Early Phase of Allergic Airway Inflammation in Diabetic Rats: Role of Insulin on the Signaling Pathways and Mediators

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Key Words

Diabetes mellitus • Insulin • Asthma • NF- κ B • MAPK • Akt • PKC • iNOS • COX-2 • Nitric oxide • PGE₂ • IL-6 • CINC-2

Abstract

Background: Allergic lung inflammation is impaired in diabetic rats and is restored by insulin treatment. In the present study we investigated the effect of insulin on the signaling pathways triggered by allergic inflammation in the lung and the release of selected mediators. **Methods:** Diabetic male Wistar rats (alloxan, 42 mg/kg, i.v., 10 days) and matching controls were sensitized by s.c. injections of ovalbumin (OA) in aluminium hydroxide, 14 days before OA (1 mg/0.4 ml) or saline intratracheal challenge. A group of diabetic rats were treated with neutral protamine Hagedorn insulin (NPH, 4 IU, s.c.), 2 h before the OA challenge. Six hours after the challenge, bronchoalveolar lavage (BAL) was performed for mediator release and lung tissue was homogenized for Western blotting analysis of signaling pathways. **Results:** Relative to non-diabetic rats, the diabetic rats exhibited a significant reduction in OA-induced phosphorylation of the extracellular signal-regulated kinase

(ERK, 59%), p38 (53%), protein kinase B (Akt, 46%), protein kinase C (PKC)- α (63%) and PKC- δ (38%) in lung homogenates following the antigen challenge. Activation of the NF- κ B p65 subunit and phosphorylation of I κ B α were almost suppressed in diabetic rats. Reduced expression of inducible nitric oxide synthase (iNOS, 32%) and cyclooxygenase-2 (COX-2, 46%) in the lung homogenates was also observed. The BAL concentration of prostaglandin (PG)-E₂, nitric oxide (NO) and interleukin (IL)-6 was reduced in diabetic rats (74%, 44% and 65%, respectively), whereas the cytokine-induced neutrophil chemoattractant (CINC)-2 concentration was not different from the control animals. Treatment of diabetic rats with insulin completely or partially restored all of these parameters. This protocol of insulin treatment only partially reduced the blood glucose levels. **Conclusion:** The data presented show that insulin regulates MAPK, PI3K, PKC and NF- κ B pathways, the expression of the inducible enzymes iNOS and COX-2, and the levels of NO, PGE₂ and IL-6 in the early phase of allergic lung inflammation in diabetic rats. It is suggested that insulin is required for optimal transduction of the intracellular signals that follow allergic stimulation.

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Introduction

There is evidence that the inflammatory response is impaired in diabetic rats and that insulin treatment is able to reverse this condition [1-6]. In the particular case of allergic lung inflammation, this is also reduced in diabetic animals [7, 8]. We have previously reported that following an antigen challenge in the airways of sensitized rats, the diabetic rats exhibited significantly lower lung E-selectin expression, leukocyte infiltration and interleukin (IL)-1 β , tumour necrosis factor (TNF)- α concentrations in the bronchoalveolar lavage (BAL) and that the treatment of the diabetic rats with a single dose of insulin, 2 hours before antigen challenge restored all of these parameters [8].

It is known that allergic stimulation leads, at the cellular level, to the activation of intracellular signaling cascades, among them the mitogen-activated protein kinases (MAPK), which comprise the extracellular signal-regulated kinase (ERK) and p38 [9]. Protein kinase C (PKC) plays a central role in signal transduction and participates in diverse biological and biochemical functions [10, 11] and protein kinase B (Akt) is a downstream regulator of phosphatidylinositol 3'-kinase (PI3K) and is implicated in PI3K-mediated regulation of nuclear factor- κ B (NF- κ B) [12], an important transcription factor for pro-inflammatory mediators. Insulin has the potential to interfere with these pathways via its effects at the cellular level, which include glucose transport, glycogen synthesis, mitogenesis [13], the stimulation of protein kinases and the activation or repression of gene transcription [14]. Previous studies from our group established a protocol of insulin treatment for alloxan-induced diabetic rats in which the dose of insulin was chosen as the lowest dose able to significantly reverse the inflammatory parameters that were reduced in diabetic rats [1-8]. Although this dose of insulin only partially reduces the blood glucose, it maintains elevated blood insulin levels throughout the length of the experiment. It is plausible that the observed effects would be primarily due to the increased levels of insulin rather than to the reduction of glycaemia.

