Endocrinology. First published June 10, 2004 as doi:10.1210/en.2004-0592

Insulin attenuates the systemic inflammatory response in endotoxemic rats

Running Title: Insulin attenuates the endotoxemic response

Marc G Jeschke MD PhD[*], Dagmar Klein PhD[*], Ulrich Bolder MD PhD[†], Ralf Einspanier PhD[§]

[*] Department of Surgery. Friedrich-Alexander University of Erlangen, Germany.

[†] Department of Surgery. University of Regensburg, Germany.

[§] Institute of Veterinary Biochemistry, Free University Berlin, Germany.

Corresponding author:

Marc G Jeschke, MD PhD.

Shriners Hospital for Children

Galveston Burns Unit

815 Market Street

Galveston, TX 77550

USA

Phone: 001-409-770-6731

e-mail: Mcjeschke@hotmail.com

Key Words: sepsis, endotoxemia, infection, cytokines, SIRS

Abstract

Insulin decreases the mortality and prevents the incidence of infection and sepsis in critically ill patients. The molecular and cellular mechanisms by which insulin improves survival have not been defined. The purpose of the present study was to determine the effect of insulin on the inflammatory reaction during endotoxemia. Endotoxemic rats were randomly divided into two groups to receive either saline or insulin. The effect of insulin on hepatic signal transcription factor mRNA expression, pro-inflammatory and anti-inflammatory cytokine mRNA and protein concentration was determined. Insulin administration did not change glucose or electrolyte levels but significantly decreased pro-inflammatory signal transcription factors (C/EBPβ, STAT-3 and-5, RANTES) and cytokine expression in the liver and serum levels of IL-1β, IL-6, MIF and TNF-α. Insulin administration further decreased HMG-1 in the serum compared to controls. In addition, insulin increased anti-inflammatory cytokine expression in the liver and serum levels of IL-2, IL-4 and IL-10, and hepatic SOCS-3 mRNA expression. Insulin modulates the inflammatory response by decreasing the pro-inflammatory and increasing the anti-inflammatory cascade. As glucose and electrolyte levels did not differ between insulin and control we hypothesize that the effects are direct anti-inflammatory mechanisms of insulin rather than indirect through modulation of glucose or electrolyte metabolism.

Introduction

Severe sepsis and septic shock are associated with substantial mortality and health care resources (1). There are an estimated 750,000 cases per year of sepsis or septic shock in the United States and in 20-40% the cases are lethal (2). In elderly people the incidence of sepsis or septic shock and the associated lethality rates are vastly higher than those in younger people (1, 2). Given the increasing number of elderly the cases of sepsis will increase of about 934,000 to over one million per year in the year 2010 to 2020 (2). These numbers indicate the clinical pivotal role for a successful treatment of sepsis and septic shock.

The pathophysiologic cascade is mediated by pro-inflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6), macrophage inhibitory factor (MIF) or tumor necrosis factor (TNF) that are potentially detrimental (3, 4, 5). Pro-inflammatory mediators are balanced by anti-inflammatory cytokines, such as interleukin-2 (IL-2), interleukin-4 (IL-4), or interleukin-10 (IL-10) (6, 7). Recently it was shown that the expression and synthesis of pro- and anti-inflammatory cytokines are controlled by several pro- and anti-inflammatory signal transcription factors. Signal transducer and activator of transcription (STAT-3 and -5) and CCCAT-enhancer binding protein-β (C/EBP-β) belong to the pro-inflammatory signal transcription factors (8, 9, 10). Suppressors of cytokine signaling (SOCS-1, -2, -3) belong to the group of signal transcription factors that suppresses pro-inflammatory cytokine expression (11, 12, 13). RANTES (regulated on activation, normally T cell-expressed and secreted) exerts an indifferent role during the inflammatory cascade (13). Recently the group of Tracey et al. showed that high mobility group 1 protein (HMG-1) is a late mediator of lethality during a septic state and possibly involved in the clinical outcome of septic patients (14).

