evans blue/g dry weight. Results presented as a function of dry weight of tissue avoid underevaluation of changes due to edema formation [26].

### Data analysis

All data are presented as means  $\pm$  SEM, and analysed by Student *t* test or one-way analysis of variance followed by the Tukey-Kramer multiple comparisons test when appropriate. Differences were considered statistically significant at  $P < 0.05$ .

## Results

### Characteristics of study groups

Table 1 summarizes the general characteristics of 8 weeks old nSTZ diabetic rats and matching controls. Diabetic rats presented a significant increase in 24-h water intake and urine volume compared to control rats. Despite no differences in food intake, diabetic rats exhibited a reduction in body weight gain. Plasma glucose levels were 20 % above normal values, and glucose in urine was  $>50$  mmol/l. There were no differences in the levels of plasma insulin compared to control rats. nSTZ diabetic rats submitted to oGTT showed an increase in blood glucose levels from (mean  $\pm$  SEM)  $4.4 \pm 0.1$  mmol/l to  $9.6 \pm 0.5$  mmol/l ( $n = 17$ ,  $P < 0.001$ ). Values attained in control rats were  $4.5 \pm 0.1$  mmol/l and  $5.6 \pm 0.1$  mmol/l ( $n = 12$ ,  $P > 0.05$ ) before and after glucose intake, respectively.

### ivGTT and insulin resistance

As illustrated in Figure 1, immediately before glucose challenge (at 0 time) glycemic values were higher in diabetic rats than in controls. Plasma glucose levels increased during ivGTT, peaking at 5 min in both groups of animals ( $P > 0.05$ ). In control rats glycemia decreased until normalization at 60 min. In contrast, values attained in nSTZ diabetic rats were significantly higher up to 60 min. Plasma insulin levels, which did not differ between groups at 0 time, increased sharply in control animals peaking at 5 min and normalizing at 60 min. However, in nSTZ diabetic rats levels of insulin did not change during ivGTT. The homeostasis model assessment of insulin resistance ( $HOMA_{ir}$ ) was calculated to determine the insulin resistance level in the nSTZ group according to the equation:  $HOMA_{ir} = \text{fasting plasma insulin } (\mu\text{U/ml}) \times \text{fasting plasma glucose (mmol/l)} / 22.5$  [27]. Values were (mean  $\pm$  SEM)  $10.53 \pm 1.07$  and  $22.17 \pm 2.14$  ( $n = 7$ ,  $P < 0.05$ ) in control and diabetic rats, respectively, suggesting the presence of insulin resistance in the nSTZ diabetic group.

### OA-induced pleurisy in nSTZ diabetic rats

Data presented in Figure 2 showed that there was a 5-fold increase in the number of leukocytes migrating into the pleural cavity of OA challenged control rats, compared to saline treated rats during the 4-h OA pleurisy. Neutrophils accounted for  $\sim 45\%$  of migrated cells, mononuclear leukocytes were  $\sim 50\%$  and eosinophils  $\sim 5\%$ . When control rats were submitted to 24-h OA pleurisy, number of migrated leukocytes was comparatively reduced. There was a 3-fold increase in the

**Table 1.** General characteristics of eight week-old animals

|                                     | Animals                |                         |
|-------------------------------------|------------------------|-------------------------|
|                                     | Control rats           | nSTZ diabetic rats      |
| Food intake/24 h (g)                | $31 \pm 1$<br>(26)     | $34 \pm 2$<br>(23)      |
| Water intake/24 h (ml)              | $44 \pm 2$<br>(26)     | $95 \pm 6$<br>(23)      |
| Urine volume/24 h (ml)              | $6.0 \pm 0.6$<br>(26)  | $39.0 \pm 4.2$<br>(23)  |
| Body weight (g)                     | $189 \pm 2$<br>(26)    | $175 \pm 2$<br>(23)     |
| Plasma glucose (mmol/l)             | $5.7 \pm 0.1$<br>(06)  | $7.0 \pm 0.2$ †<br>(08) |
| Plasma insulin ( $\mu\text{U/ml}$ ) | $47.2 \pm 8.9$<br>(06) | $50.2 \pm 12.3$<br>(08) |

Data are means  $\pm$  SEM. Figures in parentheses indicate the number of animals used.

