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Insulin Attenuates Apoptosis and Exerts Anti-Inflammatory Effects in Endotoxemic Human Macrophages

Mareike Leffler, M.D.,* Thomas Hrach, M.S.,* Michael Stuerzl, Ph.D.,† Raymund E. Horch, M.D.,* David N. Herndon, M.D.,‡ and Marc G. Jeschke, M.D., Ph.D.‡,[1]

**Department of Plastic and Hand Surgery and †Department of Molecular and Experimental Surgery, University Hospital Erlangen-Nürnberg, Erlangen, Germany; ‡Shriners Hospitals for Children, Galveston Burns Unit and Department of Surgery, University Texas Medical Branch, Galveston, Texas*

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Background. **Insulin decreases the incidence of sepsis and improves mortality of critically ill patients. In endotoxemic as well as in thermally injured rats, insulin attenuates the systemic inflammatory response by decreasing the proinflammatory and increasing the antiinflammatory cascade. The aim of the present study was to determine the effects of insulin on cell survival, cell activity, apoptosis, and proinflammatory response in a human macrophage-like cell line (THP-1 cells) stressed with lipopolysaccharide (LPS).**

Materials and methods. **Human macrophages were stressed with LPS and received either saline or insulin. Cell viability was analyzed by MTS, apoptosis was detected using JC-1 and terminal deoxynucleotidyl transferase-mediated nick end labeling-staining, and to elucidate on the signaling pathway, we used wortmannin as a phosphatidylinositol-3-kinase inhibitor. Tumor necrosis factor (TNF) and interleukin-1beta (IL-1β) were measured to determine the effect of insulin on proinflammatory cytokine expression.**

Results. **Insulin caused a significant increase in cell viability and significantly reduced apoptosis in LPS-stimulated human macrophages in a dose-dependent manner. The antiapoptotic effect of insulin could be completely blocked with the addition of wortmannin. Insulin significantly decreased TNF and IL-1β in endotoxemic human macrophages.**

Conclusions. **Our results indicate that insulin exerts antiapoptotic effects and reduces the expression of proinflammatory cytokines in endotoxemic human macrophages. The antiapoptotic effects are mediated via the phospatidylinositol-3-kinase-pathway.**

[1] To whom correspondence and reprint requests should be addressed at Department of Surgery, Shriners Hospitals for Children, 815 Market Street, Galveston, TX 77550. E-mail: majeschk@utmb.edu.

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INTRODUCTION

The systemic inflammatory response can lead to severe sepsis and septic shock. Despite advances in the diagnosis and supportive treatments, sepsis is associated with high morbidity and mortality [1]. In the United States, there are an estimated 750,000 cases of sepsis or septic shock per year, and 20 to 40% are lethal [2]. During a systemic inflammatory response, hypermetabolism and catabolism occur [3]. The pathophysiological mediators include pro- and anti-inflammatory mediators such as cytokines and signal proteins [4–6]. Recently, Van den Berghe *et al.* [7, 8] showed that intensive insulin therapy decreases morbidity and mortality in critically ill patients. Insulin, given at a dose to maintain tight euglycemia (<110 mg/dL), significantly improved incidence of infection, shortened hospital stay, and improved survival of critically ill patients [8]. The inflammatory response is a major contributor to morbidity and mortality in critically ill patients, thus attenuation would be beneficial. Recent studies suggested an anti-inflammatory effect of insulin by increasing the anti- and decreasing the proinflammatory cascade and thereby homeostasis restoration in thermally injured and endotoxemic rats. In addition, insulin prevented liver damage and preserved liver function in these animals [9–12]. Some studies suggest that a tight glucose control rather than the admission of insulin is the key mechanism of the survival improvement [13–15]. On the other hand,

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there is evidence that insulin may act directly as an anti-inflammatory agent [16–19].

The first cells that respond to pathological stimuli before the B-cell mediated-humoral response is initiated are activated macrophages. Lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, is one of the main pathogenic factors during the systemic inflammatory response. Recent studies suggest that LPS mediated cell death could be an underlying mechanism of hyporesponsiveness and dysfunction of macrophages in the late phase of septic shock [20]. It has already been shown that insulin prevents cell death in oligodendrocytes, epithelial cells, or carcinoma cell lines [21–23]. These antiapoptotic effects are suggested to be mediated via the phosphatidylinositol-3-kinase (PI3K) signaling pathway [24]. The effects of insulin on macrophages are not entirely defined. Therefore, the purpose of this study was determine whether insulin has an effect on macrophages exposed to endotoxin (LPS) in terms of apoptosis, proliferation, cell viability, and proinflammatory cytokine expression. We hypothesized that insulin improves macrophage viability and decreases macrophage apoptosis, which is associated with an activation of the PI3K signaling pathway. We further hypothesized that insulin administration decreases proinflammatory cytokine production.