The present study was designed to investigate the effect of insulin treatment on the signaling pathways and the mediators released in the early phase of allergic lung inflammation in alloxan-induced diabetic rats.

Materials and Methods

Animals

Specific-pathogen-free male Wistar rats weighing 200 ± 20 g at the beginning of the experiments were used. The animals were maintained at 23 ± 2 °C under a 12 hours light:dark cycle and were allowed access to food and water *ad libitum*. The animal care and research protocols were in accordance with the principles and guidelines adopted by the Brazilian College of Animal Experimentation (COBEA) and were approved by The Ethical Committee for Animal Research of the Biomedical Sciences Institute, University of São Paulo.

Induction of diabetes mellitus and insulin treatment

Diabetes mellitus was induced by an intravenous injection of 42 mg/kg of alloxan monohydrate (Sigma Chemical Co., St. Louis, MO, USA) dissolved in physiological saline (0.9% NaCl). The control rats were injected with physiological saline only. After 10 days blood glucose concentrations were determined and the animals which presented glucose above 200 mg/dl were used. Blood samples were obtained from the cut tip of the rat tail and glucose was determined with the aid of a blood glucose monitor (Eli Lilly, São Paulo, SP, Brazil). Relative to the controls, alloxan-treated diabetic rats exhibited a significant reduction in body weight gain (values, mean $\pm$ SEM, control: 147 ± 12 g, $n=9$; diabetic: 11 ± 11 g, $n=9$, $p<0.001$) and sharply elevated blood glucose levels (control: 87 ± 2 mg/dl, $n=9$; diabetic: 531 ± 26 mg/dl, $n=9$, $p<0.0001$) during the 24-day period of the experiment.

A group of diabetic rats received 4 IU of neutral protamine Hagedorn (NPH) insulin (Eli Lilly, São Paulo, SP, Brazil) subcutaneously (s.c.) 2 hours before the ovalbumin (OA, grade III; Sigma Chemical Co.) challenge. As the maximum serum concentration (C_{max}) of NPH insulin is reached between 6 and 8 hours after subcutaneous administration, we decided to give insulin to the animals 2 hours before the OA challenge as was done in previous study of this nature [8]. After treatment with a single dose of NPH insulin, the diabetic rats exhibited a reduction in blood glucose levels (257 ± 15 mg/dl, $n=9$, $p<0.0001$) but they were still hyperglycaemic.

Sensitization to ovalbumin and the antigen challenge

The animals were actively immunized with OA by subcutaneous injection of 0.2 ml sterile physiological saline containing 0.1 mg OA and 8 mg of aluminium hydroxide [$Al(OH)_3$]. The animals were anaesthetized 14 days thereafter with an intraperitoneal injection (150 mg/kg) of S-(+)-ketamine hydrochloride (Ketamin-S(+), Cristalia, Itapira, SP, Brazil) and the trachea of each animal was exposed through a midline ventral incision of approximately 0.5 cm length in the neck. Physiological saline solution (0.9% NaCl; 0.4 ml) containing 1 mg OA was instilled into the airways. The incision was closed with sutures and the animals were returned to their cages.

Bronchoalveolar lavage

The BAL was performed 6 hours after the intratracheal administration of OA or saline. The animals were anaesthetized, as described above, and the abdominal cavity was opened up for blood exsanguination from the abdominal aorta. The lungs were then lavaged by instillation of 10 ml of phosphate buffered saline (PBS), at room temperature, through a polyethylene tube (1 mm in diameter) inserted into the trachea. The first 10 ml instilled into the lungs was withdrawn and re-instilled twice more. After centrifugation (500 g for 15 min), the supernatant was frozen at -70 °C for inflammatory mediator measurements. The lungs were further lavaged with 15 ml PBS (3 x 5 ml) to harvest the leukocytes. The BAL was not used if the retrieved volume was less than 85% of the 25 ml instilled. Total cell counts were determined using an automatic hemacytometer (CELM, São Paulo, SP, Brazil). Differential cell counts were carried out on haematoxylin-eosin-stained slides under oil immersion microscopy. A total of 100 cells were counted and classified as neutrophils, eosinophils or mononuclear cells based on morphological criteria.

