

Insulin Inhibits LPS-Induced Signaling Pathways in Alveolar Macrophages

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Key Words

Insulin • LPS • Macrophages • ERK • p38 • Akt • PKC • TNF

Abstract

The systemic inflammatory response syndrome (SIRS) is triggered by lipopolysaccharide (LPS) from Gram-negative bacteria. Insulin was shown to have a protective role in SIRS related to sepsis. Lungs are particularly affected in this condition and provide a second wave of mediators/cytokines which amplifies SIRS. The aim of the present study was to investigate the effect of insulin on the signaling pathways elicited by LPS in alveolar macrophages (AMs) and its consequence in cellular response to LPS measured as production of tumor necrosis factor (TNF). To this purpose, resident AMs from male Wistar rats were obtained by lung lavage and stimulated by LPS (100 ng/mL). Insulin (1 mU/mL) was added 10 min before LPS. Activation (phosphorylation) of signaling molecules by LPS was analyzed by western blot, 30 min after LPS stimulation. TNF was measured in the AMs culture supernatants by bioassay using L-929 tumor cells. Relative to controls, LPS induced a

significant increase in the activation of ERK (3.6-fold), p38 (4.4-fold), Tyr-326 Akt (4.7-fold), Ser-473 Akt (6.9-fold), PKC α (4.7-fold) and PKC δ (2.3-fold). Treatment of AMs with insulin before LPS stimulation, significantly reduced the activation of ERK (54%), p38 (48%), Tyr-326 Akt (64%), Ser-473 Akt (41%), PKC α (62%) and PKC δ (39%). LPS induced TNF production in AMs which was also inhibited by insulin (60%). These results show that insulin down-regulates MAPK, PI3K and PKCs and inhibits a downstream effect of LPS, TNF production, in rat AMs stimulated with LPS and suggest that the protective effect of insulin in sepsis could be through modulation of signal transduction pathways elicited by LPS in lung macrophages.

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Introduction

The systemic inflammatory response syndrome (SIRS) is triggered by lipopolysaccharide (LPS) from Gram-negative bacteria that affects many organs and

may lead to death. Insulin was shown to attenuate SIRS in endotoxemic rats [1], to modulate inflammatory response in diabetic rats [2-4] and to decrease mortality and incidence of sepsis in critically ill patients [5]. Lungs are particularly affected by SIRS related to sepsis and provide a second wave of mediators/cytokines amplifying SIRS and the mortality associated to this condition. Thus, one possibility to explain the protective effect of insulin in SIRS would be that it reduces the release of mediators/cytokines by the lungs. This could be achieved by an effect of insulin on LPS-induced signaling pathways in lung macrophages. It is well established that LPS/LBP (LPS-binding protein) complex acts on toll-like receptor (TLR)-4 through CD14 [6] to activate inflammatory genes expression in macrophages and release of a plethora of mediators/cytokines which are involved in SIRS such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF), nitric oxide (NO), leukotrienes and platelet activating factor (PAF). These effects of LPS are consequent to activation of intracellular signaling cascades among them the mitogen-activated protein kinases (MAPK), which comprises the extracellular signal-regulated kinase (ERK) and p38. In macrophages, LPS was shown to activate both pathways [7, 8]. LPS also induces activation of PKC α and PKC δ in mouse peritoneal macrophages, both involved in macrophage functions such as phagocytosis, respiratory burst and cytokines secretion [9]. Akt is a downstream regulator of phosphatidylinositol 3'-kinase (PI3K) and is implicated on PI3K-mediated regulation of NF- κ B [10] an important transcription factor for pro-inflammatory mediators. Insulin has the potential to interfere with these pathways: its effects at cellular level includes glucose transport, glycogen synthesis, mitogenesis [11-13]; stimulation of protein kinases and activation or repression of genes transcription [14].