The magnitude and duration of the systemic inflammatory response syndrome (SIRS) determines the development and degree of tissue damage, multi organ failure or even death (14, 15). Despite recent advances in the understanding of the molecular cascade of the systemic inflammatory response syndrome the lack of an effective treatment still remains a clinical problem. Intensive insulin therapy was shown to decrease mortality in critically ill patients (16). Insulin given at doses to maintain blood glucose below 110 mg/dl prevented the incidence of multi organ failure and thus improved clinical outcome and

rehabilitation of mainly patients undergoing thoracic surgery (16). Insulin administration furthermore decreased the incidence of sepsis and septic events in these patients (16). The mechanisms by which insulin improves survival in critically ill patients are not known. Insulin was shown to alter inflammatory mediators and improve liver morphology and function after a thermal injury (17, 18). But hepatic signaling and cytokine synthesis are different during the aftermath of a burn and endotoxemia (19). Therefore the aim of the present study was to determine whether insulin administration modulates the systemic inflammatory response during endotoxemia.

Material and Methods

Male Sprague-Dawley rats (350-375 g) were placed in wire-bottom cages housed in a temperature-controlled room with a 12-hour light-dark cycle. Rats were acclimatized to their environment for 7 days before the study. All rats received water ad libitum throughout the study. Rats were equal for gender (all males), weight (350-375 grams) and age. Prior to the study animals were randomly divided into one of the experimental groups, to either receive an intraperitoneal injection of LPS of 3 mg/kg body weight plus insulin at a dose of 5 International Units/kg body weight s.c. injected (n=32, LPS+insulin), or intraperitoneal injection of LPS of 3 mg/kg body weight plus NaCl s.c. injected (n=32, LPS). Animals were sacrificed at 1, 2, 5 and 7 days after LPS injection by an overdose of anesthesia. Blood was collected by punction of the Vena cava inferior and centrifuged at 1000 g for 15 minutes. The serum was stored at -73^{o}C. Samples of liver were harvested, fragmented, snap-frozen in liquid nitrogen, and stored at -73^{o}C for analysis.

Twelve rats received no injury, no treatment, no anesthesia, and no analgesia. Rats received the same chow and were pair-fed to the treatment animals. Animals were sacrificed at the same time points as treated animals 1, 2, 5 and 7 days. These rats served as non-endotoxemic-untreated, time-matched sham rats to establish baseline levels in the present study.

LPS at a dose of 3 mg/kg body weight was chosen, because this represents half of the LD 50 for LPS, but induces a severe endotoxemic reaction. The insulin used was protamine-insulin (Berlininsulin H, Berlin-Chemie AG, Berlin, Germany), a form of insulin that is released over a 24 hour period. The dose of 5 IU/kg body weight was determined in a dose response study measuring the effect of various insulin doses on IL-1β and TNF-α (data not shown).

Nutrition

Rats were fed with a standard rat diet composed as follows: 64% carbohydrates, 5% fat and 14% protein (Teklad 2014, Harlan, USA). The average food intake was 26±3 grams per day. Both groups of rats were pair-fed according to the caloric intake. The feeding protocol was as follows: 25 calories on the day of endotoxemia, 51 calories on the first day, 76 calories on the second, and 101 calories from the third day on. It was ensured that the nutritional intake was the same in all groups.

Serum glucose and electrolytes

Serum glucose and electrolytes levels were determined by standard laboratory techniques (Böhringer, Ingelheim, Germany).

Hepatic signal transcription factor mRNA and cytokine mRNA expression

Isolation of RNA: Total RNA was prepared from rat liver samples according to the method of Chomcyznski and Sacchi using Trizol reagent (Gibco BRL, Gaithersburg, USA) and analysis was performed as previously published (19, 20). Total RNA was quantified spectroscopically (OD 260 nm) or fluorometrically using Pico-green-dye, and equilibrated to an absolute quantity of 0.5 µg/µl. Subsequently Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was performed. Total liver RNA (0.5 µg) was introduced to synthesize cDNA in a 60 µl reaction mixture using 2.5 µM random hexamers (Amersham Pharmacia, Freiburg, Germany) and Superscript II reverse transcriptase (Gibco BRL). The following primers were used to amplify specific rat transcripts for:

18S rRNA (QuantumRNA, Ambion, Austin, USA) (488 bp) forward 5' - TCAAGAACGAAAGTCGGAGG-3', reverse 5'-GGACATCTAAGGGCATCACA-3'.