Quantification of mediators in the BAL

Interleukin-6 and CINC-2 concentrations in the BAL supernatant were determined by enzyme-linked immunosorbent assay (ELISA) using commercially available kits according to the manufacturer's instructions (R & D Systems Inc., Minneapolis, MN, USA). The sensitivity of the assay was of 15 pg/ml.

The nitrite concentration in the BAL was measured using the standard Griess reaction. Briefly, 50 µl of the BAL was mixed with 50 µl of Griess Reagent (1% sulphanilamide, 0.1% naphthylethylene diamine dihydrochloride, 2.5% H₃PO₄) for 10 min at room temperature. The absorbance was measured at 540 nm using a 620 nm reference filter in a Dynatech microplate reader (Dynatech Laboratories, Chantilly, VA, USA), and the nitrite concentration was determined using a standard curve of sodium nitrite. All assays were done in triplicate. The results are presented as µM NO₂.

For extraction and quantification of prostaglandin (PG)-E₂, the cell-free BAL was acidified with HCl 1N to pH 3.4-3.6 and slowly passed through an octadecylsilyl silica column (Sep Pak C 18 column) pre-washed with 10 ml ethanol and 10 ml water. After washing the column with 10 ml water and 1 ml ethanol (35%), the eicosanoid was eluted from the column with 2 ml absolute ethanol and the samples dried under a stream of nitrogen. The PGE₂ concentration was determined by using an enzyme immunoassay (EIA) kit (Cayman Chemical Co., Ann Arbor, MI, USA). The sensitivity of the assay was 5 pg/ml.

Western blotting analysis

The lungs were homogenized in PBS containing 1% of a protease inhibitor cocktail according to the manufacturer's instructions (Sigma Chemical Co.). Samples containing 20 µg protein were separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE 10%) and transferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA, USA). The membranes were incubated in Tris-Buffered Saline Tween-20 (TBS-T, 150 mM NaCl, 20 mM Tris, 1% Tween 20, pH 7.4) con-

taining 5% non-fat dried milk for 60 min. After this, the blots were washed with TBS-T and probed with antibodies (Cell Signaling Technology, INC., Beverly, CA, USA; 1:500 dilution) directed against phospho-ERK 1/2 MAPK (Thr183/Tyr185), phospho-p38 MAPK (Thr180/Tyr182), phospho-Akt (Ser473), phospho-PKCα (Thr638/641) and phospho-PKCδ (Thr505) (Cell Signaling Technology), and against COX-2 or iNOS (Cayman Chemical, Ann Arbor, MI, USA, 1:500) for 120 min at room temperature. The membranes were washed with TBS-T and incubated with peroxidase-conjugated monoclonal anti-rabbit IgG (1:2000) for 60 min at room temperature. The immunocomplexed peroxidase-labelled antibodies were visualized by an enhanced chemiluminescent (ECL) kit following the manufacturer's instructions (Amersham, Piscataway, NJ, USA) and exposed to photographic films. Finally, blots were stripped and reprobed for β-actin. The band densities were determined by densitometric analysis using the AlphaEaseFC™ program (Alpha Innotech, San Leandro, CA, USA). Density values of the bands were normalized to the total β-actin present in each lane and expressed as a percentage of the control.

To determine the nuclear NF-κB p65 subunit and cytoplasmic I-κBα phosphorylation, the extraction of lung nuclear and cytoplasmic proteins was performed using the nuclear and cytoplasmic extraction reagent kit (NE-PER®, Pierce Chemical, Rockford, IL, USA) containing a 1% of protease inhibitor cocktail according to the manufacturer's instructions (Sigma Chemical Co.). The protein content in the supernatant of the lysed lungs was determined using the bicinchoninic acid (BCA) protein assay reagent kit (Pierce Chemical), according to the manufacturer's protocol. In separate experiments, phosphorylation of I-κBα (cytoplasmic extracts) or activation of p65 NF-κB (nuclear extracts) containing 10 µg of protein per sample were suspended in sodium dodecylsulphate (SDS) sample buffer (Invitrogen, Carlsbad, CA, USA) and collected by boiling the sample at 100°C for 5 min. Western blot was performed as described above with primary antibodies specific for phosphorylated I-κBα (1:250) or phosphorylated NF-κB p65 (1:500). The data are expressed as total pixels or the percentage of the control after adjustment for the density of the respective control bands.