†  $P < 0.05$ ,  $P < 0.0001$

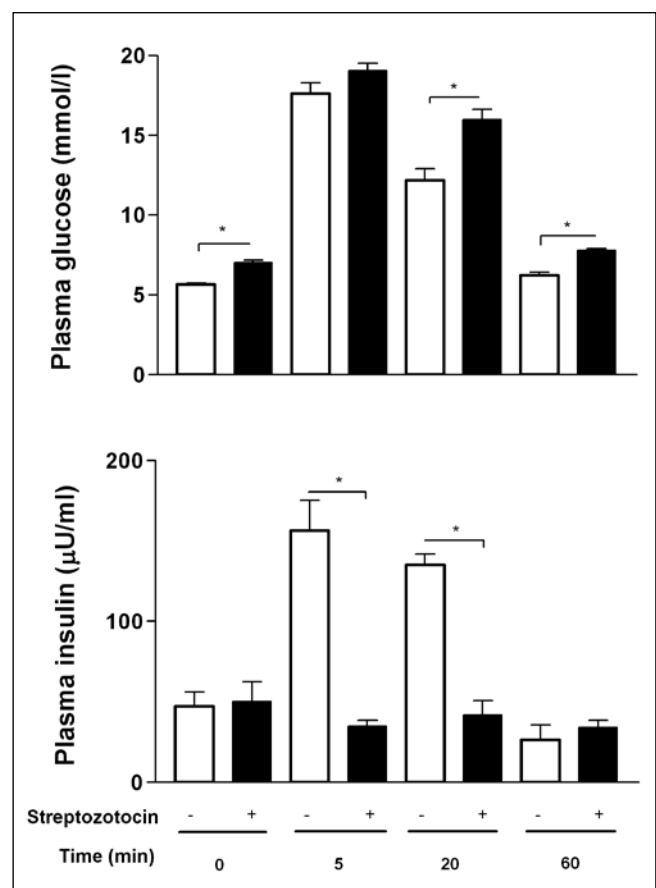


**Fig. 1.** Plasma glucose (mmol/l) and insulin ( $\mu\text{U/ml}$ ) levels during intravenous glucose tolerance test (ivGTT), eight weeks after streptozotocin injection (160 mg/kg, i.p.). Glucose (1 g/kg, i.v.) was administered to control rats (open bars) and neonatal streptozotocin-induced diabetic rats (black bars). Values are means  $\pm$  SEM for 6 to 8 animals. \*  $p < 0.05$  vs corresponding values in control group.

