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INSULIN REGULATES CYTOKINES AND INTERCELLULAR ADHESION MOLECULE-1 GENE EXPRESSION THROUGH NUCLEAR FACTOR-κB ACTIVATION IN LPS-INDUCED ACUTE LUNG INJURY IN RATS

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ABSTRACT—Diabetic patients have increased susceptibility to infection, which may be related to impaired inflammatory response observed in experimental models of diabetes, and restored by insulin treatment. The goal of this study was to investigate whether insulin regulates transcription of cytokines and intercellular adhesion molecule 1 (ICAM-1) via nuclear factor-κB (NF-κB) signaling pathway in *Escherichia coli* LPS–induced lung inflammation. Diabetic male Wistar rats (alloxan, 42 mg/kg, i.v., 10 days) and controls were instilled intratracheally with saline containing LPS (750 μg/0.4 mL) or saline only. Some diabetic rats were given neutral protamine Hagedorn insulin (4 IU, s.c.) 2 h before LPS. Analyses performed 6 h after LPS included: (*a*) lung and mesenteric lymph node IL-1β, TNF-α, IL-10, and ICAM-1 messenger RNA (mRNA) were quantified by real-time reverse transcriptase–polymerase chain reaction; (*b*) number of neutrophils in the bronchoalveolar lavage (BAL) fluid, and concentrations of IL-1β, TNF-α, and IL-10 in the BAL were determined by the enzyme-linked immunosorbent assay; and (*c*) activation of NF-κB p65 subunit and phosphorylation of I-κBα were quantified by Western blot analysis. Relative to controls, diabetic rats exhibited a reduction in lung and mesenteric lymph node IL-1β (40%), TNF-α (~30%), and IL-10 (~40%) mRNA levels and reduced concentrations of IL-1β (52%), TNF-α (62%), IL-10 (43%), and neutrophil counts (72%) in the BAL. Activation of NF-κB p65 subunit and phosphorylation of I-κBα were almost suppressed in diabetic rats. Treatment of diabetic rats with insulin completely restored mRNA and protein levels of these cytokines and potentiated lung ICAM-1 mRNA levels (30%) and number of neutrophils (72%) in the BAL. Activation of NF-κB p65 subunit and phosphorylation of I-κBα were partially restored by insulin treatment. In conclusion, data presented suggest that insulin regulates transcription of proinflammatory (IL-1β, TNF-α) and anti-inflammatory (IL-10) cytokines, and expression of ICAM-1 via the NF-κB signaling pathway.

KEYWORDS—Diabetes mellitus, insulin, LPS, cytokines, ICAM-1, real-time RT-PCR

INTRODUCTION

It has long been recognized that poorly controlled diabetic patients have a worse prognosis once infection is established (1). Several aspects have been shown to be impaired during inflammation in diabetes mellitus, including a reduction in the number of leukocytes in inflammatory lesions (2–5), reduced mast cell degranulation (6), and lymph node retention capacity (7). These abnormalities might contribute to the increased susceptibility and severity of infection in the diabetic host. Reversal of the impaired responses is attained by treatment of the animals with insulin (2, 3, 5–7). Therefore, insulin through direct or indirect effects regulates leukocyte behavior in inflammation. In addition, intensive insulin therapy reduces morbidity and mortality among critically ill patients, even in those who are not diabetic (8).

Experimental acute lung injury induced by intratracheal instillation of LPS, a component of the gram-negative bacterial cell wall, shares similarities with acute respiratory distress syndrome seen in sepsis caused by gram-negative bacteria (9–12). LPS is known to induce a global activation of inflammatory responses. Binding of LPS to toll-like receptors triggers a complex sequence of events leading to increased expression of specific genes through nuclear factor-κB (NF-κB) and mitogen-activated protein kinase signaling (13). Nuclear factor-κB is an inducible transcription factor that mediates signal transduction between cytoplasm and nucleus in many cell types. In resting cells, NF-κB is localized in the cytoplasm as a heterodimer composed of two polypeptides of 50 kd (p50) and 65 kd (p65), which are noncovalently associated with cytoplasmic inhibitory proteins, including the inhibitor of κBα (I-κBα) (14). Upon cell stimulation by a variety of agents, I-κB kinases specifically phosphorylate I-κB proteins, marking them for ubiquitination and proteolysis, leading to NF-κB complex migration into the nucleus and subsequent binding to DNA recognition sites in the regulatory regions of the target genes (14).

