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Insulin Suppresses LPS-induced iNOS and COX-2 Expression and NF-κB Activation in Alveolar Macrophages

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Key Words

Insulin • LPS • Macrophages • NO • iNOS • PGE_2 • COX-2 • NF-κB • IκBα

Abstract

The development of septic shock is a common and frequently lethal consequence of gram-negative infection. Mediators released by lung macrophages activated by bacterial products such as lipopolysaccharide (LPS) contribute to shock symptoms. We have shown that insulin down-regulates LPS-induced TNF production by alveolar macrophages (AMs). In the present study, we investigated the effect of insulin on the LPS-induced production of nitric oxide (NO) and prostaglandin (PG)-E_2, on the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2, and on nuclear factor kappa B (NF-κB) activation in AMs. Resident AMs from male Wistar rats were stimulated with LPS (100 ng/mL) for 30 minutes. Insulin (1 mU/mL) was added 10 min before LPS. Enzymes expression, NF-κB p65 activation and inhibitor of kappa B (I-κB)α phosphorylation were assessed by immunobloting; NO by Griess reaction and PGE_2 by enzyme immunoassay (EIA). LPS induced in AMs the expression of iNOS and COX-2 proteins and production of NO and PGE_2, and, in parallel, NF-κB p65 activation and cytoplasmic I-κBα phosphorylation. Administration of insulin before LPS suppressed the expression of iNOS and COX-2, of NO and PGE_2 production and Nuclear NF-κB p65 activation. Insulin also prevented cytoplasmic I-κBα phosphorylation. These results show that in AMs stimulated by LPS, insulin prevents nuclear translocation of NF-κB, possibly by blocking I-κBα degradation, and supresses the production of NO and PGE_2, two molecules that contribute to septic shock.

Introduction

The development of septic shock is a common and highly lethal consequence triggered by lipopolysaccharide (LPS) from gram-negative bacteria that affects many organs and may lead to death. Insulin was shown to modulate inflammatory response in diabetic rats [1-3] and to decrease mortality and incidence of sepsis in critically

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ill patients [4]. Lungs are particularly affected during sepsis and provide a second wave of mediators/cytokines, amplifying the systemic inflammatory response and the mortality associated to this condition. LPS activates several signaling pathways in macrophages by acting on toll-like receptor (TLR)-4 through CD14 [5] to activate inflammatory gene expression and release of mediators/cytokines such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF), nitric oxide (NO), leukotrienes and platelet activating factor (PAF).

Activated macrophages produce a vast array of bioactive molecules, among them is NO, derived from the amino acid L-arginine by enzymatic activity of nitric oxide synthase (iNOS). Prostaglandins (PGs) are product of arachidonic acid metabolism via cyclooxygenases (COX). After stimulation with LPS, macrophages express the inducible forms of these enzymes (iNOS and COX-2), which are responsible for the production of large amounts of NO and PGs [6]. The promoter region of COX-2 genes in mice [7], rats [8], and humans [9] has been cloned and sequenced. This promoter region contains various putative transcriptional regulatory elements such as cyclic AMP response element (CRE), GATA box, nuclear factor-kappa B (NF-κB) and NF-IL-6. Amongst these elements, NF-κB and NF-IL-6 act as positive regulatory elements for the COX-2 transcription in some cell lines [10].

NF-κB is an inducible transcription factor that mediates signal transduction between cytoplasm and nucleus in many cell types [11]. In resting cells, NF-κB is localized in the cytoplasm as a heterodimer composed of two polypeptides of 50 kDa (p50) and 65 kDa (p65), which are non-covalently associated with cytoplasmic inhibitory proteins, including inhibitor of kappa B (I-κB)α. Upon cell stimulation by a variety of agents, the NF-κB complex migrates into the nucleus and binds DNA recognition sites in the regulatory regions of the target genes [12]. Activation of NF-κB by LPS induces a cascade of events leading to the phosphorylation of I-κBα and its futher proteolytic degradation [13].

We have recently shown that insulin down-regulates mitogen-activated protein kinases (MAPK), phosphotidylinositol 3'-kinase (PI3K) and protein kinase C (PKC)-α and PKC-δ and inhibits TNF production, in rat alveolar macrophages (AMs) stimulated with LPS [14].