Statistical analysis

The data are presented as means ± SEM and were analysed by the Student's t-test or ANOVA followed by the Tukey-Kramer multiple comparisons test when appropriate, and p<0.05 was considered significant.

Results

Insulin modulates OA-induced activation of signaling pathways

Six hours after the OA challenge, the lungs were removed and processed as described in the Materials and methods. Figure 1 shows that whereas in the non-diabetic rats the OA challenge increased the phosphoryla-

Fig. 1. The effect of insulin on OA-induced ERK 1/2 (A) and p38 MAPK (B) phosphorylation in the lung tissue of control non-diabetic rats (C, open bars) and diabetic rats (D, black bars) 6 hours after saline or the OA challenge. Insulin (NPH, 4 IU/rat) was given to diabetic rats (DI, grey bars) 2 hours before the OA challenge. The phosphorylation of ERK and p38 MAPK was assessed by Western blot analysis. The antibodies recognized the phosphorylated residues Thr183/Tyr185 of p44/42 MAPK and Thr180/Tyr182 of p38 MAPK. The illustrations of the Western blot represent one out of four independent experiments. Protein expression was quantified using the AlphaEaseFC™ software. Values are means $\pm$ SEM of four animals per group. [†] $p < 0.001$ vs the other groups.

tion of ERK (3.3-fold) and p38 (2.9-fold), this was not observed in the diabetic rats. Treatment of the diabetic rats with a single dose of NPH insulin 2 hours before the OA challenge restored lung ERK and p38 phosphorylation.

Fig. 2. The effect of insulin on OA-induced Akt phosphorylation in the lung tissue of control non-diabetic rats (C, open bars) and diabetic rats (D, black bars) 6 hours after saline or the OA challenge. Insulin (NPH, 4 IU/rat) was given to diabetic rats (DI, grey bars) 2 hours before the OA challenge. The phosphorylation of Akt was assessed by Western blot analysis. The antibody recognized the phosphorylated Ser-473 residues of Akt. The illustrations of the Western blot represent one out of four independent experiments. Protein expression was quantified using the AlphaEaseFC™ software. Values are means $\pm$ SEM of four animals per group. [†] $p < 0.001$ vs the other groups. ^{*} $p < 0.05$ vs the saline control group.

Figure 2 shows that in the non-diabetic rats the OA challenge increased the phosphorylation of Akt (3.4-fold) whereas in the diabetic rats the increase was only 1.8-fold. Treatment of the diabetic rats with a single dose of NPH insulin, 2 hours before the OA challenge, restored phosphorylation of Ser-473 residues of Akt.

Figure 3 shows that the OA challenge induced a significant increase in the phosphorylation of PKC- α (4.3-fold) and PKC- δ (2.2-fold) in the lungs of non-diabetic rats compared to saline-instilled rats. In contrast, no significant increase was observed in the diabetic rats. Treatment of the diabetic rats with a single dose of NPH insulin 2 hours before the OA challenge restored lung PKC- α and PKC- δ phosphorylation.

The results illustrated in Figure 4 show that the OA challenge induced the activation of the NF- κ B p65 subunit and phosphorylation of cytoplasmic I- κ B α in non-diabetic rats. In contrast, in the diabetic rats much lower levels of NF- κ B p65 phosphorylation were observed and this was partially restored by insulin treatment. Increased phos-

Fig. 3. The effect of insulin on OA-induced PKC α (A) and PKC δ (B) phosphorylation in the lung tissue of control non-diabetic rats (C, open bars) and diabetic rats (D, black bars) 6 hours after saline or the OA challenge. Insulin (NPH, 4 IU/rat) was given to diabetic rats (DI, grey bars) 2 hours before the OA challenge. The phosphorylation of PKC α and PKC δ was assessed by Western blot analysis. The antibodies recognized the phosphorylated residues Thr638/641 of PKC α and Thr505 of PKC δ . The illustrations of the Western blot represent one out of four independent experiments. Protein expression was quantified using the AlphaEaseFC™ software. Values are means $\pm$ SEM of four animals per group. [†] $p < 0.001$ vs the other groups.