It has been previously shown that superoxide generation (15), production/release of cytokines such as TNF-α (12, 13), IL-1β, and IL-10 (16), protein expression of cell adhesion

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molecules (16), and generation of prostaglandin E_2 (17) are reduced in LPS-instilled diabetic rats. The levels of these inflammatory mediators are normalized after treatment of the animals with insulin (15–17).

The present study was designed to investigate the effects of insulin on cytokine and *ICAM-1* gene transcription and NF-κB signaling pathway.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 200 ± 20 g at the beginning of the experiments were used. The animals were maintained at 23°C ± 2°C under a 12-h light/dark cycle and were allowed access to food and water *ad libitum*. All experiments were in accordance with the ethical principles in animal research adopted by the Brazilian College of Animal Experimentation. Approval of the Animal Subject Committee of the Heart Institute (InCor), University of São Paulo Medical School, was obtained before initiating the experiments.

Alloxan-induced diabetes

Diabetes mellitus was induced by an i.v. injection of 42 mg/kg of alloxan monohydrate (Sigma Chemical Co, St Louis, Mo) dissolved in physiologic saline (0.9% NaCl). Control rats were injected with physiologic saline only. Ten days thereafter, the presence of diabetes was verified by blood glucose concentrations above 200 mg/dL determined with the aid of a blood glucose monitor (Eli Lilly, São Paulo, Brazil) in samples obtained from the cut tip of the rat tail.

Insulin treatment

A group of diabetic rats received 4 IU of neutral protamine Hagedorn (NPH) insulin (Eli Lilly) s.c., 2 h before LPS instillation.

LPS-induced acute lung injury

The animals were anesthetized by an intraperitoneal injection (150 mg/kg) of S (+)-ketamine hydrochloride (Ketamin-S[+], Cristalia, Itapira, São Paulo, Brazil), and the trachea was exposed through a midline ventral incision of approximately 0.5-cm length in the neck. Physiologic saline solution (0.9% NaCl, 0.4 mL) containing 750 μg of *Escherichia coli* LPS (serotype 055:B5, Sigma Chemical Co) was instilled into the airways. Control animals received physiologic saline only by the same route. The incision was closed with sutures, and the animals were returned to their cages.

Experimental protocol

Six hours after LPS instillation, the animals were anesthetized, as described previously, and exsanguinated from the abdominal aorta. Two sets of experiments were performed: (*a*) the lungs and mesenteric lymph nodes were removed, rinsed in cold physiologic saline, immediately immersed in liquid nitrogen, and stored at −70°C until use for quantitative real-time reverse transcriptase–polymerase chain reaction (RT-PCR) of cytokine messenger RNA (mRNA) and ICAM-1 mRNA (lungs), or Western blot analysis of NF-κB p65 subunit and I-κBα phosphorylation (lungs); and (*b*) bronchoalveolar lavage (BAL) was performed by instillation of phosphate buffered saline (PBS) into the lungs for leukocyte counts, and cytokine measurements were performed using the enzyme-linked immunosorbent assay.