C/EBP-ß (according to EMBL Acc. Nr. M84011) (190 bp) forward 5'-GAGCGACGAGTACAAGA-3'; reverse 5'-CTGCTTGAACAAGTTCCG-3'.

RANTES (according to EMBL Acc. Nr. U06436) (178 bp) forward 5'-TGCCTCCCCATATGGCTC-3', reverse 5'-AACCCACTTCTTCTCTGGGTTG-3'.

STAT-3 (according to EMBL Acc. Nr. X91810) (436 bp) forward 5'–TGGACCAGATGCGGAGAAG, reverse 5'–AATTTGACCAGCAACCTGAC.

STAT-5 (according to EMBL Acc. Nr. X91988) (317 bp) forward 5'-TCATCATCGAGAAGCAGCC-3', reverse 5'-TTCCGTCACAGACTCTGCAC-3'.

SOCS-3 (according to EMBL Acc. Nr. AJ249240) (300 bp) forward 5'-AAGACCTTCAGCTCCAAGAGC-3',reverse 5'- CTTGAGTACACAGTCAAAGCGG-3'.

IL-1 (305 bp) forward 5' - CTTCCTTGTGCAAGTGTCTGAAGC - 3', reverse 5' – AAGAAGGTCCTTG GGTCCTCATCC - 3'.

IL-6 (559 bp) forward 5' - AGCCCACCAGGAACGAAAGTC - 3', reverse 5' – TGGAAGTTGGGGTA GGAAGGA - 3'.

TNF (209 bp) forward 5’ – TGCCTCAGCCTCTTCTCATT – 3’, reverse 5’ – GCTTGGTGGTTTG CTACGAC – 3’.

MIF (470 bp) forward 5' - CGGCCGTCGTTCGCAGTCTC - 3', reverse 5' – CCGGAAGGTGGCC ATCATTACG - 3'.

IL-2 (190 bp) forward 5' - CAGCGTGTGTTGGATTTGAC - 3', reverse 5' – TGATGCTTTGACA GATGGCTA - 3'.

IL-10 (417 bp) forward 5' - GAACCACCCGGCGTCTAC - 3', reverse 5' – AGGGATGAGGG CAAGTGAAA - 3'

IFN-γ (292 bp) forward 5' - GGCAAAAGGACGGTAACACGA - 3', reverse 5' – CGACTCCTTTTCC GCTTCCTT - 3'.

The predicted size of each RT-PCR product is assigned in parentheses. Each PCR was initially performed in a thermal cycler (Biometra, Göttingen, Germany) as previously described using standardized amplification programs. Five µl of each reaction was subsequently subjected to agarose gel electrophoresis followed by ethidium bromide staining. Absolute transcript concentrations were quantified introducing external cDNA standards by use of a real-time PCR cycler (Light Cycler, Roche Diagnostics, Mannheim, Germany). Each gene-specific standard was prepared using the corresponding gel-purified amplicon followed by a spectroscopic nucleic acid concentration determination. After serial dilutions of resulting DNA standards final sensitivity levels between 0.1 pg and 1 ng specific transcript per sample were performed during real-time PCR as follows: Using 1 µl of each cDNA the Master SYBR Green protocol was performed (Roche Diagnostics, Mannheim, Germany) in 10µl sample volume in glass capillaries using the experimental conditions as follows: a) 95°C 10 min pre-incubation, b) amplification 95°C 5 sec, 55°C 10 sec with fluorescence detection, 72°C 18 sec, 45 cycles, c) melting curve: 94°C 10 sec, 50°C 60 sec, than 0.1°C/sec up to 90°C under continuous fluorescence detection. Confirmation of each amplicon identity was obtained through melting curve analysis as well as by sequencing of resulting RT-PCR products (TOBLAP, Munich, Germany). Sequence analysis of each PCR product confirmed a 100% homology to the published rat sequences. As negative controls, water instead of RNA was used.