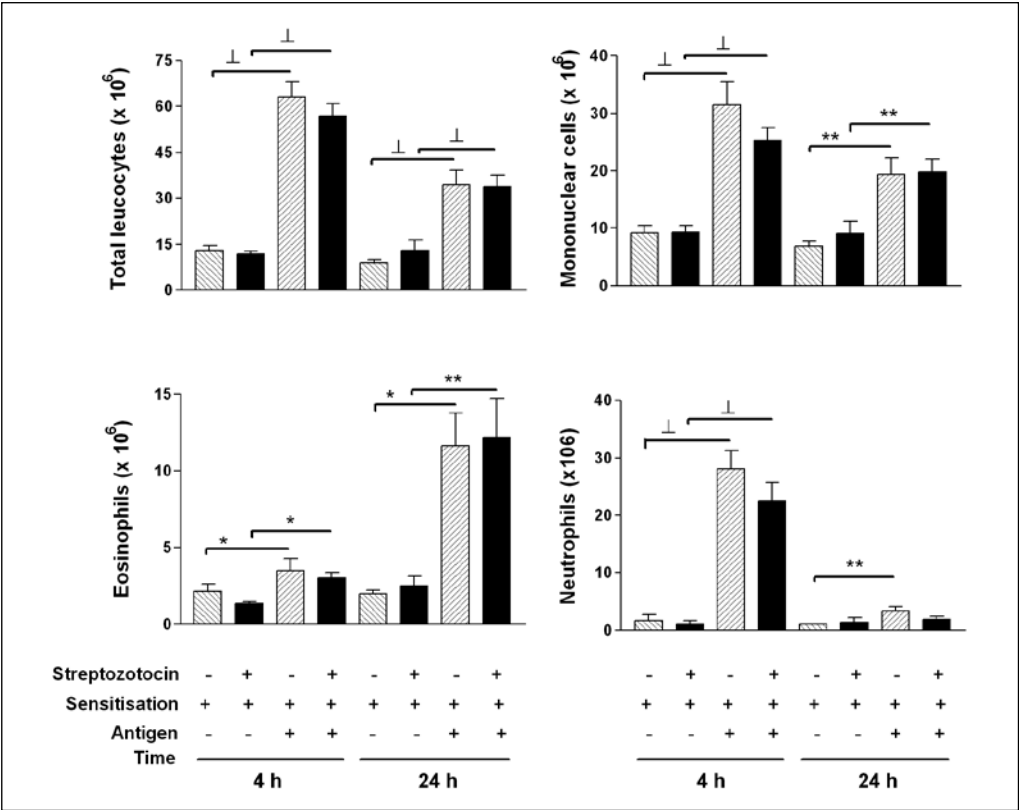


**Fig. 2.** Number of leukocytes into inflammatory exudate (pleurisy) of control (hatched bars) and neonatal streptozotocin-induced diabetic rats (black bars), 4 h and 24 h after saline or antigen challenge (12 µg ovalbumin/0.1 ml saline). Values are means ± SEM for 9 to 14 rats in control group, and 13 to 18 rats in the diabetic group. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; †  $p < 0.001$  vs corresponding values in saline-treated animals.

number of total cells in OA challenged control rats, compared to saline treated rats. Eosinophils were ~34 %, mononuclear cells, ~56 % and neutrophils, ~10 %. Similar results were observed in nSTZ diabetic rats at 4 and 24 h after OA challenge. There were no differences in the number of mast cells in the pleural cavity of nSTZ and control rats. Values were (mean ± SEM):  $651 \pm 81 \times 10^3$  cells in nSTZ ( $n = 7$ ), and  $589 \pm 82 \times 10^3$  cells in control rats ( $n = 6$ ,  $P > 0.05$ ).

*OA-induced airway inflammation in nSTZ diabetic rats*

Relative to saline instilled rats, OA challenged control rats presented, 48 h thereafter, an increase in the number of leukocytes migrated into the airways. Eosinophils accounted for 41 % of the migrated cells, neutrophils were 15 %, and mononuclear leukocytes 43 %. Similar results were observed in nSTZ diabetic rats 48 h after OA challenge. Results are summarized in Table 2.

*Lung vascular permeability in nSTZ diabetic rats*

Lung vascular permeability evaluated by Evans blue dye extravasation showed that there were no differences in the response to saline challenge between nSTZ diabetic rats and matching controls (Fig. 3). Lung vascular permeability was 2 to 3.5-fold increased in all samples analyzed, including trachea, lung parenchyma, main and intrapulmonary bronchi, after OA challenge of control rats compared to saline challenged rats. In contrast, vascular permeability responses of

nSTZ diabetic rats to OA were significantly reduced in airway segments, including the trachea (50 %), lung parenchyma (46 %), main bronchus (33 %), and intrapulmonary bronchus (49 %), compared to corresponding values observed in OA challenged control rats. Data are illustrated in Figure 3.

**Discussion**

The present study examined the course of the allergic reaction assessed by OA-induced airway inflammation and OA-induced pleurisy models in adult rats made diabetic by treatment with STZ during the neonatal period. Data presented suggest that type 2 diabetes is associated with a reduction in protein leakage but not with leukocyte recruitment in allergen-induced inflammation. The suggestion is supported by the following observations. First, nSTZ diabetic rats exhibited a 30 to 50 % reduction in protein leakage as indicated by Evans blue extravasation into the lungs. Second, the number of leukocytes harvested from the bronchoalveolar lavage fluid of nSTZ diabetic rats did not differ from those observed in matching control rats. Third, similar results were observed in allergen-induced pleurisy. There were no differences in the number of leukocytes recovered from the pleural cavity of both groups of animals in response to OA challenge.

Direct measurements of water consumption, diuresis, glycosuria, presence of moderate hyperglycemia, and a clinical picture of glucose intolerance are in agreement with the classical signs that characterize type 2 diabetes in humans. These characteristics were observed in eight-week old nSTZ rats compared to age-matched controls. nSTZ rats exhibited

**Table 2.** Inflammatory cells in bronchoalveolar lavage fluid of OA-challenged rats

| Groups        | Treatment  | Cells (x10 <sup>6</sup> ) |                   |              |              |
|---------------|------------|---------------------------|-------------------|--------------|--------------|
|               |            | Total                     | Mononuclear cells | Neutrophils  | Eosinophils  |
| Control       | Saline (5) | 0.53 ± 0.16               | 0.52 ± 0.16       | 0.01 ± 0.01  | 0 ± 0        |
|               | OA (5)     | 4.84 ± 0.83*              | 2.10 ± 0.23*      | 0.75 ± 0.15* | 1.99 ± 0.54* |
| nSTZ Diabetic | Saline (4) | 0.78 ± 0.09               | 0.75 ± 0.09       | 0.02 ± 0.01  | 0 ± 0        |
|               | OA (4)     | 4.88 ± 1.46*              | 2.52 ± 1.25*      | 0.45 ± 0.17* | 1.90 ± 0.50* |

Bronchoalveolar lavage was performed 48 h after ovalbumin (OA) challenge. Data are means ± SEM. Figures in parentheses indicate the number of animals used. \*P<0.05 vs corresponding saline-treated rats.