Fig. 4. The effect of insulin on the OA-induced nuclear NF-kB p65 subunit (A) and cytoplasmic I-kB α phosphorylation (B). Nuclear NF-kB p65 subunit phosphorylation and cytoplasmic I-kB α phosphorylation in lung tissue were assessed by Western blot analysis. The antibodies recognized the phosphorylated Ser276 residue of the NF-kB p65 subunit (80 kDa) and the Ser32 of I-kB α (40 kDa). Protein expression was quantified using the AlphaEaseFC™ software. The density values of bands were normalized to the total β -actin (42 kDa). Samples were obtained from control non-diabetic rats (C, open bars) and diabetic rats (D, black bars) 6 hours after saline or the OA challenge. Insulin (NPH, 4 IU/rat) was given to diabetic rats (DI, grey bars) 2 hours before the OA challenge. The illustration of the Western blot represents one out of four independent experiments. Values are means $\pm$ SEM of four animals per group. [†] $p < 0.001$ vs the control saline group. ^{*} $p < 0.05$ vs the OA control and DI groups.

phorylation of cytoplasmic I-kB α was observed after the OA challenge in the non-diabetic rats, whereas in the diabetic group the OA challenge did not affect the phos-

phorylation of cytoplasmic I-kB α . Treatment of the diabetic rats with insulin partially restored the phosphorylation of cytoplasmic I-kB α .

Insulin modulates OA-induced enzyme expression and mediators release

The results illustrated in Figure 5 show that in the non-diabetic rats the OA challenge increased BAL concentrations of CINC-2 (4.6-fold) and IL-6 (20.0-fold) compared to the control group, which received saline. In the diabetic rats, the concentration of IL-6 was reduced (65%) whereas the CINC-2 levels did not differ between the OA-challenged groups (Fig. 5A). The IL-6 concentration in the BAL was normalized after treatment of the diabetic rats with insulin (Fig. 5B).

Six hours after OA challenge, lung COX-2 and iNOS protein expression was analyzed by Western blot, PGE₂ by EIA and NO release by the standard Griess reaction. Figure 6 shows that OA induced COX-2 and iNOS protein expression in control non-diabetic rats. In diabetic rats the expression of lung COX-2 and iNOS after OA challenge was significantly reduced (46% and 32%, respectively) compared to the OA-challenged control non-diabetic rats. Treatment of diabetic rats with insulin 2 hours before OA challenge restored lung COX-2 and iNOS expression (Fig. 6A and 6B). The OA challenge also increased BAL levels of PGE₂ and NO in control non-diabetic rats. Whereas in diabetic rats the levels of PGE₂ and NO were reduced (74% and 44%, respectively) compared to the OA-challenged control non-diabetic rats, concentrations of both mediators were normalized by insulin treatment (Fig. 6C and 6D).

Discussion

We have previously shown that insulin modulates the development of lung inflammation induced by the OA challenge in sensitized rats by its ability to regulate the production of IL-1 β , TNF- α and the expression of P-selectin [8]. In the present study, we investigated the effect of insulin on the OA challenge-induced signaling pathways in the lung by measuring the phosphorylation of ERK, p38, Akt, PKC- α , PKC- δ , the NF- κ B p65 subunit and I- κ B α . We also measured the expression of iNOS and COX-2 proteins and the release of the products of these enzymes, NO and PGE₂. The data presented herein shows that OA-challenged diabetic rats, compared to OA-challenged non-diabetic rats, exhibited (i) reduced phosphorylation of lung ERK, p38, Akt, PKC- α , PKC- δ , the NF- κ B p65 subunit and I- κ B α ; (ii) reduced expression of lung iNOS and COX-2, and BAL levels of NO and PGE₂; (iii) a reduced concentration of

Fig. 5. The effect of insulin on CINC-2 (A) and IL-6 (B) concentrations in the BAL of control non-diabetic rats (C, open bars) and diabetic rats (D, black bars) 6 hours after saline or the OA challenge. The CINC-2 and IL-6 concentrations in the BAL were determined by ELISA. Insulin (NPH, 4 IU/rat) was given to diabetic rats (DI, grey bars) 2 hours before the OA challenge. Values are means $\pm$ SEM for nine animals in each group. † p<0.0001 vs the saline control group. *p<0.001 vs the OA C and DI groups.