In the present study we investigated the effect of insulin on the NF-κB activation, expression of iNOS and COX-2, and production of NO and PGE_2, in AMs stimulated with LPS.

Materials and Methods

Animals

Male Wistar rats weighing 200 +/- 20g (about 9 weeks of age) were obtained from Central Laboratory Animal of the Biomedical Sciences Institute of University of São Paulo. The animals were maintained at 23 +/- 2°C under a cycle of 12 hours light: 12 hours darkness and were allowed access to food and water *ad libitum*. Animal care and research protocols were in accordance the principles and guidelines adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by The Ethical Committee for Animal Research of the Biomedical Sciences Institute, University of São Paulo.

Cell isolation and culture

Resident AMs from rats were obtained by *ex vivo* lung lavage, as previously described [15] and were resuspended in RPMI-1640. Cells were allowed to adhere in culture–treated plates for 1 hour (37°C, 5% CO_2); this was followed by one wash with warm RPMI-1640, resulting in more than 99% of adherent cells identified as AMs by staining with a modified Wright-Giemsa stain. Cells were cultured overnight in RPMI-1640 supplemented 10% FBS (fetal bovine serum) and were washed twice the next day with warm medium to remove the non-adherent cells.

Cell treatments

AMs ($1x10^6$) plated in 4-well tissue culture dishes were pre-treated or not with crystalline insulin at a final concentration of 1 mU/mL, 10 minutes before LPS from *Escherichia coli* (serotype 055:B5) stimulus *in vitro* [14]. Then, AMs were stimulated for 30 minutes with 100 ng/mL LPS and were placed on ice for 10 minutes to stop the reaction. After that, AMs were washed three times in ice-cold PBS and lysed by sonication in ice-cold lyses buffer containing 150 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, and 1 µg/mL leupeptin, followed by ultracentrifugation at 100 000 *g* for 20 minutes at 4°C, the supernatant was frozen -70ºC for immunobloting and measuring of NO and PGE_2 production [15, 16].

Measurement of nitrite and PGE_2

To evaluate NO production, nitrite concentration in the supernatants of AMs cultures was measured using the standard Griess reaction. Briefly, 50 µl of the culture supernatant was mixed with 50 µl of Griess Reagent (1% sulphanilamide, 0.1% naphthylethylene diamine dihydrochloride, 2.5% H_3PO_4 for 10 min at room temperature. The absorbance was measured at 540 nm using a 620 nm reference filter in a Dynatech microplate reader, and the nitrite concentration was calculed using a standard curve of sodium nitrite. All assays were done in triplicate.

PGE_2 levels in the supernatants of the AMs culture were measure by enzyme immunoassay (EIA) using a commercial kit from Cayman Chemical. Briefly, dilutions of the supernatants were incubated with the conjugated eicosanoid-acetylcholi-