MATERIALS AND METHODS

Cell Culture

Human-like macrophages, THP-1 cells (ATCC, USA, cat. no. TIB-202) were grown in suspension in 275-mL culture flasks (Schubert und Weiss; Labortechnik, Munich, Germany) in RPMI 1640 medium containing GlutaMAX (Gibco, London, United Kingdom, cat. no. 61870-010). Glucose concentration in the media was 2000 mg/L (11.11 mmol). Antibiotics (1% penicillin/streptomycin [10.000 U/10.000 μg/mL] [Biochrom, Berlin, Germany, cat. no. A2213]) and 10% heat-inactivated fetal bovine serum (FBS) (Biochrom, cat. no. S0115) were added to the medium. Cells were incubated in a humidified atmosphere consisting of 5% CO_2. Medium was changed every 3 to 5 d. After at least 3 d of growth, cells were harvested and counted by trypan blue staining (Biochrom, cat. no. L 6323) by the use of a Neubauer counting chamber. $1{,}5 \times 10^6$/2 mL cells were distributed into each well of a 6-well plate, 8×10^4/100 μL cells were distributed into each well of a 96-well plate and $1{,}1 \times 10^5$/500 μL cells were distributed into each chamber of a chamber slide (BD Falcon, Heidelberg, Germany), respectively. Differentiation of THP-1 monocytes into macrophages was achieved by overnight incubation with phorbol-12-myristate-13-acetate (PMA; Sigma, St. Louis, MO, cat. no. P 1585) at a concentration of 5×10^{-5} g/mL. Differentiated cells were adherent to the wells whereas undifferentiated cells remained in suspension and were removed by washing the cells with PBS, pH 7.4. New serum-free medium was added and cell treatment was performed as described below. These cells were incubated with serum-free medium alone or with lipopolysaccharide (LPS; Sigma, cat. no. L 7895) at different concentrations (100 ng/mL, 1, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 μg/mL) to determine the optimal dosage of LPS and the dose-effect relation curve.

To study the effects of insulin on cell survival, induction of apoptosis and expression of proinflammatory cytokines cells were treated with insulin (Insuman Rapid Optiset; Aventis, Frankfurt, Germany) at different concentrations (1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 IU/mL) alone or in combination with LPS for 24 h. To determine whether the effects of insulin are mediated via the PI3K pathway the PI3K-inhibitor wortmannin (Sigma, Sigma-Aldrich Chemie Gmbh, Munich, Germany, cat. no. 075K4050) was used. Cells were cultured in chamber slides and were treated with a combination of 10 μg/mL LPS and 5 IU/mL insulin as described above. Wortmannin was added to the medium at different concentrations (1, 10, 50, 1000, 200 nM). As a control group, cells were treated with wortmannin alone or the combination of LPS and wortmannin without the addition of insulin.

After 24 h of treatment, the cells were stained with trypan blue (Biochrom, Germany, cat. no. L6323). Cells were counted under light microscopy and the percentage of dead cells was determined. At least three different cell assays were stained for trypan blue.

Cell Viability

For measurements of the cell activity, the CellTiter 96 aqueous nonradioactive cell proliferation assay (Promega, Madison, WI, cat. no. G5421) was performed according to the kit guidelines. The CellTiter 96 assay uses the tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and the electron coupling reagent, phenazine methosulfate (PMS). MTS is chemically reduced by cells into formazan, which is soluble in tissue culture medium. The measurement of the absorbance of the formazan can be carried out using 96-well microplates at 492 nm. The assay measures dehydrogenase enzyme activity found in metabolically active cells. As the production of formazan is proportional to the number of living cells, the intensity of the produced color is a good indication of cell viability. MTS solutions were prepared according to the manufacturer's instructions. Stock PMS (Sigma) was dissolved in PBS at a concentration of 0.92 mg/mL DPBS (0.92 mg/mL PMS in DPBS is also included with the CellTiter 96 Assay System, Promega, USA, cat. no. G5421). The assay was performed according to the manufacturer's guidelines. Briefly, cells were treated with serum-free medium alone (control), with LPS or insulin alone, or with the combination of LPS and insulin as described above. After 24 h, the conversion of a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, MTS) into aqueous, soluble formazan by metabolically active cells was measured. The amount of soluble formazan was determined by measuring the absorbance at 492 nm. The experiments were carried out in two triples ($n = 6$) and only cells from one passage were used.

Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL)

TUNEL-staining was performed for the detection of apoptosis (in situ cell death detection kit, TMR red; Roche, Basel, Switzerland, cat. no. 2156792). During apoptosis, DNase activity not only generates double-stranded, low molecular weight DNA fragments (mono- and oligonucleosomes), but also introduces strand breaks ("nicks") into the high molecular weight DNA. These processes can be identified by labeling the free 3′-OH termini with terminal transferase (TdT), which attaches labeled nucleotides to all 3′OH-ends (TUNEL reaction; TdT-mediated dUTP nick end labeling) [25, 26]. Prior to staining, the cells were cultured in chamber slides and treated with medium alone (control), with LPS or insulin alone, or with a combination of LPS and insulin as described above. Twenty-four hours after LPS or insulin administration, TUNEL-staining was performed according to the kit guidelines and were repeated in two triples ($n = 6$). Samples were analyzed immediately after staining using fluorescence microscopy at two different wavelengths. Two fields of vision were analyzed per experiment. Apoptotic cells showed a red fluorescence while nonapoptotic cells did not show any fluorescence.