IL-6 in the BAL. Treatment of the diabetic rats with insulin restored the phosphorylation of these signaling molecules and increased enzyme expressions and mediator release.

Insulin regulates many cellular processes, such as glucose transport, glycogen synthesis and gene transcription [13, 14]. Through different signaling pathways, insulin exerts a negative effect on the transcription of a subset of genes, whilst for other genes the effect is positive [14]. In the present study, we found reduced phosphorylation of MAPK, PI3K, PKC and NF- κ B pathways in diabetic rats and that insulin restored the phosphorylation of these signaling molecules.

Fig. 6. The effect of insulin on OA-induced COX-2 (A) and iNOS (B) protein expression in the lung tissue, and PGE₂ (C) and NO (D) concentrations in the BAL of control non-diabetic rats (C, open bars) and diabetic rats (D, black bars) 6 hours after saline or the OA challenge. Insulin (NPH, 4 IU/rat) was given to diabetic rats (DI, grey bars) 2 hours before the OA challenge. The COX-2 and iNOS expressions were assessed by Western blot analysis, PGE₂ by EIA and NO levels were measured by the Griess reaction. The illustration of the Western blot represents one out of four independent experiments. Protein expression was quantified using the AlphaEaseFC™ software. Values are means $\pm$ SEM for four to nine animals in each group. $\dagger p < 0.001$ vs the saline C group. * $p < 0.05$ vs the OA C and DI groups.

The MAPK signaling cascade plays a pivotal role in the activation of inflammatory cells. It has been shown that the activity of p42/44 MAPK (also known as ERK) in the lungs was significantly higher in asthmatics compared to healthy controls [9]. This pathway is involved in the activation of several transcription factors that control gene activity and expression. Activation of p21^{ras} leads to activation of all members of the MAPK family, and this effect is mediated by specific MEK kinases [15, 16], including ERK and p38. It has been shown that insulin modulates ERK and p38 in alveolar macrophages [17]. The results presented herein show that in diabetic rats, the OA-induced ERK and p38 phosphorylation in the lung is reduced compared to non-diabetic rats. However, the treatment of diabetic rats with insulin restored the phosphorylation of these signaling molecules to the levels of the non-diabetic animals. Insulin has been shown to activate the p21^{ras}/MAPK pathway through the binding

of growth factor receptor-bound protein (GRB)-2 to the tyrosine-phosphorylated insulin receptor substrate (IRS)-1 [18]. Several IRS-1-associated proteins have already been identified, including PI3K [19]. The PI3K protein is composed of two subunits, a 110 kDa (p110) catalytic subunit and an 85 kDa (p85) regulatory subunit [20]. A direct interaction between p21^{ras} and p110, and the demonstration that p21^{ras} regulates PI3'-kinase activity [21], suggest that activation of p21^{ras} via IRS-1 could initiate both the MAPK and PI3K signaling pathways. The data presented herein show that insulin restored the phosphorylation of Ser-473 Akt which was reduced in diabetic rats. This suggests that insulin could act directly on p21^{ras}, which favours the hypothesis that the immune hyporesponsiveness in diabetic patients is due to the modulation of cellular signal transcription factors [17, 22] rather than to changes in metabolism and blood glucose levels [1-8].

Insulin modulates the inflammatory cell migration into the airways during the course of allergic reactions [7]. We have previously described that, in the same experimental model used in the present study, the number of neutrophils and mononuclear cells in the BAL of diabetic rats was markedly reduced in response to the OA challenge. Reversal of the impaired response was attained by treatment of the diabetic rats with insulin [8]. Protein kinase C belongs to a family of multifunctional serine/threonine kinases, which is involved in insulin action, resistance and secretion [23], and is also largely involved in leukocyte migration through the airway epithelium during the course of allergic lung inflammation [24]. The results presented herein show that phosphorylation of PKC α and PKC δ were reduced in the lung of OA challenged diabetic rats compared to OA challenged non-diabetic rats, and that the treatment of diabetic rats with insulin restored the phosphorylation of these signaling molecules. It can be speculated that insulin increases leukocyte transmigration in the lung via the activation of PKC and ERK pathways.