In the present study we investigated the effect of insulin on the signaling pathways induced by LPS in alveolar macrophages focusing on ERK, p38, Akt and PKC proteins activation. The effect of insulin on a downstream effect of LPS, production of TNF, was also investigated.

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% CO₂); 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 (1x10⁶) 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 stimulus *in vitro* [16]. 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 μ g/mL leupeptin, followed by ultracentrifugation at 100 000 g for 20 minutes at 4°C, the supernatant was frozen -70°C for immunoblotting [15, 17].

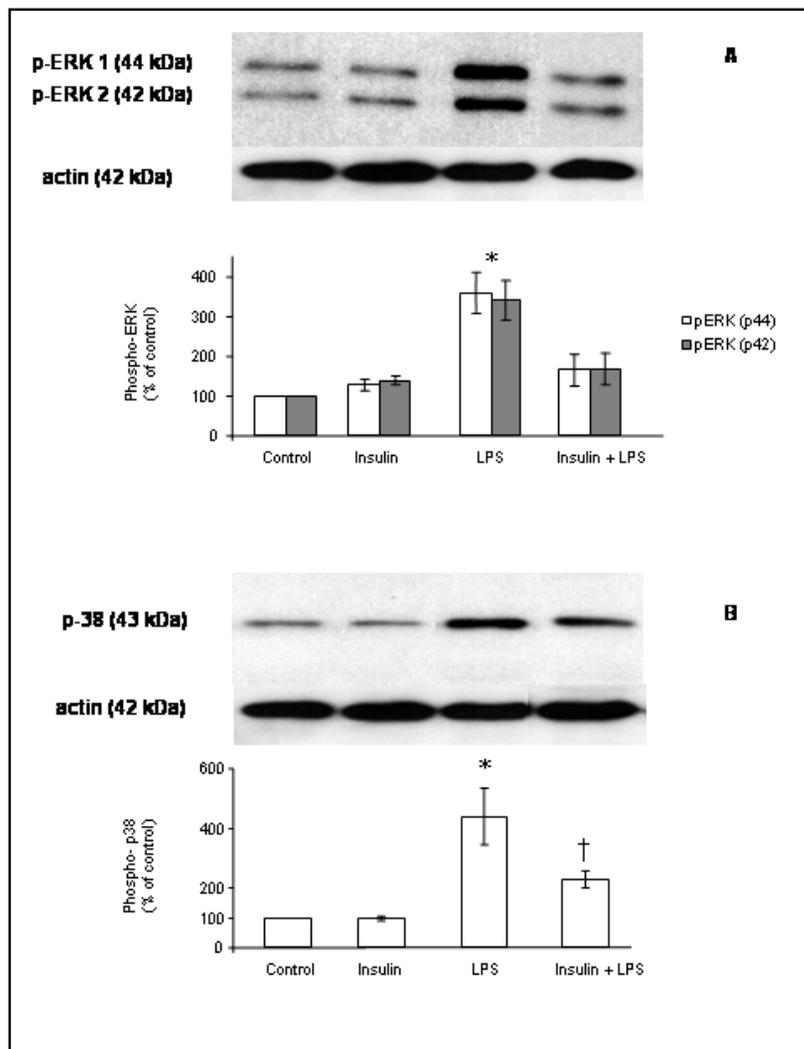
Insulin treatment

AMs (1x10⁶) plated in tissue culture dishes were treated with insulin 10 min before, concomitantly or 10 min after LPS stimulation. Different doses of crystalline insulin (0.1, 1, 10 mU/mL) were used. Then, AMs were stimulated for 30 minutes with 100 ng/mL LPS from *Escherichia coli* (serotype 055:B5). After that, the supernatant was frozen -70°C for measuring of TNF production.