RNA isolation and RT-PCR

Total RNA was extracted from 100 μg of lung or mesenteric lymph nodes using TRIzol Reagent. RNA samples (30 μg) were DNAse I treated (Promega) and then purified (RNeasy Kit, Qiagen) according to manufacturer's instructions. Samples were quantified spectrophotometrically at 260 nm, and their integrity was verified by 1% agarose gel. Then, 2 μg of treated total RNA were used in a 20-μL complementary DNA (cDNA) reaction (cDNA Archive Kit, Applied Biosystems). Quantitative real-time RT-PCR of lung IL-1β, TNF-α, IL-10, and ICAM-1 mRNA was performed on the Taqman assay using an ABI Prism 7700 Sequence Detector (Applied Biosystems) and using a combination of forward and reverse primers and a specific nonquenched FAM-labeled probe. The amplicon obtained in all cases laid from 58 to 79 base pairs. Data and results were normalized for β-actin expression and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Primers and probes were obtained from Applied Biosystems (assay-on-demand) IL-1β (Assay ID Rn00580432_m1), TNF-α (Assay ID Rn00562055_m1), IL-10 (Assay ID Rn00563409_m1), ICAM-1 (Assay ID Rn00564227_m1), β-actin (Assay ID Rn00667869_m1), and GAPDH (Assay ID Rn99999916_s1). Initially, amplification efficiency for each set of primers and probes was tested by using serial dilutions (5, 10, 20, and 200 times) of a rat LV control mRNA through Ef (%) = $[10^{(-1/\text{slope})}-1] \times 100$ equation, and then the best cDNA dilution was used. Each experiment was performed in triplicate, and cDNA templates were diluted 20 times for IL-1β, TNF-α, IL-10, ICAM-1, β-actin, and GAPDH. Gene expression was analyzed using ΔCt method for *IL-1β*, *TNF-α*, *IL-10*, *ICAM-1*, *β-actin*, or *GAPDH* genes. Because the ΔCt method for quantitating mRNA levels by RT-PCR also requires the control displayed expression of the corresponding mRNA, values used were those obtained from noninflamed saline-treated rats.

NF-κB Western blot analysis (p65 and I-κBα)

To determine NF-κB p65 subunit phosphorylation and cytoplasmic I-κBα phosphorylation, the extraction of lung nuclear and cytoplasmic proteins was performed using NE-PER nuclear and cytoplasmic extraction reagent kit (Pierce Chemical, Rockford, Ill) containing 1% of inhibitor cocktail according to manufacturer's instructions (Pierce Chemical). Protein content in the supernatant of the lysed lungs was determined using the BCA protein assay reagent kit (Pierce Chemical) according to the manufacturer's protocol. In separate experiments, cytoplasmic extracts from phosphorylation of I-κBα or nuclear extracts from activation of p65 NF-κB containing 10 μg of protein per sample were suspended in sodium dodecyl sulfate sample buffer (Invitrogen, Carlsbad, Calif) and collected by boiling the sample at 100°C for 5 min. After that, the samples were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and transferred to nitrocellulose membrane (Invitrogen) using the BioRad Mini-Gel system and Transblot SD-semidry Transfer cells. For immunoblotting, the nitrocellulose membranes were incubated in Tris–Buffered Saline–Tween (TBS–T) buffer (150 mM NaCl, 20 mM Tris, 1% Tween 20, pH 7.4) containing 5% nonfat dried milk for 1 h. The blot was treated with primary antibodies (Cell Signaling Technology, Inc, Beverly, Calif) specific for phosphorylated I-κBα (1:250) or phosphorylated NF-κB p65 (1:500) for 2 h at room temperature, then were washed three times with TBS-T, and incubated with