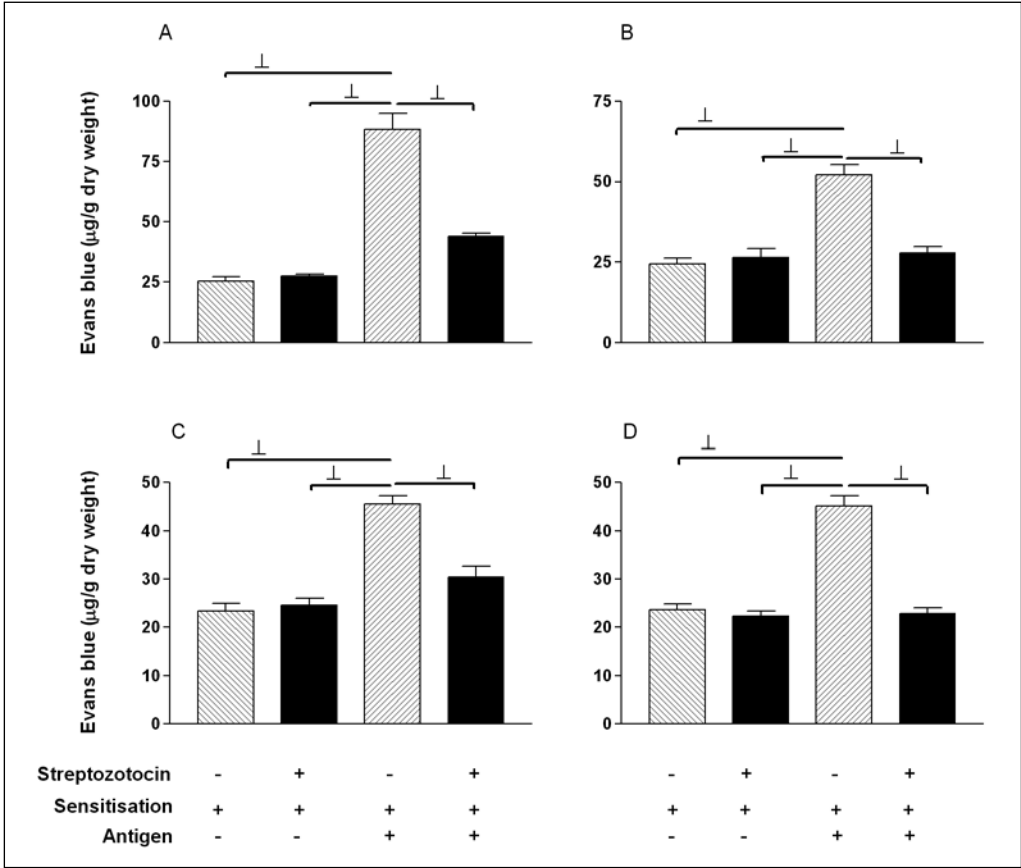


**Fig. 3.** Evans blue dye extravasation (µg/g dry weight tissue) in trachea (A), lung parenchyma (B), main bronchus (C), and intrapulmonary bronchus (D) obtained from control rats (hatched bars) and neonatal streptozotocin-induced diabetic rats (black bars), 15 min after saline or antigen challenge (10 mg ovalbumin/1 ml saline). Evans blue dye (20 mg/kg, i. v.) was injected immediately before saline or ovalbumin aerosol. Values are means ± SEM for 5 to 7 animals in each group. †: p < 0.001 of OA challenge control rats vs other groups.