TNF bioassay

The TNF levels were determined in the supernatants of macrophage cultures by a bioassay with L929 tumor cells [18, 19]. Briefly, L929 cells were plated in 96-well flat-bottom microplates in a concentration of 5x10⁴ cells/100 mL of complete medium and incubated for 24 h until obtaining a monolayer. The samples were serially diluted and added to the L929 monolayers in presence of actinomycin D (2 μ g/mL). After 24 h of incubation at 37°C in humidified 5% CO₂ incubator, the L929 monolayers were stained with crystal violet (0.5% in acetic acid 30%) for 10 min. After this, the plates were washed with distilled water and left to dry at room temperature. A volume of 100 μ L of absolute methanol was added to dissolve the stain and the absorbance was read at 620 nm in a Dynatech microplate reader. One unit of TNF was referred to as the reciprocal of the dilution that induces 50% of L-929 cell lyses.

Fig. 1. Insulin inhibition of LPS-induced ERK (A) and p38 MAPK (B) activation. Alveolar macrophages (1×10^6) 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. ERK and p38 MAPK phosphorylation were assessed by western blot analysis. The antibodies recognized phosphorylated residues of Thr183/Tyr185 of p44/42 rat MAP Kinase and the Thr180/Tyr182 of p38 MAP Kinase. Illustrations of the western blot represent one out of 6 independent experiments. Expression of protein was quantified by AlphaEaseFC™ software. Values are means \pm SEM of 6 animals per group. * $P < 0.001$ vs other groups; † $P < 0.05$ vs LPS group.



Western blotting

Protein concentration was determined by BCA protein assay reagent kit. Samples containing 20 μ g protein were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose membrane using the Biorad 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. After that, the blots were washed with TSB-T buffer three times for 5 min and were probed with antibodies (1:500 dilution) directed against phospho-ERK 1/2 MAP Kinase (Thr183/Tyr185), phospho-p38 MAP Kinase (Thr180/Tyr182), phospho-Akt (Tyr326), phospho-Akt (Ser473), phospho-PKC α (Thr638/641) and phospho-PKC δ (Thr505), for 90 min, followed by anti-rabbit secondary antibody (1:2000; for 1 h). Blots were developed using enhanced chemiluminescence (ECL) detection 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 % of control.