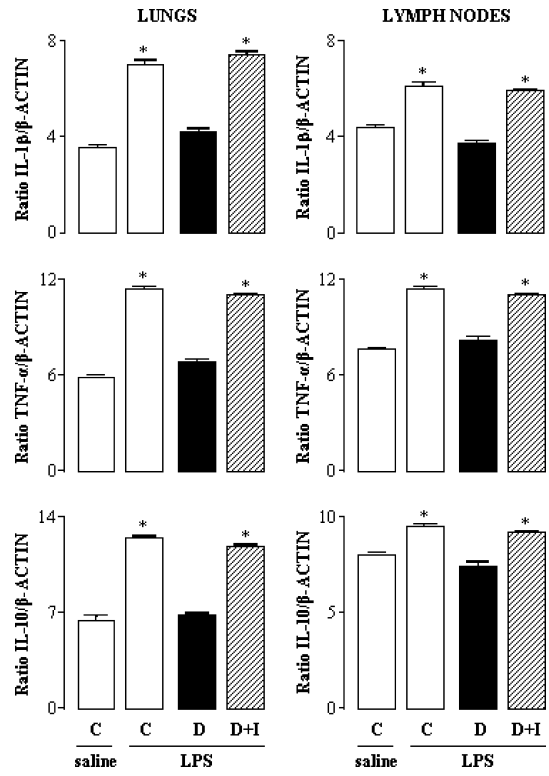

FIG. 1. **IL-1β, TNF-α, and IL-10 mRNA signals expressed as a ratio to β-actin mRNA in lung tissue and mesenteric lymph nodes of control (C, open bars) and diabetic rats (D, black bars) 6 h after saline or LPS instillation**. Insulin (NPH, 4 IU/rat) was given to diabetic rats (DI, hatched bars) 2 h before LPS instillation. Values are presented as mean ± SEM of triplicate determinations for five animals in each group. $^*P < 0.001$ vs. other groups.

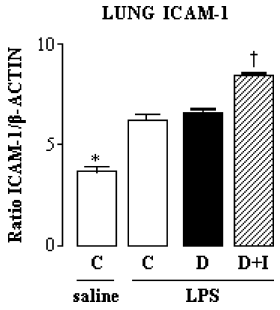

FIG. 2. **Intercellular adhesion molecule 1 mRNA signals expressed as a ratio to β-actin mRNA in lung tissue of control (C, open bars) and diabetic rats (D, black bars) 6 h after saline or LPS instillation**. Insulin (NPH, 4 IU/rat) was given to diabetic rats (DI, hatched bars) 2 h before LPS instillation. Values are presented as mean ± SEM of triplicate determinations for five animals in each group. $^{*}P < 0.0001$ vs. other groups; $^{\dagger}P < 0.001$ vs. LPS-treated groups.

peroxidase-conjugated monoclonal anti–rabbit immunoglobulin G (1:2,000) for 1 h at room temperature. Protein bands at 80 kd (NF-κB p65) or at 40 kd (I-κBα) were identified by comparison with Rainbow protein molecular weight markers (Amersham, Piscataway, NJ). The immunocomplexed peroxidase-labeled antibodies were visualized by an enhanced chemiluminescence kit following manufacturer's instructions (Amersham) and exposed to photographic film. Finally, blots were stripped with 200 mM glycine, pH 3.0, for 10 min, washed with TBS-T three times for 30 min each, and reprobed with β-actin (Sigma-Chemical Co) diluted 1:10.000, followed by anti–mouse secondary antibody (1:2,000). The band densities were determined by densitometric analysis using the AlphaEaseFC program. Density values of bands were normalized to the total β-actin present in each lane, and data are expressed as total pixel or the percent of control after adjustment for the density of its respective control band.

Bronchoalveolar lavage

The animals were anesthetized and exsanguinated as described previously. Bronchoalveolar lavage was performed by instillation of 10 mL of PBS, at room temperature, through a polyethylene tube (1 mm in diameter) that was inserted into the trachea. The first 10 mL instilled into the lungs was withdrawn and reinstilled twice. After centrifugation (500*g* for 15 min), the supernatant was kept at −70°C until use for cytokine measurements. The lungs were further lavaged with 15 mL PBS (3 × 5 mL) to harvest leukocytes. The BAL fluid was not used if the retrieved volume was less than 85% of the 25 mL instilled. Total cell counts were determined by using an automatic hemacytometer (CELM, São Paulo, Brazil). Differential cell counts were carried out on 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.

Enzyme-linked immunosorbent assays

Concentrations of IL-1β, TNF-α, and IL-10 in the BAL supernatant were determined by the enzyme-linked immunosorbent assay using commercially available kits according to the manufacturer's instructions (R & D Systems Inc, Minneapolis, Minn). The sensitivity of the assay was of 15 pg/mL.

Statistical analysis

Data are presented as means ± SEM and analyzed by Student *t* test or ANOVA followed by the Tukey-Kramer multiple comparisons test when appropriate. $P < 0.05$ was considered significant.