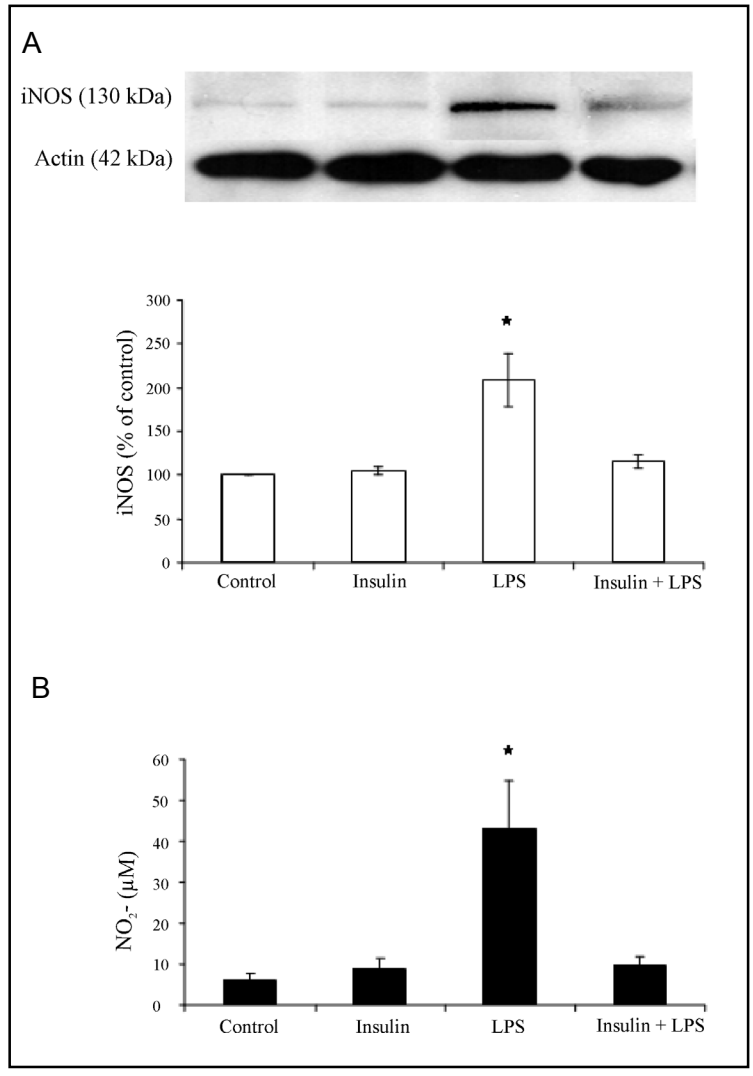

Fig. 1. Insulin suppresses LPS-induced iNOS expression (A) and NO production (B). Alveolar macrophages ($1x10^6$) were obtained by *ex vivo* lung lavage from male Wistar rats and incubated with insulin (1 mU/mL) 10 minutes before LPS stimulation (100 ng/mL) for 30 minutes. iNOS expression was assessed by western blot analysis and NO levels measured by Griess reaction. Ilustration of the western blot represent one out of 4 independent experiments. Expression of protein was quantified by AlphaEaseFC™ software. Values are means ± SEM of 4 animals per group. *P<0.001 *vs* other groups.

nesterase and with the specific antiserum in 96-well plates precoated with anti-rabbit immunoglobulin G antibodies. After overnight incubation at 4ºC, the plates were washed and the enzyme substrate (Ellman's reagent) was added for 60 to 120 min at 25ºC. The optical density of the samples was determined at 412 nm in a microplate reader, and the concentration of eicosanoids was calculated from standard curve. All assays were done in duplicate.

iNOS and COX-2 expression

Protein content in the supernatant of the lysed AMs was determined using the BCA protein assay reagent kit (Pierce), according to the manufacture´s protocol. Samples containing 20 µg protein were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose membrane using the Biorrad Mini-Gel system and trans-blot® SD-semidry Transfer cells. For immunoblotting, the nitrocellulose membranes were incubated in TSB-T buffer (150 mM NaCl, 20 mM Tris, 1% Tween 20, pH 7.4) containing 5% non-fat milk dried milk, for 1 h. The blot was treated with 1:1000 dilution of rabbit polyclonal antibodies to COX-2 or rabbit antiserum iNOS for 2 h at room temperature, then were washed three times with TBS-T, and incubated with 1:2000 dilution of peroxidase-conjugated monoclonal anti-rabbit IgG for 1 h at room temperature. Protein bands at 72 kDa (COX-2) or at 130 kDa (iNOS) were identified by comparison with Rainbow™ protein molecular weight markers. The immunocomplexed peroxidase-labeled antibodies were visualized by an ECL chemiluminescence kit following manufacturer´s instruction (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 (1:10.000), followed by anti-mouse secondary antibody (1:2000). 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 expressed in percentagem of control.

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

To determine cytoplasmic I-κBα phosphorylation and NF-kB p65 subunit phosphorylation, the extraction of AMs cytoplasmic and nuclear proteins was perfomed using NE-PER nuclear and cytoplasmic extraction reagents containing 1% of