JC-1

In addition to TUNEL-staining, we attempted to confirm apoptotic rates by measuring the mitochondrial membrane potential changes using JC-1-staining (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide; 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolo-carbocyanine iodide CBIC2 [3], Mobitec, Goettingen, Germany, cat. no. T3168). The loss of mitochondrial membrane potential is an early indicator for apoptosis [27, 28]. JC-1 staining solution was freshly prepared in a concentration of 10 μg/mL prior to use with RPMI medium without phenol red. Cells were cultured in chamber slides and treated with LPS and insulin as described above ($n = 6$). After washing with PBS, pH 7.4, 300 μL staining solution was added to each chamber and the cells were incubated for 10 min. After another washing step, fluorescence microscopy of the cells was performed. Four high power fields were analyzed per sample. In apoptotic cells, the ratio of red to green fluorescence decreases if the mitochondrial membrane depolarizes. In nonapoptotic cells, the polarized mitochondria are marked by punctuate orange-red fluorescent staining.

Cytokines

Proinflammatory cytokines were determined using an interleukin-1beta (IL-1β) and tumor necrosis factor (TNF) enzyme-linked immunosorbent assay (ELISA) (BenderMedSystem, Burlingame, CA, cat. no. BMS224/2TEN [IL-1β], cat. no. BMS223/3TENCE [TNF-α]). The cells were cultured in 6-well plates and treated with medium alone, LPS or insulin alone, or with the combination of LPS and insulin as described above. Cell culture supernatants were harvested after 4, 8, 12, and 24 h. All experiments were repeated six times. The supernatants were used either diluted or pure and measurements were taken according to the kit guidelines.

Statistical Analysis

Data were expressed as means ± standard error of the mean (SEM). Statistical analysis was performed using ANOVA with post-hoc Bonferroni correction. Significance was accepted at $P < 0.05$ (*), but we further set a second significance level, which we considered highly significant at $P < 0.001$ (**).

RESULTS

Cell Viability

Cell treatment of human macrophages with LPS for 24 h resulted in a significant reduction of cell viability in a dose-dependent manner (Fig. 1A). Five μg/mL LPS had no significant influence on the viability of human macrophages whereas 10 μg/mL and more significantly decreased cell viability when compared with the control group, $P < 0.05$.

Insulin alone, without LPS, at a dose between 1 and 15 IU/mL had no influence on cell viability, however, at 25 IU/mL insulin resulted in an almost complete death of the cells (data not shown). In LPS stimulated cells insulin significantly attenuated LPS-induced cell death in human macrophages in a dose-dependent manner (insulin dosage between 1 and 15 IU/mL, Fig. 1B). The addition of insulin to low-dose LPS (10 to 30 μg/mL) stimulated macrophages resulted in a 50% decrease of cell death. However, at a higher LPS dose (>40 μg/mL) the "rescue effect" of insulin was abolished (Fig. 1B).

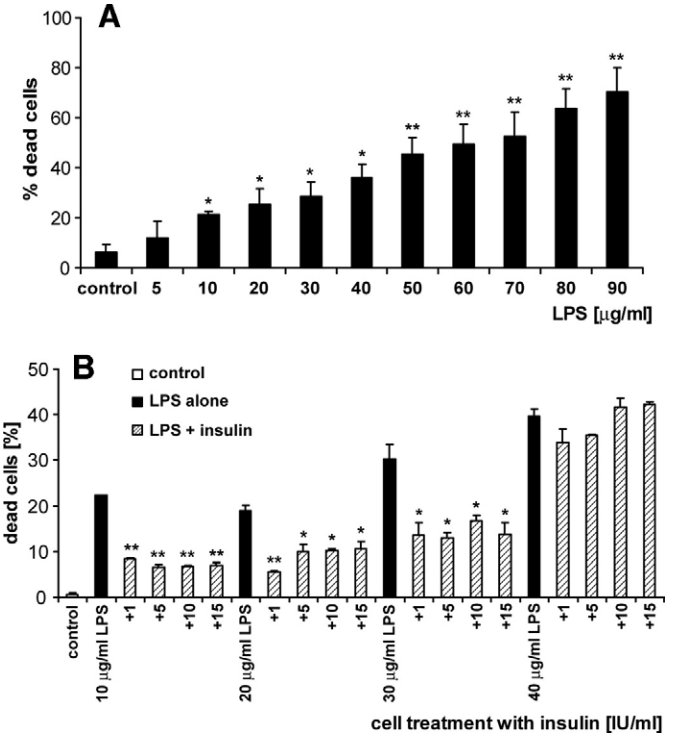

FIG. 1. (A) Trypan blue staining of macrophages after various doses of LPS administration. LPS at 10 μg/mL and greater led to a significant increase of cell death as compared to the control group. Significant differences compared to control $^*P < 0.05$, $^{**}P < 0.001$. (B) Trypan blue staining of macrophages after LPS plus insulin administration. The addition of insulin to LPS-treated macrophages resulted in a significant decrease of cell death. Significant differences compared with LPS $^*P < 0.05$, $^{**}P < 0.001$.