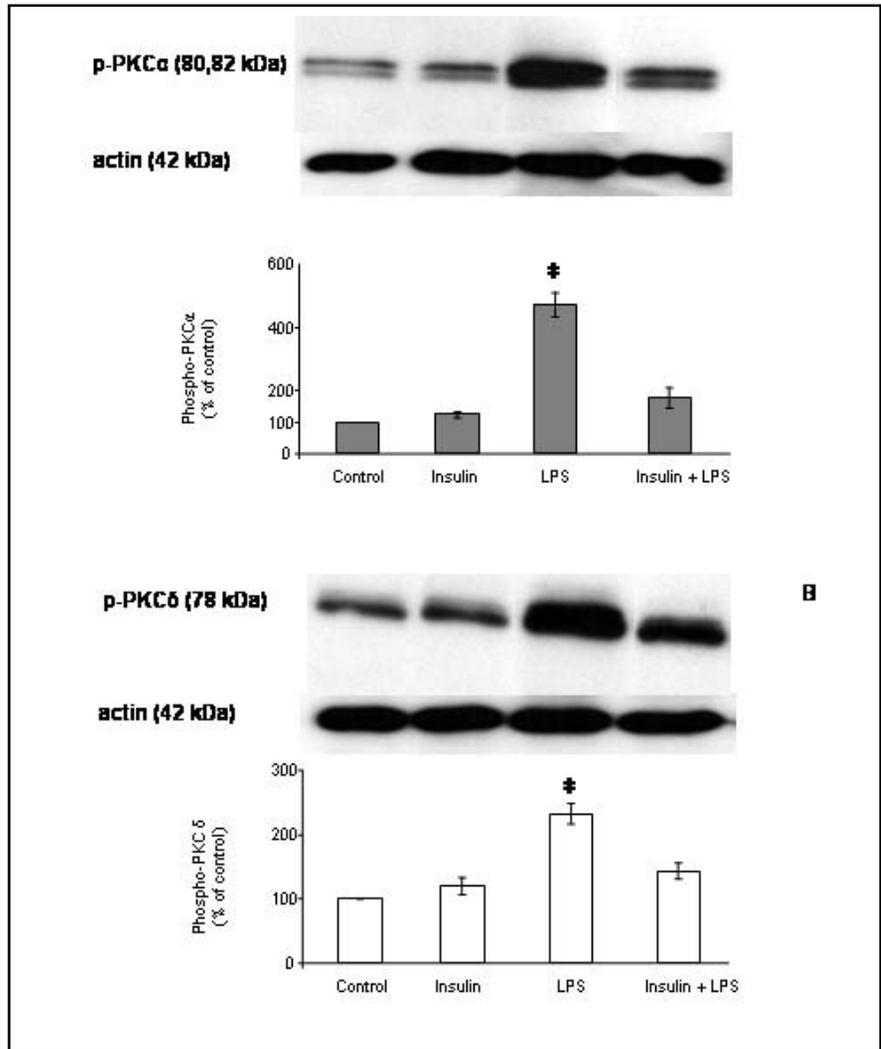
Drugs and reagents

RPMI-1640 and FBS (Gibco-Invitrogen, Carlsbad, CA, USA); Wright-Giemsa stain (Diff-Quik; American Scientific Products, McGraw Park, IL, USA); Methanol (Merck, São Paulo, Brazil); Crystalline insulin (Biobrás, São Paulo, Brazil); LPS, actinomycin D, crystal violet and β -actin (Sigma, Chemical Co, St Louis, Mo, USA); BCA protein assay reagent kit (Pierce Chemical, Rockford, IL, USA); chemiluminescence (ECL) detection (Amersham, Piscataway, NJ, USA); Antibodies all from Cell Signaling technology, INC. Beverly, MA, USA.

Statistical analysis

Data are presented as mean \pm 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. A p values lower than 0.05 were considered statistically significant. TNF data are presented as median of the titles and differences of at least two dilutions were considered significant.

Fig. 2. Insulin inhibition of LPS-induced PKC α (A) and PKC β (B) activation. Alveolar macrophages (1×10^6) 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. PKC α and PKC δ phosphorylation were assessed by western blot analysis. The antibodies recognized phosphorylated residues of Thr638/641 of PKC α and the Thr505 of PKC δ . Illustrations of the western blot represent one out of 6 independent experiments. Expression of protein was quantified by AlphaEaseFC™ software. Values are means \pm SEM of 6 animals per group. * $P < 0.001$ vs other groups.



Results

Insulin inhibits LPS-induced ERK and p38 MAPK activation

Resident AMs from male Wistar rats were obtained by *ex vivo* lung lavage and stimulated with LPS (100 ng/mL) for 30 min. Activation (phosphorylation) of signaling of MAPK by LPS was analyzed by western blot. Figure 1 shows that, relative to controls, LPS induced a significant increase in the activation of ERK (3.6 fold) and p38 (4.4 fold). 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 [16]. Insulin significantly reduced the activation of ERK (54%) and p38 (48%). These data show that insulin down-regulates the MAPK cascade in AMs stimulated with LPS.

Insulin inhibits LPS-induced PKC α and PKC δ activation

Activation (phosphorylation) of PKC α and PKC δ by LPS was analyzed by western blot. Figure 2 shows that, relative to controls, LPS induced a significant increase in the activation of PKC α (4.7 fold) and PKC δ (2.3 fold) in AMs. Treatment of AMs with insulin (1 mU/mL), 10 minutes before LPS stimulation, significantly reduced the activation of PKC α (62%) and PKC δ (39%). These data suggest that the insulin down-regulates PKC's activation in AMs activated with LPS.