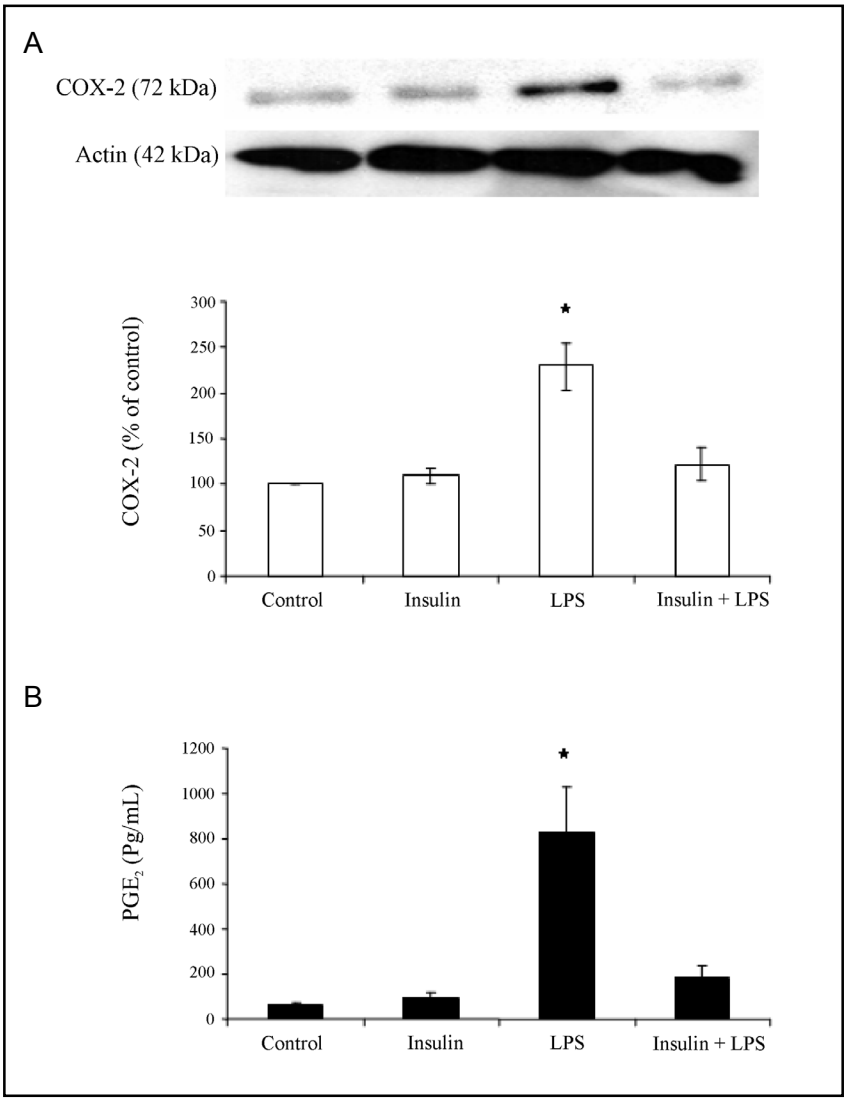

Fig. 2. Insulin suppresses LPS-induced COX-2 expression (A) and PGE_2 production (B). Alveolar macrophages ($1x10^6$) were obtained by *ex vivo* lung lavage from male Wistar rats and incubated with insulin (1 mU/mL) 10 minutes before LPS stimulation (100 ng/mL) for 30 minutes. COX-2 expression was assessed by western blot analysis and PGE_2 levels measured by EIA. Ilustration of the western blot represent one out of 4 independent experiments. Expression of protein was quantified by AlphaEaseFC™ software. Values are means ± SEM of 4 animals per group. *P<0.001 *vs* other groups.

inhibitor cocktail per the manufacture´s instructions (Pierce). Protein content in the supernatant of the lysed AMs was determined as described above. In separate experiments, phosphorylation of I-κBα (cytoplasmic extracts) or activation of p65 NF-κB (nuclear extracts) containg 10 μg of protein per sample were suspended in SDS sample buffer (Invitrogen) and collected by boiling the sample at 100ºC for 5 min. Western blot was analysed as described above with primary antibodies specific for phosphorylated I-κBα (1:250) or phosphorylated NF-κB p65 (1:500). Data are expressed as pixel total or the percent of control after adjustment for the density of its respective control band.