Cell viability was further determined by MTS assay. Conforming to the trypan blue data, the MTS assay showed decreased cell viability with increasing doses of LPS. LPS at a dose between 1 and 30 μg/mL did not affect cell viability; cell viability significantly decreased at 40 μg/mL of LPS, $P < 0.05$ (Fig. 2A). Insulin administration to THP-1 cells without LPS showed a dose-dependent influence on cell viability (Fig. 2B). If insulin was added at a dose between 5 and 15 IU/mL, no changes in cell viability were found, whereas a higher dose of insulin (20 to 25 IU/mL) resulted in a significant decrease of viability (Fig. 2B). The combination of LPS (10 μg/mL) plus insulin reduced cell viability in THP-1 cells (Fig. 2C). No change in cell viability was found in lower insulin doses; however, insulin at 15 IU/mL significantly decreased cell viability (Fig. 2C). The same effect could be seen when 20 or 30 μg/mL LPS were used (data not shown).

Apoptosis

LPS-induced endotoxemia of human macrophages resulted in a significant induction of apoptosis in a dose-dependent manner as shown by TUNEL (Fig. 3). The combination of LPS and insulin (dosage 1 to 15 IU/mL)

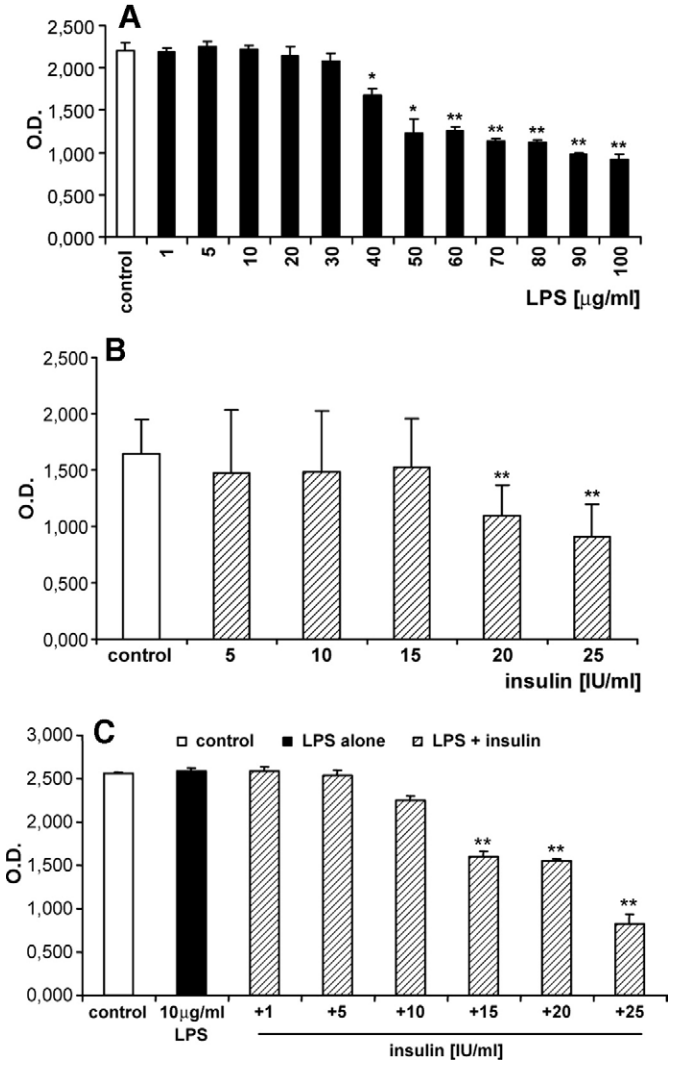

FIG. 2. (A) Cell viability after LPS administration at various doses. Beginning 40 μg/mL of LPS cell viability decreased significantly. Significant differences compared to control $^{*}P < 0.05$, $^{**}P < 0.001$. (B) THP-1 cell viability insulin administration at different concentrations. Insulin a lower doses had no influence on cell viability, while higher doses significantly reduced THP-1 cell viability. Significant differences compared with control $^{**}P < 0.001$. (C) Cell viability of THP-1 cells stressed with 10 μg/mL of LPS and different insulin doses. LPS plus 15 IU of insulin significantly decreased cell viability. Significant differences compared with control $^{*}P < 0.05$, $^{**}P < 0.001$.

resulted in a significant decrease of LPS-induced apoptosis in human macrophages as compared with endotoxemia alone (Fig. 3). THP-1 cells received LPS at 10 μg/mL and insulin in various doses. Insulin decreased the number of apoptotic cells to a number that did not differ from the control group (control: 8.5 $\pm$ 0.96%, 10 μg/mL LPS: 36.5 $\pm$ 0.13%, LPS +1 IU/mL insulin: 12 $\pm$ 0.17%), $P < 0.001$. Addition of insulin to endotoxemic cells resulted in a more than 50% reduction of apoptosis even if a high dosage of LPS was used (Fig. 3G) (50 μg/mL LPS: 84.3 $\pm$ 0.40%, LPS +1 IU/mL insulin: 43.1 $\pm$ 1.51%). Treatment of the cells with insulin alone in a dose between 1 and 15 IU/mL did not show any influence on apoptosis induction in human macrophages (data not shown).

Results obtained with the TUNEL assay could be confirmed by JC-1 staining (Fig. 4A, B, C, D, E, and F). LPS with a dosage between 5 and 50 μg/mL resulted in a significant induction of apoptosis in a dose-dependent manner (Fig. 4G). The addition of insulin resulted in a significant reduction of LPS-induced apoptosis if a concentration of 10 μg/mL LPS was used, $P < 0.05$ (Fig. 5).