Insulin inhibits LPS-induced Akt activation

Activation (phosphorylation) of Akt in AMs stimulated with LPS was analyzed by western blot. Figure 3 shows that LPS increased phosphorylation of both Ser-473 and Tyr-326 Akt residues. Relative to the

Fig. 3. Insulin inhibition of LPS-induced Akt activation. Alveolar macrophages (1×10^6) 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. Akt phosphorylation was assessed by western blot analysis. The antibodies used recognize phosphorylated residues of the Tyr-326 or Ser-473 of Akt. Illustration of the western blot represents one out of 6 (for Tyr-326) or 3 (for Ser-473) of Akt independent experiments. Expression of protein was quantified by AlphaEaseFC™ software. Values are means \pm SEM of 6 or 3 animals per group. * $P < 0.001$ vs other groups; † $P < 0.05$ vs LPS group; ‡ $P < 0.05$ vs control.

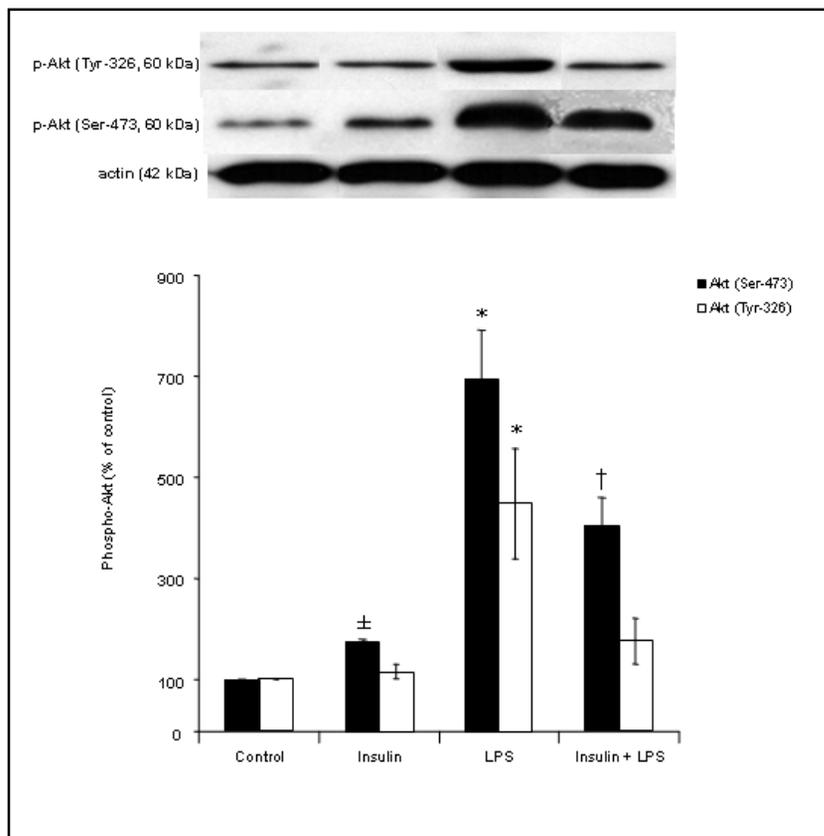


Table 1. Insulin inhibition of LPS-induced TNF production. Alveolar macrophages (1×10^6) were obtained by *ex vivo* lung lavage from male Wistar rats. Insulin (0.1; 1; 10 mU/mL) was given 10 minutes before, together or 10 minutes after LPS stimulation. TNF levels were measured in supernatants by bioassay using L929 cells 30 minutes after LPS. The median of TNF (U/mL) and standard deviation (SD) of each group are shown. Differences of two or more dilutions were considered as significant. * $P < 0.001$.