Hepatic and serum cytokine protein concentration

Hepatic and serum pro-inflammatory cytokines IL-1β, IL-6, MIF and TNF-α, and anti-inflammatory cytokines IL-2, IL-4 and IL-10 were determined by enzyme linked immunosorbent assay (R&D Systems Inc, Minneapolis, MN). Serum was used either pure or diluted, and measurements performed according to kit guidelines. Liver was completely homogenized in a lysis buffer (HEPES, sucrose, CHAPS, DTT, PMSF, Leupeptin, EDTA, Pepstatin) in a ratio 1:6. The exact concentration was HEPES 100 mM, Succrose 10%, CHAPS 0.1%. On 10 ml of this solution one tablet Complete Mini was added and stored on ice. After homogenization samples were centrifuged at 4^{o}C, 14000 rpm for 10 minutes. The clean supernatant was then used to determine cytokine protein concentration.

Serum HMG-1 was determined by pooling the sample according to time points and treatment groups. Samples were then analyzed by Western blotting (SDS-page gel) as described (3). Briefly, serum samples were ultrafiltered with Centricon 100 (milipore). The elute was fractionated by SDS/Page, transferred to a polyvinylidene difluoride immunoblot membrane (Bio-Rad) and probed with either specific HMG-1 antiserum (Dr. KJ Tracey, 1:250 dilution) or purified IgG from anti-HMG-1 antiserum (Dr. KJ Tracey, 5µl/ml) for Western blot analysis. Polyclonal anti-HMG-1 IgG was purified by using protein A agarose according to the manufacturers instruction (Pierce). Western blots were scanned with a silver image scanner (Silverscanner II, Lacie Limited, Beaverton, OR), and the relative band intensity was quantified by the NIH IMAGR 1.59 software. The levels of HMG-1 were determined by reference to standard curves generated with purified HMG-1. The determination of HMG-1 was performed at Dr Kevin J Tracey's laboratory, North Shore, Long Island, USA.

Ethics and Statistics

These studies were reviewed and approved by the Animal Care and Use Committee assuring that all animal received humane care. Statistical comparisons were made by analysis of variance (ANOVA) and Student's t-test with Bonferroni's correction. Data are expressed as mean±standard deviation (SD) or means±standard error of the mean (SEM), where appropriate. Significance was accepted at $p<0.05$.

Results

Serum glucose and electrolytes

Insulin administration at a dose of 5 units/kg body weight did not change blood glucose concentration compared to endotoxemic animals receiving normal saline (Table 1). Furthermore, there were no significant differences in serum sodium, potassium, calcium and phosphate levels between endotoxemia and insulin at one, two, five and seven days after the induction of endotoxemia (Table 1).

Hepatic signal transcription factor mRNA expression

Induction of endotoxemia by LPS administration caused an immediate increase in hepatic C/EBPβ mRNA concentration. Insulin significantly decreased hepatic mRNA expression on day one, two and five after LPS administration when compared to endotoxemia, $p<0.05$ (Figure 1a). Endotoxemia caused a 10 to 15 fold increase in hepatic STAT-3 mRNA expression compared to normal animals. Insulin treated animals demonstrated significantly decreased hepatic STAT-3 mRNA levels, which were two days after the induction of endotoxemia in the normal range. Insulin decreased significantly hepatic STAT-3 mRNA expression at 1, 2, and 5 days after LPS administration compared to endotoxemia, $p<0.05$ (Figure 1b). Similar to STAT-3, insulin decreased hepatic STAT-5 mRNA levels. Insulin administration significantly decreased hepatic STAT-5 mRNA on days 1, 2 and 5 after LPS administration when compared to endotoxemic animals, $p<0.05$ (Figure 1c).

Hepatic RANTES mRNA levels were increased by two fold after LPS injection. Insulin decreased hepatic RANTES mRNA at days 1, 2 and 5 after LPS injection compared to endotoxemic animals, $p<0.05$ (Figure 1d). Hepatic SOCS-3 mRNA levels were increased after LPS injection in the endotoxemia and insulin group compared to normal (Figure 1e). Insulin treatment significantly increased hepatic SOCS-3 mRNA expression at 5 and 7 days after LPS when compared to endotoxemia, $p<0.05$ (Figure 1e).