To determine whether the antiapoptotic effect of insulin is mediated via the PI3K-pathway, cells were treated with a combination of 10 μg/mL LPS, 5 IU/mL insulin, and the specific PI3K-inhibitor wortmannin at different concentrations (1, 10, 100 nM). Apoptosis was detected using the JC-1-staining. The antiapoptotic effect of insulin in endotoxemic human macrophages was completely abolished if wortmannin was added to the culture medium (Fig. 6). The addition of wortmannin (concentration between 1 and 200 nM) alone had no influence on apoptosis induction (data not shown).

Cytokines

LPS administration resulted in a significant increase of TNF in human macrophages in a dose-dependent manner (Fig. 7A). The addition of insulin at a concentration between 1 and 10 IU/mL did not show any significant influence on TNF protein expression compared to LPS-treatment alone (Fig. 7B). The addition of 1 IU/mL insulin resulted in an increase of TNF protein expression at all time-points, but this increase was not significant. However, the addition of 15 IU/mL insulin resulted in a significant decrease of TNF protein concentration in endotoxemic human macrophages at all time-points (Fig. 7B). The addition of insulin alone (dosage between 1 and 15 IU/mL) did not affect TNF in human macrophages (data not shown).

Similar results were obtained after investigation of the influence of endotoxemia on IL-1β protein expression of human macrophages. One μg/mL LPS resulted in a significant increase of IL-1β compared with the control at four different time-points (Fig. 7C). The addition of insulin resulted in a decrease of IL-1β protein expression, but this reduction was significant if insulin at a dose of 15 IU/mL was added to the medium (Fig. 7D). The addition of insulin alone (dosage between 1 and 15 IU/mL) did not effect IL-1β protein expression in human macrophages (data not shown).

DISCUSSION

In the present study, we showed that LPS causes, in a dose-dependent manner, macrophage apoptosis and decreased cell viability. Insulin caused a significant increase in cell viability and significantly reduced apoptosis in LPS-stimulated human macrophages in a