| Groups | Median of TNF (U/mL) | SD |
|----------------------|----------------------|------|
| Control | 4 [*] | 0 |
| LPS | 32 | 18.5 |
| Insulin before LPS | | |
| 0.1 | 32 | 9.2 |
| 1 | 4 [*] | 2.3 |
| 10 | 6 | 0 |
| Insulin together LPS | | |
| 0.1 | 32 | 9.2 |
| 1 | 4 [*] | 2.3 |
| 10 | 4 [*] | 2.3 |
| Insulin after LPS | | |
| 0.1 | 32 | 0 |
| 1 | 8 [*] | 2.3 |
| 10 | 8 [*] | 0 |

controls, the Tyr-326 phosphorylation was 4.7-fold and of Ser-473 was 6.9-fold. At the concentration of 1 mU/ml insulin did not increase significantly the phosphorylation of the Tyr-326 residue although it did cause a small increase (1.8-fold, $p < 0.05$ relative to control) in the Ser-473 residue phosphorylation. Treatment of AMs with insulin (1 mU/mL), 10 minutes before LPS stimulation, significantly reduced the activation of Tyr-326 Akt (64%) and of Ser-473 Akt (41%). These data show that insulin exerts an inhibitory effect on PI3K activation in AMs stimulated with LPS as verified by phosphorylation of both Tyr-326 and Ser-473 residues of Akt.

Insulin inhibits LPS-induced TNF production

TNF release was measured by a cytotoxicity assay using L-929 tumor cells. AMs were pre-treated with insulin (0.1, 1, 10 mU/mL) 10 min before stimulation with LPS and TNF levels determined in the culture supernatants 30 min later. We found that 0.1 mU/mL has no effect,

and that 1 and 10 mU/mL of insulin abolished TNF release. We also assayed the effect of adding insulin concomitantly with LPS and after 10 min. There is no difference between pre and concomitant treatment on LPS-induced TNF. The post treatment with insulin was also inhibitory but relative to pre and concomitant treatments, showed a tendency to increase but the difference was not significant (table 1). These data show that insulin is able to markedly affect a macrophage function and confirm that the lowest concentration of insulin to achieve this effect was 1 mU/mL.

Discussion

Data presented here show that LPS added to rat AMs enhance phosphorylation of ERK 1/2, p38 MAPK, Akt, PKC α PKC δ and that the phosphorylation of these molecules decreased when insulin was given before LPS stimulation. Insulin alone did not activate these pathways, in rat AMs at the experimental condition used in this study. We also showed a downstream effect of insulin e.g. inhibition of TNF release. Insulin is a key hormone regulating the control of metabolism and maintenance of normoglycemia and normolipidemia. Our results are consistent with an inhibitory effect of insulin and corroborate previous findings showing a protective effect of insulin on systemic inflammation related the sepsis [1]. In this study, we focused on the ERK 1/2, p38 MAPK, Akt, PKC α and PKC δ , which are kinases involved with the regulation of a number of macrophage functions including phagocytosis, respiratory burst, and secretion of several cytokines, such as TNF.

The presence of insulin receptors has been demonstrated in monocytes/macrophages [20, 21] but not in alveolar macrophages. However, there are several studies showing that insulin exerts clear effects on these macrophages [22, 23], which suggests that they do express insulin receptors. In the present study we wanted to use the lower dose of insulin able to interfere with LPS-induced signaling without having a significant effect by itself on the molecules analyzed. At 1mU/ml, insulin alone did not increase basal levels of phosphorylation of most molecules analyzed but it clearly interfered with the LPS-induced signaling cascades. Although it is well known that insulin activates Akt [reviewed in 24] in our experimental conditions it only caused a small increase in the phosphorylation of Ser-473 but not Tyr-326 residues of Akt. We believe that higher doses of insulin will have more marked effect. Grupe et al. [25] showed that insulin

receptor phosphorylation was dose-dependent; the dose of 1 mM was minimally active and the dose of 10 mM induced a sharp increase. Taken together these results suggest that the inhibitory effect of insulin on LPS-induced signaling in AMs is a receptor-dependent phenomenon.