RESULTS

General characteristics of the animals

Relative to controls, alloxan-treated diabetic rats exhibited a significant reduction in body weight gain (control, 60 ± 2 g, n = 31; diabetic, 13 ± 6 g, n = 26; $P < 0.001$) during a 10-day period and sharply elevated blood glucose levels (control, 83 ± 1 mg/dL, n = 31; diabetic, 455 ± 22 mg/dL, n = 26; $P < 0.0001$). After treatment with a single dose of NPH insulin, diabetic rats exhibited a significant reduction in blood glucose levels from 489 ± 9 mg/dL to 259 ± 14 mg/dL (n = 12, $P < 0.0001$), not sufficient to reduce glycemia to control values.

Effect of insulin on cytokine gene expression

Twofold increases in lung IL-1β, TNF-α, and IL-10 mRNA levels were observed 6 h after exposure of control rats to LPS compared with saline-treated rats. In contrast, diabetic rats presented a significant reduction in the level of lung IL-1β (40%), TNF-α (34%), and IL-10 (50%) mRNA after LPS instillation compared with LPS-treated control rats. Results are illustrated in Figure 1. Treatment of diabetic rats with a single dose of NPH insulin 2 h before LPS exposure completely restored lung IL-1β, TNF-α, and IL-10 mRNA levels (Fig. 1). A mean 1.4-fold increase in mesenteric lymph node IL-1β, TNF-α, and IL-10 mRNA levels was observed after exposure of control rats to LPS compared with saline-treated rats. However, reduced IL-1β (40%), TNF-α (30%), and IL-10 (20%) mRNA levels were observed in diabetic rats after LPS instillation. Treatment of the animals with a single dose of NPH insulin 2 h before LPS exposure restored mesenteric lymph node cytokine gene expression. Results are illustrated in Figure 1.

Effect of insulin on lung ICAM-1 *gene expression*

Lung ICAM-1 mRNA levels increased 1.8-fold in LPS-treated control rats compared with saline-treated rats. Despite

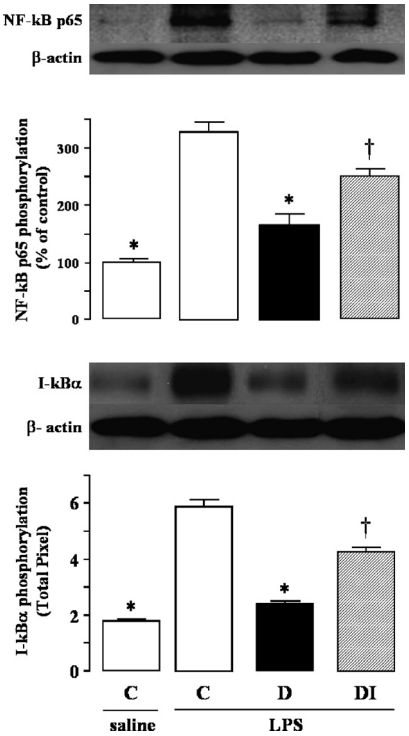

FIG. 3. **Nuclear NF-κB p65 subunit phosphorylation and cytoplasmic I-κBα phosphorylation in lung tissue were assessed by Western blot analysis**. The antibodies recognized phosphorylated residues of the Ser276 of NF-κB p65 subunit (80 kd) and the Ser32 of I-κBα (40 kd). Expression of protein was quantified by AlphaEaseFC software. Density values of bands were normalized to the total β-actin (42 kd). Samples were obtained from control (C, open bars) and diabetic rats (D, black bars) 6 h after saline or LPS instillation. Insulin (NPH, 4 IU/rat) was given to diabetic rats (DI, hatched bars) 2 h before LPS instillation. Illustration of the Western blot represents one out of three independent experiments. Values are presented as mean ± SEM of three animals per group. $^{*}P < 0.001$ vs. other groups; $^{\dagger}P < 0.01$ vs. LPS control.