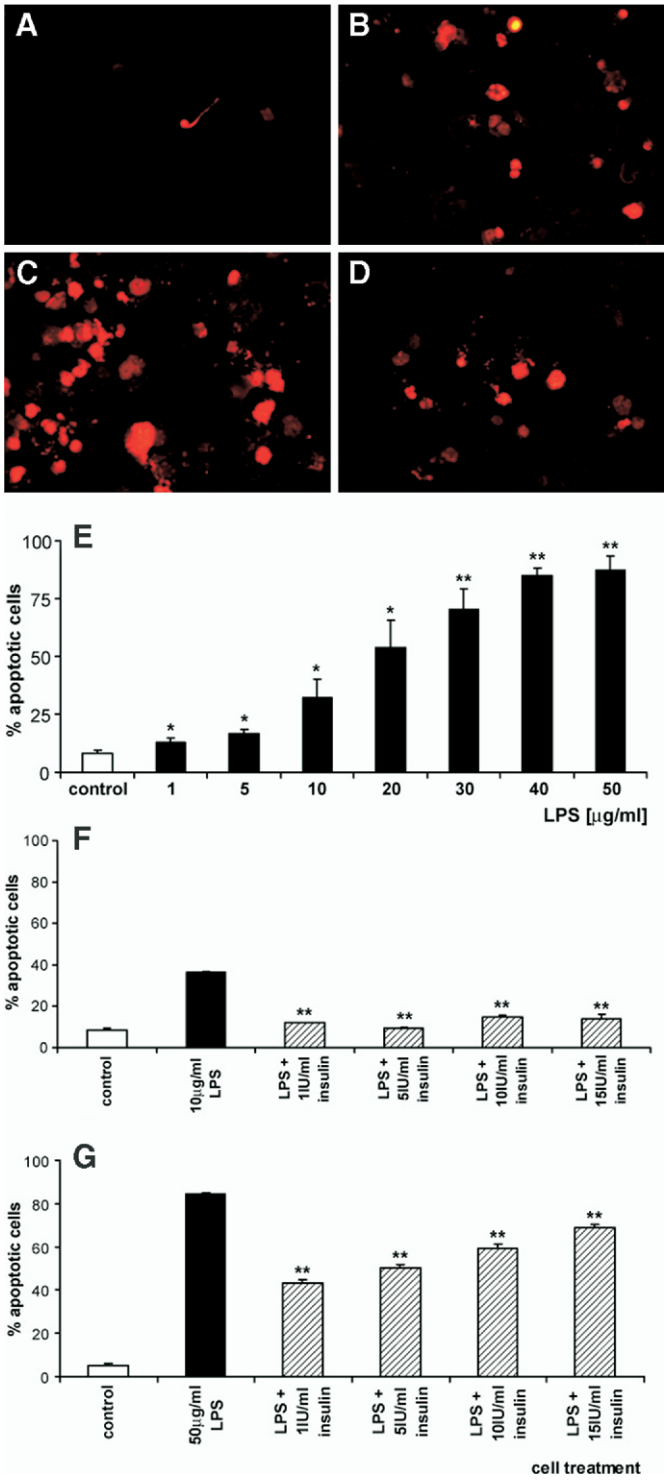

FIG. 3. (A) Microscopic representative view of human THP-1 cells after TUNEL staining. Apoptotic cells show a red fluorescence while nonapoptotic cells do not show any fluorescence. Untreated cells (control group). (B) Cells were treated with 10 μg/mL LPS. (C) THP-1 cells treated with 50 μg/mL LPS. (D) THP-1 cells treated with 50 μg/mL LPS and 5 IU/mL insulin. The number of apoptotic cells decreased as compared to LPS-treatment alone. (E) Endotoxemia resulted in a significant induction of apoptosis in human macrophages in a dose-dependent manner as compared to the control. Significant differences compared with control $^*P < 0.05$, $^{**}P < 0.001$. (F) LPS at 10 μg/mL induced THP-1 cell apoptosis which is attenuated with various doses of insulin. Significant differences compared with LPS, $^{**}P < 0.001$. (G) LPS at a dose of 50 μg/mL induced THP-1 cell apoptosis which was attenuated with insulin. Significant differences compared to LPS, $^{**}P < 0.001$.

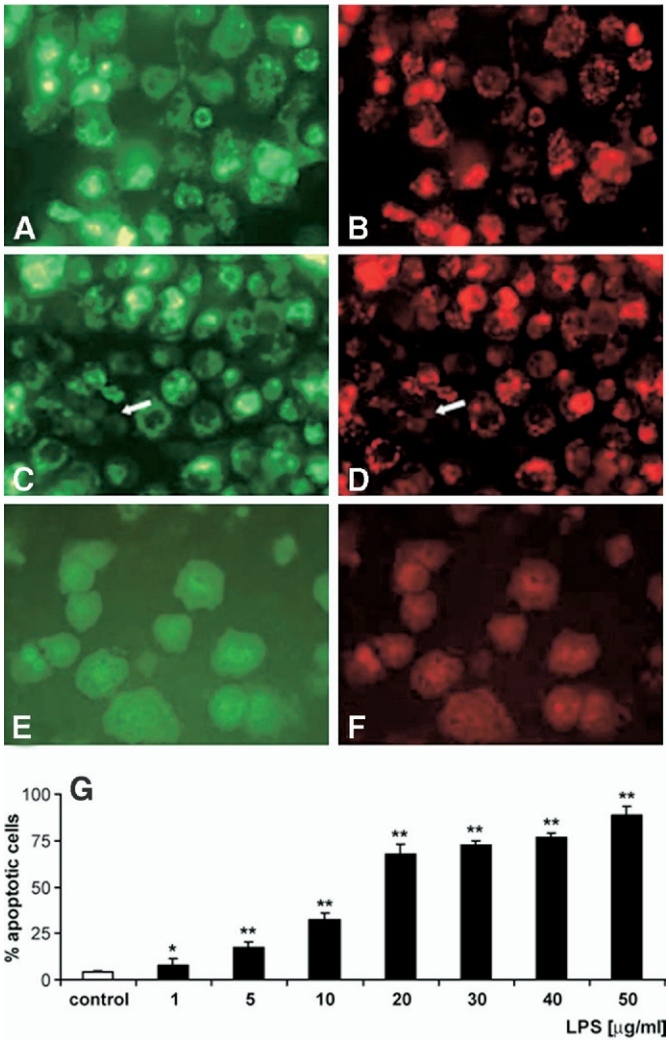

FIG. 4. Microscopic representative view of human THP-1 cells with JC-1 staining. Almost all untreated cells (control group) show a green (A) and red (B) fluorescence as a sign for a polarized mitochondrial membrane. (C) About one-third of the cell that were treated with 10 μg/mL LPS showed a diffuse green fluorescence (white arrow = apoptotic cell [diffuse green fluorescence]) and no punctuate red fluorescence (D), white arrow = apoptotic cell). (E), (F) THP-1 cells treated with 50 μg/mL of LPS0 μg/mL; approximately 90% of the THP-1 cells showed depolarization of the mitochondrial membrane potential as a clear sign for apoptosis. (G) Quantification of apoptotic THP-1 cells. Endotoxemia resulted in a significant induction of apoptosis in a dose-dependent manner. Significant differences compared to control $^*P < 0.05$, $^{**}P < 0.001$.

dose-dependent manner. The antiapoptotic effect of insulin could be completely blocked with the addition of wortmannin, a PI3K inhibitor. Insulin furthermore,

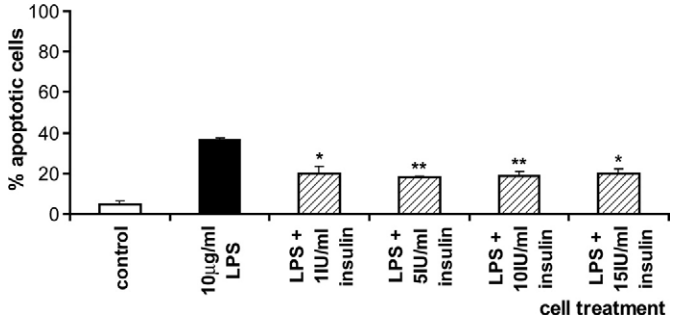

FIG. 5. THP-1 cells were stressed with 10 μg/mL LPS and received insulin in various doses. Apoptotic cells were determined by JC-1 staining. Insulin significantly decreased THP-1 cell apoptosis in a dose-dependent fashion. Significant differences compared to LPS $^{*}P < 0.05$, $^{**}P < 0.001$.

significantly decreased TNF and IL-1β in endotoxemic human macrophages. We concluded from this study that insulin exerts antiapoptotic effects and reduces the expression of proinflammatory cytokines in endotoxemic human macrophages. The antiapoptotic effects are mediated via the phospatidylinositol-3-kinase pathway.