The MAPK 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 MAP kinase family, and this effect is mediated by specific MEK kinases [26, 27], including ERK and p38. Our results show that LPS-induced increase in ERK and p38 phosphorylation was down-regulated by insulin, which could suggest that insulin acts directly on p21^{ras}. Insulin was shown to activate the p21^{ras}/MAP kinase pathway through the binding of growth factor receptor-bound protein (GRB)-2 to tyrosine-phosphorylated insulin receptor substrate (IRS)-1 [28]. In addition, several IRS-1-associated proteins have already been identified including PI3K [29]. Our results show that LPS increased Akt phosphorylation at both Tyr-326 and Ser-473 residues. The increase was more prominent at the Ser-473 residue (6.9-fold relative to control) than on Tyr-326 (4.7-fold relative to control).

PI3K is composed of two subunits, a 100 kDa (p110) catalytic subunit and an 85 kDa (p85) regulatory subunit [30]. A direct interaction between p21^{ras} and p110, and the demonstration that p21^{ras} regulates PI 3'-kinase activity [31], suggests that activation of p21^{ras} via IRS-1 could initiate both the MAP kinase and PI3K signaling pathway.

Jeschke et al. [1] reported that in endotoxemic rats, insulin treatment increased the serum level of anti-inflammatory cytokines and decreased the proinflammatory ones, helping to restore systemic homeostasis. TNF is a well-characterized pro-inflammatory cytokine and may contribute to complex clinical problems encountered in the sepsis syndrome [32]. MAPKs are a group of signaling molecules that appear to play important roles in inflammatory processes and LPS has been reported to activate MAPK pathways [8]. We decided to measure TNF production in order to evaluate a downstream effect of insulin in LPS-induced signaling and found that insulin abolished TNF production. We have no direct evidence that this effect of insulin is related to inhibition of the signaling molecules studied. However, others have demonstrated that selective pharmacological inhibitors of p38 and ERK pathways reduced TNF production by murine macrophages [33]. In our experiments, insulin down-regulated both p38 and ERK pathways and TNF production elicited by LPS.

These observations favor the hypothesis that the protective effect of insulin in sepsis is due to modulation of cellular signal transcription factors rather than to changes in metabolism and blood glucose. In addition, although the insulin receptor and the enzymes whose activity is altered by insulin treatment have been well characterized, the initial steps following insulin binding to its receptor that leads to a change in the activity of a specific enzyme remains to be elucidated [34].

PKC is a family of multifunctional serine/threonine kinases also involved with the regulation of macrophage functions. Treatment of mouse peritoneal macrophages with LPS stimulates activation of PKC α and PKC δ in a dose- and time-dependent manner [9]. In agreement, our results show that increase of both, PKC α and PKC δ phosphorylation in LPS-activated AMs was down-regulated by pre-treatment of AMs with insulin. Since PKC plays a role in macrophage functions such as phagocytosis and insulin inhibits PKC, thus insulin should reduce phagocytosis and consequently, the host defense against infection. However, host defense during sepsis, is dependent primarily on circulating neutrophils and it is

not known what effect has insulin on phagocytosis and killing by neutrophils. Also we do not know what is the effect of the low dose of insulin that we used (1 mU/mL) on the macrophages microbicidal activity (reactive oxygen intermediate - ROI, NO production, etc). At much higher concentration (200 mU/mL), Costa Rosa et al. [22], found that insulin enhanced phagocytosis and production of H₂O₂ by macrophages. Thus, the effect of insulin seems to be dependent on the dose, time and type of cell.

In conclusion, this report shows that insulin inhibits the LPS-induced ERK 1/2, p38, Akt, PKC α and PKC δ phosphorylation and TNF release in AMs *in vitro*. Thus, it is possible that the protective effect of insulin in sepsis reported by others is due to inhibition of the secondary wave of mediators released by the lungs during this condition.

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