Table 1. **Cytokine concentrations and number of neutrophils in the BAL fluid**

Groups	Treatment	n	Neutrophils, $\times 10^6$	IL-1β, pg/mL	TNF-α, pg/mL	IL-10, pg/mL
Control	Saline	10	0.2 ± 0.1	64.0 ± 3.4	48.0 ± 3.5	31.2 ± 3.7
Control	LPS	11	26.9 ± 1.0*	474.4 ± 62.2†,‡	404.2 ± 46.7*	69.0 ± 2.5*
Diabetic	LPS	9	7.4 ± 0.4§	226.0 ± 15.2	155.3 ± 7.7	39.5 ± 1.9
Diabetic + insulin	LPS	7	46.2 ± 2.8*	470.9 ± 49.1†,‡	349.0 ± 30.1*	61.4 ± 1.4*

Neutrophils, IL-1β, TNF-α, and IL-10 concentrations in the BAL fluid of control and diabetic rats 6 h after LPS or saline instillation. Rats were rendered diabetic by the injection of alloxan (42 mg/kg, i.v.) 10 days before LPS instillation. Insulin (NPH, 4 IU/rat, s.c.) was administered 2 h before the intratracheal instillation procedure. Values are presented as mean ± SEM. n indicates the number of animals in each group.
*$P < 0.001$ vs. other groups.
†$P < 0.001$ vs. saline-control rats.
‡$P < 0.01$ vs. LPS-diabetic rats.
§$P < 0.01$ vs. saline-control rats.

no significant differences between LPS-treated control and LPS-treated diabetic rats, lung ICAM-1 mRNA levels further increased after insulin treatment of diabetic rats (Fig. 2).

Insulin modulates LPS-induced NF-κB activation and I-κBα phosphorylation

Activation of NF-κB p65 subunit and phosphorylation of cytoplasmic I-κBα in lungs after instillation with LPS was analyzed by Western blot. Results, illustrated in Figure 3, showed that LPS induced the activation of NF-κB p65 subunit and phosphorylation of cytoplasmic I-κBα 6 h after exposure of control nondiabetic rats to LPS compared with saline-treated rats. In contrast, diabetic rats presented a significant reduction in the activation of NF-κB p65 subunit and phosphorylation of cytoplasmic I-κBα after LPS instillation compared with LPS-instilled control rats. Treatment of diabetic rats with a single dose of NPH insulin 2 h before LPS exposure partially restored the activation of NF-κB p65 subunit and phosphorylation of cytoplasmic I-κBα (Fig. 3).

Effect of insulin on cytokine release and neutrophil migration

Results, summarized in Table 1, showed that increases in BAL concentrations of IL-1β (7.4-fold), TNF-α (8.4-fold), and IL-10 (2.2-fold) were observed after instillation of control rats with LPS compared with saline-treated rats. This was accompanied by a massive and acute migration of neutrophils into the lungs. Cytokine release and neutrophil migration were comparatively reduced in LPS-treated diabetic rats. Interestingly, whereas cytokine concentrations in the BAL fluid were normalized after treatment of diabetic rats with insulin, the number of neutrophils increased 72% more than values observed in LPS-treated control rats.

DISCUSSION

Data presented herein suggest that insulin modulates the development of LPS-induced acute lung injury by its ability to mediate gene expression of proinflammatory and anti-inflammatory cytokines as well as expression of the adhesion molecule ICAM-1 via NF-κB signaling pathway. This is supported by the following observations: (*a*) relative to LPS-treated control rats, LPS-treated diabetic rats exhibited reduced IL-1β, TNF-α, and IL-10 mRNA levels in both lung and mesenteric lymph nodes; (*b*) treatment of diabetic rats with a single dose of insulin completely restored the expression of these genes. Despite no significant differences in lung *ICAM-1* gene expression between LPS-diabetic and LPS-control rats, an increase in the expression of this adhesion molecule was observed after insulin treatment; and (*c*) relative to LPS-treated control rats, the activation of NF-κB p65 subunit and phosphorylation of I-κBα were not observed in LPS-treated diabetic rats. Activation of NF-κB signaling pathway was partially restored after treatment of diabetic rats with insulin.