Drugs and reagents

RPMI-1640 and FBS (Gibco-Invitrogen, Carlsbad, CA, USA); Cristalline insulin (Biobrás, São Paulo, Brazil); LPS and β-actin (Sigma, Chemical Co, St Louis, Mo, USA); NE-PER nuclear and cytoplasmic extraction reagents, inhibitor cocktail and BCA protein assay reagent kit (Pierce Chemical, Rockford, IL, USA); chemiluminescence (ECL) detection and Rainbow™ protein molecular weight markers (Amersham, Piscataway, NJ, USA); COX-2 and iNOS from Cayman Chemical, Ann Arbor, MI, USA. Phospho-I-κBα (Ser32), phospho-NF-kB p65 (Ser276), ani-rabbit and anti-mouse IgG antibody are from Cell Signaling technology, INC. Beverly, MA, USA.

Statistical analysis

Data are presented as mean ± SEM of at least four experiments, statistical analysis was performed using the GraphPad Software (San Diego, CA, USA), and compared by analysis of variance (ANOVA) followed by the Bonferroni test. P< 0.05 were considered statistically significant.

Results

Insulin inhibits LPS-induced iNOS and COX-2 protein expression and NO and PGE_2 release

Resident AMs from male Wistar rats were obtained by *ex vivo* lung lavage and stimulated with LPS (100 ng/mL) for 30 min. Protein expression of iNOS was analyzed