The systemic inflammatory response after trauma, surgery, or burn leads to a stress response including protein degradation, catabolism, and hypermetabolism. As a consequence, the structure and function of essential organs are compromised and contribute to multiorgan failure and mortality [3, 29–31]. Proinflammatory mediators such as cytokines, proteins, and signal transcription factors, which have been shown to partially originate from macrophages, mediate this inflammatory and catabolic event and are thought to be the major molecular signals that control the pathophysiologic cascade. A sustained inflammatory response can be potentially life-threatening with the uncontrolled and prolonged action of proinflammatory cytokines and acute-phase proteins contributing to multiorgan failure, hypermetabolism, morbidity, and mortality [32, 33]. Multiple trials have been undertaken in an attempt to attenuate the overexpression of proinflammatory cytokines and were successful *in vitro*, but failed *in vivo* [5, 34, 35]. We and others [7–12, 36–39] chose a different approach by investigating the hypothesis that an endogenous hormone, e.g., insulin, must be locally or systemically present to attenuate the proinflammatory cascade. Insulin is becoming more and more attractive as an agent to improve outcome of critically ill, surgical, trauma, or burn patients. This hypothesis was further supported as several studies found that hyperglycemia may be detrimental for survival of critically ill patients [13–15]. In critical illness and burns, alterations in glucose metabolism occur, including hyperglycemia associated with insulin resistance [40]. Hyperglycemia is associated with increased levels of inflammatory markers, enhanced expression of cytotoxic T-cells, and reduced expression of T-cells, which are implicated in limiting the immune process during myocardial infarction [41]. Recent *in vitro* and *in vivo* studies investigated the effect of glucose on the signaling cascade and cytokines in smooth muscle and endothelial cells. Data suggest that hyperglycemia increases reactive oxygen species (ROS) resulting in increased phospholipase A2 and C, and nuclear factor-kappaB (NF-κB). Increased NF-κB stimulates TNF expression, which in an autocrine fashion increases ROS. TNF, IL-1, and IL-6 also inhibit insulin signaling which exacerbates inflammation and ROS formation [19, 42–44].

In the present study, we showed that insulin administration without glucose alteration had multiple beneficial effects on macrophages stimulated with LPS. We conducted a large-dose response for LPS and insulin and found that LPS has itself multiple effects on macrophages that are clearly dose dependent. Insulin, on the other hand, had multiple effects on macrophages with or without LPS stimulation. Insulin decreased proinflammatory markers in a dose-dependent fashion, thus exerting an anti-inflammatory effect. As glucose was not altered with insulin administration, this indicates that insulin has a direct effect on inflammatory markers rather than an indirect via glucose modulation. This finding is in agreement with previous studies *in vivo* from our group. Furthermore, insulin exerted antiapoptotic effects, which were detected by TUNEL and JC-1. In general, we showed that insulin has dramatic effects on cell apoptosis in macrophages and markedly reduce apoptosis in these cells. A possible question is whether the LPS and the insulin dose used in

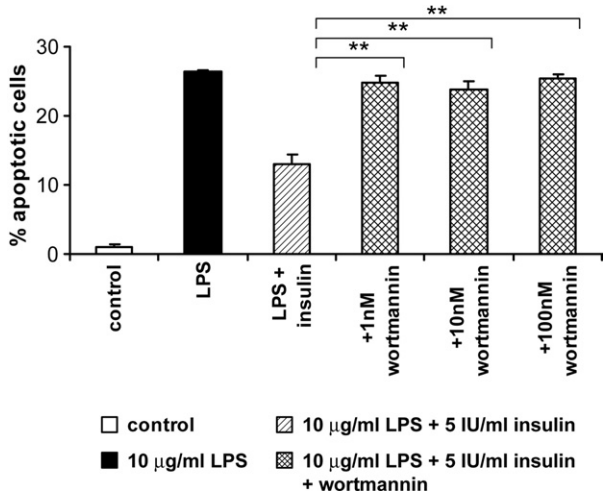

FIG. 6. THP-1 cells received 10 μg/mL LPS and 5 IU/mL insulin as well as wortmannin at different concentrations. Apoptosis induction was detected by the use of JC-1-staining. The antiapoptotic effect of insulin could be blocked using wortmannin. Significant differences compared to LPS + insulin $^{**}P < 0.001$.