Early-response cytokines, such as TNF-α and IL-1β, are released after LPS–toll-like receptor signal coupling and, in turn, amplify the host response to invading microorganisms by stimulating the release of chemokines (18), upregulation of cell adhesion molecules (19–21), and neutrophil migration into the lungs (19, 22). We have previously shown that insulin modulates the course of LPS-induced acute lung inflammation (16). The production/release of proinflammatory (IL-1β, TNF-α) and anti-inflammatory (IL-10) cytokines, as well as protein expression of adhesion molecules (ICAM-1, E-selectin), and neutrophil migration into the lungs depend on the availability of insulin (16). To investigate if insulin is involved in the transcriptional regulation of these cytokines, samples of lung and mesenteric lymph nodes were analyzed by real-time RT-PCR.

Insulin regulates many cellular processes, such as glucose transport (23), glycogen synthesis (24), mitogenesis (25), and gene transcription (26). Through different signaling pathways, insulin exerts a negative effect on the transcription of a subset of genes, and for others, the effect is positive (26). Downregulation of insulin-like growth factor binding protein 1 gene (27), insulin receptor substrate 2 gene (28), and LPS-induced signaling pathways in alveolar macrophages (29), and upregulation of insulin-like growth factor 1 gene (30) and insulin receptor gene (31) are some examples of insulin action on gene transcription.

Sensitive and quantitative measurement of mRNA is critical for accurate assessment of gene expression in normal physiological and pathological conditions. The sensitivity of RT-PCR allows rapid detection of extremely rare mRNAs, or mRNAs present in small amounts in the samples. The real-time RT-PCR, widely used to the quantification of steady-state mRNA levels, has become the method of choice for examination of gene expression, which is significantly better than

the conventional RT-PCR (32). However, the identification of a valid reference for data normalization remains the most common problem (33). In the present study, two housekeeping genes, *β-actin* and *GAPDH*, were used. β-actin mRNA is expressed in most cell types and encodes a ubiquitous cytoskeleton protein. It was one of the first RNAs to be used as an internal standard, and it is still advocated as a quantitative reference for RT-PCR assays (34). Although there are some aspects suggesting that the use of GAPDH mRNA as an internal standard is inappropriate (35), it is frequently used as an endogenous control for quantitative RT-PCR analysis because in some experimental systems, its expression is constant at different times and after experimental manipulation of the samples (36). Therefore, the use of at least two housekeeping genes at real-time RT-PCR assays must be recommended for any experiment that requires sensitive, specific, and reproducible quantification of mRNA. Although cytokine gene expression is presented herein as a ratio to β-actin mRNA, identical results were obtained with GAPDH mRNA (data not shown).

Results presented herein showed that alloxan-induced diabetic rats exhibited reduced IL-1β and TNF-α mRNA levels in the lungs and mesenteric lymph nodes after instillation with LPS when compared with nondiabetic rats. The expression of these cytokine genes was normalized after treatment of diabetic rats with insulin. So, the presence of IL-1β and TNF-α in the BAL fluid was associated with increased expression of their transcripts in the lung and mesenteric lymph nodes. These early-response cytokines amplify the inflammatory response by stimulating the release of chemoattractant factors by alveolar macrophages and airways epithelial cells, and the expression of adhesion molecules by leukocytes and the endothelium (37).

Besides the multifunctional cytokines IL-1β and TNF-α, we also evaluated the potential contribution of the anti-inflammatory cytokine IL-10, which is among the most potent anti-inflammatory agents induced in response to LPS (38). Levels of IL-10 mRNA were measured in lung and mesenteric lymph nodes as well as its concentration in the BAL fluid of the animals. As observed for IL-1β and TNF-α, there was a reduction in both IL-10 protein and mRNA levels in LPS-diabetic rats compared with LPS-control rats. The mRNA expression and the concentration of IL-10 in the BAL fluid normalized after treatment of diabetic animals with insulin. The suggestion is that gene expression of proinflammatory and anti-inflammatory cytokines might be regulated by insulin.