Experimental evidence indicates that insulin modulates mast cell degranulation [25]. Cytokines such as IL-1 β and TNF- α are released under these circumstances and are known to be involved in cell migration into inflammatory sites. We showed that in diabetic rats the levels of IL-1 β and TNF- α in the BAL are significantly lower than in control non-diabetic rats under allergic stimulation [8]. Treatment with insulin completely restores the concentrations of IL-1 β and TNF- α released by the allergic lung inflammation [8]. These cytokines amplify the inflammatory response by stimulating the release of chemoattractant factors by alveolar macrophages and airway epithelial cells, and the expression of adhesion molecules by the endothelium [26]. The results presented herein show that compared to the non-diabetic animals the diabetic rats exhibited reduced IL-6 in the BAL after the OA challenge. The concentration of this cytokine was normalized by treatment of the diabetic rats with insulin. In addition, the CINC-2 levels in the BAL were similar in diabetic and non-diabetic rats following the OA challenge and were not affected by treatment with insulin. A similar result has been previously reported by our group in relation to CINC-1 levels [8]. Taken all together, these data indicate that insulin regulates the release of pro-inflammatory cytokines into the lungs of diabetic rats, without changes in the CINC family of chemoattractant factors.

The PGE₂ and NO are important mediators of the inflammatory process and their concentration in the in-

flammatory site is dependent on the intracellular levels of the inducible enzymes COX-2 and iNOS. It has been shown that insulin modulates the expression of these enzymes in alveolar macrophages [22]. Here, we showed that the lung expression of COX-2 and iNOS, as well as the levels of PGE₂ and NO in the BAL, were significantly lower in diabetic rats compared to OA-challenged non-diabetic rats. Treatment of the diabetic rats with insulin restored the expression of both enzymes, which suggests that insulin regulates the production of PGs and NO in the early phase of allergic lung inflammation in diabetic rats.

The activity of NF- κ B is induced in many cell types by several pro-inflammatory stimuli [27]. In order to investigate the molecular mechanisms that underline the anti-inflammatory effect of insulin, activation of the NF- κ B p65 subunit and I- κ B α phosphorylation were assessed by Western blot analysis. The results, presented herein show that whereas the OA challenge induced 2.7-fold increases in NF- κ B p65 activation and in cytoplasmic I- κ B α phosphorylation in non-diabetic rats, this increase was not observed in diabetic rats. Treatment of diabetic rats with insulin before the OA challenge partially restored the activation of these transcription factors. It is suggested that the lack of insulin in diabetic rats suppresses NF- κ B activation possibly by the inhibition of nuclear translocation of NF- κ B resulting from the blockade of I- κ B α phosphorylation. Further studies into the signaling pathways by which insulin acts on leukocytes and the endothelium may improve the understanding of the role of insulin in allergic lung inflammation.

The normal course of the inflammatory process seems to depend on the availability of insulin [1-8]. It is important to mention here that the impaired response to OA in diabetics might be primarily linked to a continuing insulin deficiency rather than to secondary hyperglycaemia [7, 8]. Insulin, in adequate concentrations, seems to be required for the normal functioning of alveolar macrophages [17, 22], endothelial cells and neutrophils during the course of the inflammatory process [5]. In addition, clinical asthma appears to be less severe when diabetes mellitus is superimposed. Data from epidemiological studies in children with type-1 diabetes mellitus [28, 29] are consistent with data from previously published studies [30, 31] which reported an inverse relationship between atopy and diabetes mellitus. The suggestion is that the presence of a TH1-mediated disease may lower the prevalence of atopic diseases. However, if insulin is required for the development of allergic airway inflammation, as demonstrated herein and in previ-

ously published studies [7, 8, 25], 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.

Conclusions

The data presented show that insulin regulates MAPK, PI3K, PKC and NF- κ B pathways, the expression of the inducible enzymes COX-2 and iNOS, and the levels of PGE₂, NO and IL-6 in the early phase of aller-

gic lung inflammation in diabetic rats. These results suggest that the activation of the signaling molecules induced by allergic stimulation requires the presence of insulin.

Acknowledgements

The authors wish to thank Irene M. Gouveia and Silvana A. da Silva for expert technical help. This research was supported by FAPESP and CNPq, Brazil. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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