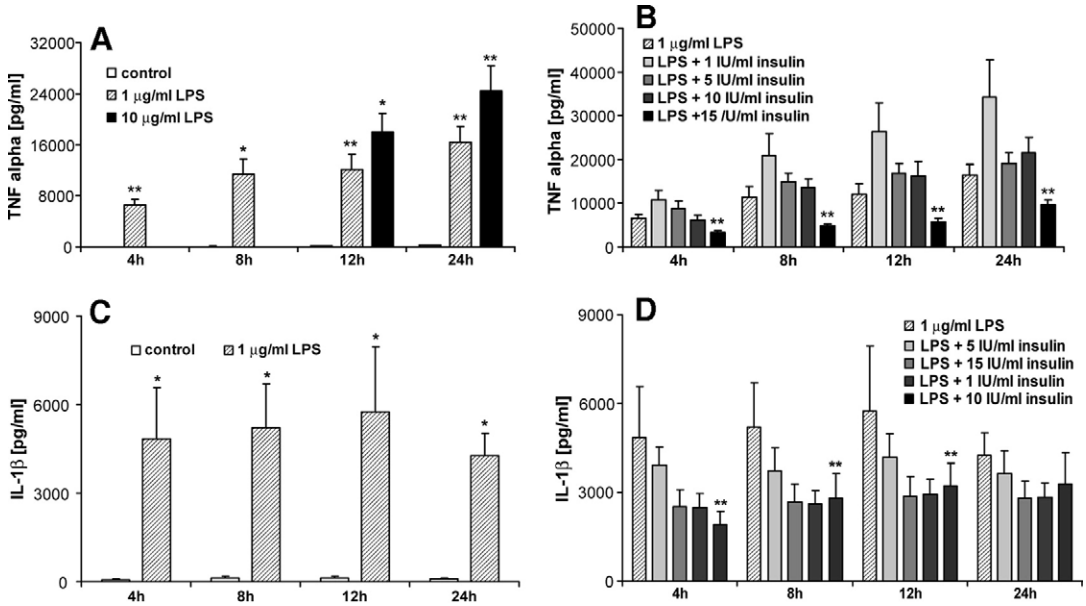

FIG. 7. (A) TNF concentration in response to LPS at various doses. TNF significantly increase in a dose- and time-dependent manner. Significant differences compared with control $^{*}P < 0.05$, $^{**}P < 0.001$. (B) TNF concentration in the supernatant. THP-1 cells received 1 μg/mL LPS and insulin at different concentrations (between 1 and 15 IU/mL). Insulin, at 15 IU/mL, significantly decreased TNF. (C) IL-1β concentration in response to 1 μg/mL LPS. IL-1β was significantly increased at all time points compared with controls. Significant differences compared to control $^{**}P < 0.001$. (D) Insulin at 15 IU/mL significantly decreased IL-1β. Significant differences compared with LPS $^{**}P < 0.001$.

this study has clinical relevance. We cannot answer the question concerning the LPS dose, but we chose this large dose as we wanted to induce a marked inflammatory response with associated metabolic changes, which resulted in these large doses. In terms of insulin, we administered insulin clinically at 0.1 IU/kg/h i.v. to our severely burned patients as a basal rate. In a 20-kg child, a dose of 48 IU is administered over 24 h. This dose may increase if the burned child becomes more insulin resistant. In our rodent studies, we used insulin at a dose of 5 IU/kg s.c. Therefore, we suggest that the use of 5 to 10 IU/mL of insulin may be a dose (or represent a dose) that is clinically used and has clinical implications.

The molecular mechanisms by which insulin administration attenuates apoptosis and inflammation and improves patient outcome are not fully determined. Recent studies from our group strongly suggest that the PI3K signaling pathway plays a major role during the aftermath of stress (unpublished data). In the present study, we found that blocking PI3K using wortmannin reversed the antiapoptotic effects of insulin entirely confirming the prosurvival effects of PI3K stimulation. PI3K and the PI3K-signaling pathway is an effective and multifactorial pathway that encompasses many signals and cascades. Once insulin binds to its receptor (IR), activation of the insulin receptor substrate (IRS) occurs. The activated insulin receptor substrate-1 (IRS-1) then phosphorylates PI3K. The PI3Ks are a family of signal transduction enzymes that are involved in regulating cellular proliferation and survival, and appear to play a major role during inflammation and sepsis [45]. A recent animal study suggested that increased PI3K expression is beneficial for survival [46]. Using a CLP mouse model, the authors found that partially blocking the PI3K pathway using wortmannin and LY294002 resulted in decreased survival. These data taken together, it appears that PI3K and the PI3K-signaling pathway improves survival and enhances cellular regeneration [46]. In the present study, we did not determine PI3K expression after insulin administration; however, in a previous study we determined the effect of insulin on hepatic PI3K mRNA postburn and found that insulin increased PI3K expression by 200% [12]. In the present study, we blocked PI3K signaling using an antibody to PI3K. This blockade of PI3K resulted in a complete reversal of the antiapoptotic effects of insulin, indicating that the antiapoptotic effects of insulin are mediated via PI3K, which is in agreement with a study conducted by Iida *et al.* [24] in which the authors found antiapoptotic effects of insulin in starved macrophages. However, one has to consider that wortmannin also inhibits part of the MAP kinase pathway, and there may be some effects due to MAP kinase inhibition. In summary, in the present study we showed that insulin markedly improves macrophage viability and survival after LPS stimulation. Improved cell viability was associated with a significantly decreased proinflammatory response. These beneficial effects appear to be mediated via PI3K, as blockade of PI3K reversed the effects of insulin.

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