ICAM-1 has been previously shown to be a requirement for neutrophil recruitment after airway instillation of LPS (16, 19–21). Furthermore, increases in whole lung ICAM-1 mRNA and protein requires the availability of IL-1β and TNF-α (20). Results presented herein showed that lung *ICAM-1* gene expression increased 41% in LPS-instilled rats compared with saline-instilled rats. This was accompanied by a massive and acute migration of neutrophils into the lungs. Despite no significant differences in lung ICAM-1 mRNA levels between LPS-diabetic rats and LPS controls, treatment of the animals with insulin potentiated *ICAM-1* gene expression. Similar results are observed regarding lung ICAM-1 protein expression, as previously described (16). Thus, upregulation of *ICAM-1* gene expression and the associated neutrophil infiltration into the lungs of insulin-treated diabetic rats might be related to the circulating levels of insulin. Accordingly, the ability of insulin to increase *ICAM-1* gene expression in the lungs of LPS-diabetic rats suggests a pathway by which insulin might modulate neutrophil migration into the lungs.

Proinflammatory stimuli such as pathogen-derived LPS and cytokines such as TNF-α are strong inducers of NF-κB activity in many cell types (14). To investigate the molecular mechanisms that underlie the effect of insulin on cytokines and *ICAM-1* gene expression, activation of NF-κB p65 subunit and I-κBα phosphorylation were assessed by Western blot analysis. Results presented herein showed that LPS induced 3-fold increases in NF-κB p65 activation and in cytoplasmic I-κBα phosphorylation in control nondiabetic rats. These alterations were not observed when LPS was given to diabetic rats. Treatment of diabetic rats with insulin before LPS stimulation partially restored the activation of these transcription factors. The suggestion is that the lack of insulin in diabetic rats suppresses NF-κB activation possibly by inhibition of nuclear translocation of NF-κB resulting from blockade of I-κBα 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 acute lung inflammation.

Despite a mean of 53% reduction in the levels of blood glucose after treatment with a single dose of insulin, diabetic rats were still hyperglycemic compared with controls. Because levels of serum corticosterone did not differ significantly between alloxan-diabetic rats and controls (16), the suggestion is that insulin seems to exert a direct effect on the transcription of cytokines and adhesion molecule genes through activation of NF-κB signaling pathway. A variety of pathogen-derived proteins have been shown to interfere with NF-κB signaling. Pathogens may benefit from downregulating NF-κB by inhibiting inflammatory gene expression, whereas activation may prevent infected cells from undergoing apoptosis (14).

Survival depends on the ability of the host to respond appropriately to pathogenic challenges. A dysregulation of the mechanisms that trigger the innate immune response against bacterial pathogens contributes to the pathophysiological consequences of bacterial sepsis (39, 40). It has long been recognized that certain infections occur almost exclusively in diabetic patients, and many of these patients have a worse prognosis once infection occurs (1, 39, 40). Adequate concentrations of insulin are essential for the normal function of endothelial cells and neutrophils during the course of the inflammatory process (1, 2, 5). The ability of insulin to mediate gene expression of inflammatory cytokines as well as adhesion molecules suggests a role of insulin in the regulation of LPS-induced acute lung injury.

In conclusion, data presented suggest that insulin regulates the transcription of proinflammatory (IL-1β, TNF-α) and anti-inflammatory (IL-10) cytokines and of the adhesion molecule ICAM-1 during the course of LPS-induced lung inflammation in diabetic rats. Increases in cytokine levels and in the number of neutrophils in the BAL fluid of the animals were associated

with increased expression of their transcripts in the lung and mesenteric lymph nodes. Regulation of cytokines and ICAM-1 transcripts by insulin might be attributed, at least in part, to the activation of NF-κB p65 subunit and phosphorylation of I-κBα. These studies contribute to a better understanding of the role of insulin in the inflammatory process, which might be of relevance for prevention of severe infection in diabetic patients.

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