a moderate hyperglycemia (20% above normal values) and a normal insulinemia before the ivGTT (at 0 time). When control and nSTZ rats were given a load of glucose by the intravenous route, and then sacrificed at varying times thereafter, glycemia increased at 5 min in both groups of animals. Whereas in control rats plasma glucose decreased until normalization (at 60 min), in nSTZ rats values were significantly higher up to 60 min. As expected, plasma insulin increased sharply after a glucose load in control rats. In contrast, in nSTZ diabetic rats levels of insulin did not change during ivGTT. Accordingly, determinations of plasma glucose and insulin concentrations during the ivGTT demonstrated the presence of glucose intolerance, and impairment of glucose-

induced insulin release in the nSTZ group, as demonstrated in previous studies [25, 28–30]. Furthermore, the HOMA<sub>ir</sub> score was 2 times higher in nSTZ diabetic rats, supporting the presence of insulin resistance in this model of type 2 diabetes, as demonstrated herein. Type 2 diabetes is characterized by a progressive decline in β cell function and chronic insulin resistance. Structural and functional abnormalities in islet tissue of type 2 diabetic subjects are associated with a loss in islet β cell mass induced by increased β cell apoptosis [31, 32]. That the major limiting factor of glucose-induced insulin release is the reduction in the insulin content of isolated pancreatic islets was also demonstrated in nSTZ diabetic rats and mice [28, 30, 33].

Data presented herein showed that protein extravasation in the airways and parenchyma of OA challenged nSTZ diabetic rats was significantly reduced compared to matching controls. In contrast, equivalent numbers of eosinophils, neutrophils, and mononuclear cells were harvested from the BAL fluid of both groups of rats. Leukocyte migration seems to follow the normal pattern was further indicated by the number of leukocytes recovered from the inflammatory exudate in OA-induced pleurisy model. Both nSTZ diabetic rats and matching controls presented equivalent increased numbers of neutrophils (4h) and eosinophils (24h) in response to allergen challenge. These findings suggest that reduction in the allergic inflammatory reaction of nSTZ diabetic rats is restricted to microvascular dysfunctions. Similar results have been reported in non-allergic inflammatory reaction. It has been shown that although vascular permeability responses of nSTZ diabetic rats to carrageenan (injected into the paw or pleural cavity) are reduced, leucocyte counts in the pleural exudate are similar in diabetic and control rats [25].

Mast cells are distributed in the proximity to blood vessels and play a significant role in inflammatory reactions through their immunomediator-secretory activities as a response to activation by both immunological and non-immunological stimuli [34]. It has been already demonstrated that alloxan-induced type 1 diabetic rats present markedly reduced inflammatory reactions to allergen challenge in the airways [19, 20] and in the pleural space [35] unrelated to changes in the number of total blood leukocytes or blood glucose levels [19], but ascribed to a reduction in mast cell degranulation upon antigen challenge [14, 15, 35].

In the present study, the impaired vascular response of nSTZ rats to antigen challenge was, apparently, not related to changes in mast cell population. First, equivalent numbers of mast cells were collected from the pleural cavity of both nSTZ diabetic rats and controls; second, there were no differences in leukocyte infiltration upon antigen challenge. Taken together, these observations suggest that endothelial cell response to inflammatory mediators released during allergen challenge might be reduced in nSTZ diabetic rats. Indeed, assessment of cutaneous vascular permeability shows that responses to serotonin or bradykinin, but not to histamine, are reduced in nSTZ rats [25].

Several lines of evidence suggest the presence of a strong association between insulin resistance and endothelial cell dysfunction, underlying the incidence of macrovascular and microvascular diseases associated with type 2 diabetes [36, 37]. Insulin receptors have been demonstrated on endothelial cells of both large and small blood vessels [38]. It has been shown that insulin-stimulated tyrosine phosphorylation of the insulin receptor and receptor substrate proteins, and the activation of phosphatidylinositol 3 kinase are impaired in aorta and microvessels of insulin-resistant obese Zucker rats [39]. Insulin-resistant individuals exhibit resistance to both metabolic and at least some of the vascular actions of insulin [36]. Endothelial function is impaired in insulin-resistant states such as obesity, hypertension, and type 2 diabetes [40].

In conclusion, our results suggest that there is a reduction of the allergic inflammatory reaction in nSTZ-induced diabetic rats, a phenomenon restricted to microvascular dysfunctions and associated, probably, with insulin resistance in the lung microvascular endothelium.

**Acknowledgments.** This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo, Fundação Carlos Chagas de Amparo à Pesquisa do Estado do Rio de Janeiro, PRONEX/CNPq, PAPES3/FIOCRUZ, Brazil. The authors wish to thank Zilma Lucia da Silva for expert technical help.

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