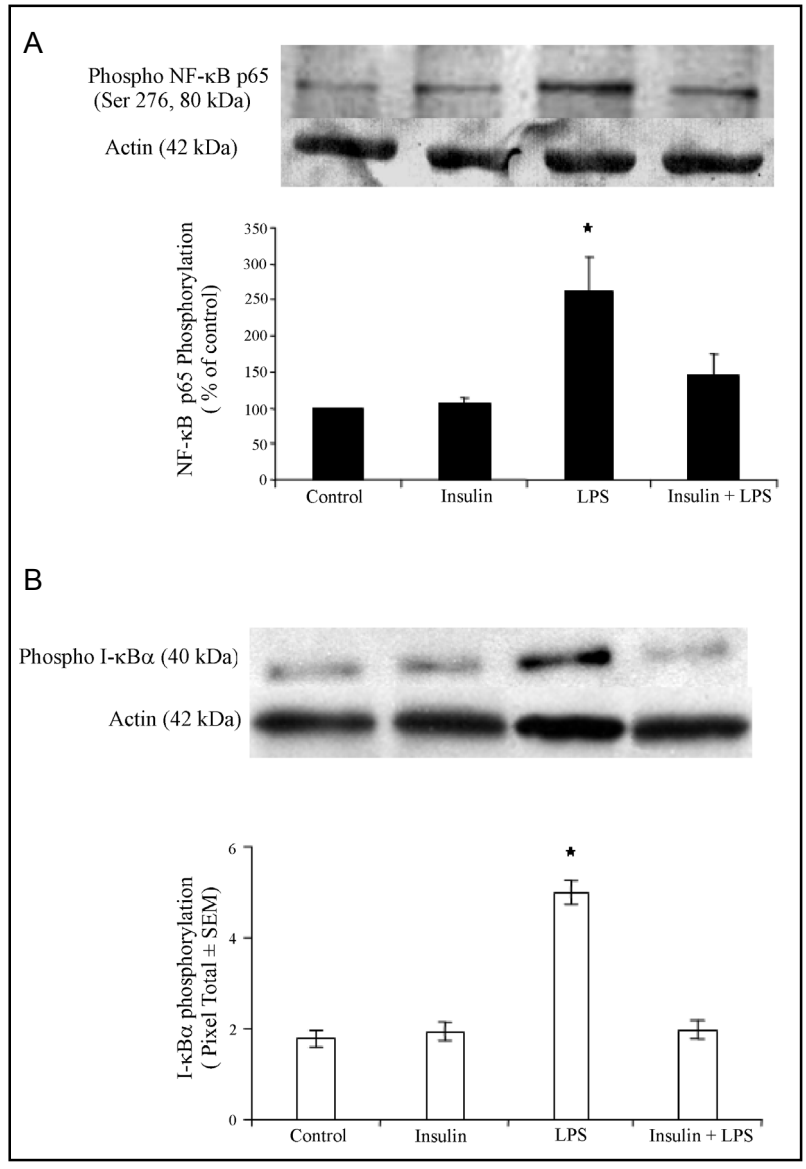

Fig. 3. Insulin modulates LPS-induced NF-kB activation and I-κBα phosphorylation. Alveolar macrophages ($1x10^6$) were obtained by *ex vivo* lung lavage from male Wistar rats and incubated with insulin (1 mU/mL) 10 minutes before LPS stimulation (100 ng/mL) for 30 minutes. Nuclear NF-kB p65 subunit phosphorylation and cytoplasmic I-kBα phosphorylation were assessed by western blot analysis. The antibodies recognized phosphorylated residue of the Ser276 of NF-kB p65 subunit and the Ser32 of I-kBα. Ilustration of the western blot represents one out of 4 independent experiments. Expression of protein was quantified by AlphaEaseFC™ software. Values are means ± SEM of 4 animals per group. *P<0.001 *vs* other groups.

by western blot and NO release by the standard Griess reaction. Figure 1 shows that LPS induced the expression of iNOS protein as well as the release of NO. A group of AMs was pre-treated with insulin (1 mU/mL), 10 minutes before LPS stimulation. This dose of insulin was taken from previous publication from our group using the same system [14]. Insulin alone did not induce iNOS but insulin supressed iNOS expression and NO generation in AMs stimulated with LPS.

COX-2 protein expression was analyzed by western blot and PGE_2 by EIA. Figure 2 shows that LPS induced the expression of COX-2 protein as well as the release of PGE_2. Insulin alone did not induce this protein or its product in rat AMs. Treatment of AMs with insulin (1 mU/mL), 10 minutes before LPS stimulation, suppressed the expression of COX-2 and release of PGE_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 AMs stimulated with LPS was analyzed by western blot. Figure 3 shows that, LPS induced the activation of NF-κB p65 subunit and phosphorylation of cytoplasmic I-κBα. Insulin alone did not induce activation of NF-κB or phosphorylation of its inhibitor in rat AMs. Treatment of AMs with insulin (1 mU/mL), 10 minutes before LPS stimulation, significantly suppressed the nuclear NF-κB p65 activation as well as the phosphorylation of cytoplasmic I-κBα. These results suggest that the suppression of NF-kB activation by insulin might be attributed to inhibition of nuclear translocation of NF-kB resulting from blockade of the phosphorylation of I-kBα in AMs activated with LPS.

Discussion

In the present work, we showed that activation of resident rat AMs with LPS *in vitro* induced the expression of iNOS and COX-2 proteins and release of the products of these enzymes, NO and PGE_2. Insulin given before LPS suppressed expression of these enzymes and mediator release. We also showed that LPS induced a significant increase in NF-κB p65 activation and in cytoplasmic I-κBα phosphorylation and that the treatment of AMs with insulin before LPS stimulation, significantly reduced these transcription factors activation. We recently showed that insulin inhibits the LPS-induced ERK 1/2, p38, Akt, PKCα and PKCδ phosphorylation and TNF release in AMs *in vitro* [14]. Our results are consistent with an inhibitory effect of insulin and corroborate previous findings showing a protective effect of insulin on systemic inflammation related to sepsis [17]. Taken together these results, suggest that the protective effect of insulin in sepsis reported by others could be, at least partially, attributed to inhibition of the secondary wave of mediators released by the lungs during this condition.

In a previous work from our group we performed a dose-response curve to insulin on LPS-induced TNF production [14]. We showed that at 1 mU/mL, insulin alone did not increase basal levels of phosphorylation of MAPK and PKC´s but it clearly interfered with the LPS-induced signaling cascades. So, in the present work we kept the same experimental conditions used in the previous work and employed the lower dose of insulin able to interfere with the LPS-induced signaling cascades and TNF production.

Intracellular levels of iNOS and COX-2 play a central role in determining NO and PGE_2 production rates in macrophages, two important mediators of the systemic inflammation during sepsis. We showed here that insulin suppressed LPS-induced iNOS and COX-2 protein expression as well as the release of the NO and PGE_2. These observations favor the hypothesis that the protective effect of insulin in sepsis could be due to modulation of cellular signal transcription factors and downstream effects of LPS on TNF [14] NO and PGE_2 production rather than to changes in metabolism and blood glucose.

One critical event that triggers sepsis/septic shock is the nuclear translocation of NF-kB and induction of NF-kB-dependent effector genes [18]. Böhrer *et al.* [19], observed that all patients with septic shock showed increased NF-kB binding activity in peripheral blood mononuclear cells (PBMC), and those in whom the binding activity exceed 200% of day 1, died. They also showed that somatic gene transfer with an expression plasmid coding for I-kBα reduced LPS-mediated NF-kB activation and increased mice survival after LPS administration. This suggests that NF-kB mediates mortality in animal models of sepsis. They also reported that gene transfer with I-kBα was not effective when given simultaneously with or after LPS, suggesting that gene transfer has to be done before the cells is stimulated to release mediator critical for the pathophysiology of sepsis. The central role of NF-kB in mediating inflammatory processes is evident from both the importance of its target genes and from the phenotypes of mice lacking the NF-kB p65 subunit [20]. Therefore, compounds inhibiting NF-kB are potentially of great interest for developing therapeutic agents for the treatment of acute and chronic inflammation. In our study LPS activation of AMs induced alterations in the activation of NF-kB p65 subunit and I-kBα phosphorylation. These alterations induced by LPS were reversed by insulin. Support for this finding comes from a study demonstrating that insulin has a potent acute anti-inflammatory effect including reduction in intranuclear NF-kB and increase in I-kB in mononuclear cells in obese subjects [21]. Thus, this acute anti-inflammatory effect, if demonstrated in the long term, as described by them, may be beneficial in sepsis.

NF-kB is also a crucial transcription factor for mRNA expression of iNOS [22] and COX-2 [23] and this report shows that insulin suppresses NF-kB activation possibly by inhibition of nuclear translocation of NF-kB resulting from blockade of phosphorylation of I-kBα. Regarding the intracellular mechanism of action mediated by insulin to decrease NF-kB activation, insulin has the potential to interfere with the stimulation of protein kinases and activation or repression of genes transcription [24]. We have shown in a previous paper [14] that insulin inhibits Akt and this molecule is a downstream regulator of PI3K and is implicated on PI3K-mediated regulation of NF-kB [25]. Another possibility is that insulin would act on the I-kB kinase (IKK), a protein kinase which activates the common pathways to NF-kB activation that is based on inducible I-kB degradation. In fact we showed here that insulin suppressed the nuclear NF-κB p65 activation as well as the phosphorylation of cytoplasmic I-κBα.

In Type II diabetes, the plasma levels of insulin are increased but the patient condition is rather associated with an inflammatory picture. In this condition, the presence of hyperglycemia is, at least in part, responsible for the proinflammatory state. In addition, obesity and type II diabetes presents insulin-resistance. Insulin exerts

an anti-inflammatory effect at the cellular and molecular level in vitro and in vivo [26]. A low dose infusion of insulin (2.5 IU/h) reduces reactive oxygen species (ROS) generation by mononuclear cells, suppresses NADPH oxidase expression and intranuclear NF-kB binding, induces I-kB expression and suppresses plasma intercellular adhesion molecule-1 (ICAM-1) and monocyte chemotactic protein-1 (MCP-1) concentrations. It also suppresses the proinflammatory transcription factor early growth response gene-1 (Egr-1), plasminogen activator inhibitor-1 (PAI-1) and MCP-1 concentrations [21, 27]. Another possibility is that an interruption/ alteration of insulin signal transduction in diabetes type II would prevent the anti-inflammatory effect of insulin from being exerted.

Hyperglycemia and insulin resistance are common in severe illness and are associated with adverse outcomes [28]. Van den Berghe et al. [4] showed in a study conducted in an intensive care unit (ICU) that strict control of blood glucose levels with insulin reduced morbidity and mortality. More recently, they showed that intensive insulin therapy significantly reduced morbidity but not mortality among all patients in the ICU [29]. It has been discussed that in critically ill patients several mediators are involved, and a single drug is unlikely to be of significant benefit. However, in some particular situations or, in some patients, insulin is beneficial. In sepsis, Das [30] discussed that a combination of naturally occuring endogenous anti-inflammatory molecules such as insulin, is one additional tool that can be used in the management of patients with sepsis, a condition for which no adequate therapy is available at present.

In conclusion, the results presented here and in the previous paper [14] suggest that the protective effect of insulin in sepsis, reported by others, could be due to inhibition of the secondary wave of mediators released by the lung macrophages.

Acknowledgements

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