Hepatic cytokine mRNA expression

Endotoxemia caused an increase in hepatic IL-1β mRNA expression in the LPS and the LPS+insulin group. Insulin administration, however, significantly decreased hepatic IL-1β mRNA at days one and two after LPS injection when compared to endotoxemic rats receiving no insulin, $p<0.05$

(Figure 2a). Hepatic IL-6 mRNA expression increased after LPS injection compared to normal values. Insulin treatment decreased hepatic IL-6 mRNA concentration on days 2 and 7 after LPS injection compared to endotoxemia, $p<0.05$ (Figure 2b). Insulin administration increased hepatic IL-10 mRNA expression on day 1 after LPS injection compared to controls, $p<0.05$ (Figure 2c). LPS caused an increase in hepatic TNF-α mRNA throughout the entire study period (day 1: LPS: 0.05±0.004, LPS+insulin: 0.06±0.011, normal: 0.001±0.0008; day 2: LPS: 0.027±0.003, LPS+insulin: 0.024±0.002, normal 0.002+0.0003; day 5: LPS: 0.016±0.003, LPS+insulin: 0.014±0.003, normal: 0.006±0.003; day 7: LPS: 0.02±0.0032, LPS+insulin: 0.024±0.0035, normal: 0.008±0.004). There were no significant differences between endotoxemia and insulin for hepatic mRNA expression of TNF-α. Hepatic MIF mRNA was increased with LPS administration (day 1: LPS: 0.023±0.0021, LPS+insulin: 0.025±0.0025, normal: 0.0062±0.0003; day 2: LPS: 0.025±0.0055, LPS+insulin: 0.034±0.004, normal 0.0068+0.0003; day 5: LPS: 0.038±0.0049, LPS+insulin: 0.033±0.006, normal: 0.0066±0.0003; day 7: LPS: 0.04±0.0091, LPS+insulin: 0.044±0.006, normal: 0.0067±0.004). There were no significant differences between endotoxemia and insulin for hepatic mRNA expression of MIF. Hepatic IFN-γ mRNA decreased with LPS administration (day 1: LPS: 0.017±0.003, LPS+insulin: 0.012±0.003, normal: 0.07±0.02; day 2: LPS: 0.022±0.006, LPS+insulin: 0.017±0.003, normal 0.09+0.04; day 5: LPS: 0.022±0.008, LPS+insulin: 0.015±0.002, normal: 0.10±0.05; day 7: LPS: 0.024±0.0054, LPS+insulin: 0.019±0.0021, normal: 0.09±0.04). There were no significant differences between endotoxemia and insulin for hepatic mRNA expression of IFN-γ. Hepatic IL-2 mRNA decreased in the LPS and LPS+insulin group. IL-2 mRNA was almost 10 folds decreased compared to normal IL-2 throughout the entire study period. There were no significant differences between LPS vs. LPS+insulin.

Hepatic cytokine protein concentration

LPS administration increased hepatic IL-1β protein concentration by 20 fold. Insulin administration significantly decreased hepatic IL-1β expression on days 2 and 5 after induction of endotoxemia compared to the endotoxemic animals, $p<0.05$ (Figure 3a). Hepatic TNF-α protein concentration demonstrated a different pattern. TNF-α demonstrated an increase over the study period and reached its maximum in the endotoxemia and insulin group seven days after LPS application. Insulin decreased hepatic TNF expression on day one after LPS application compared to endotoxemia, $p<0.05$

(Figure 3b). Endotoxemia increased hepatic IL-6 protein concentration immediately after LPS injection. Insulin decreased hepatic IL-6 protein on days one and two after LPS injection compared to endotoxemia, $p<0.05$ (Figure 3c). Hepatic MIF concentration was increased after LPS administration. Insulin decreased hepatic MIF expression two days after LPS administration compared to endotoxemia, $p<0.05$ (day 2: LPS: 294±8 pg/ml homogenate, LPS+insulin: 200±10 pg/ml homogenate). There were no significant differences between LPS and LPS+insulin at other time points.

Hepatic IL-10 protein concentration remained in the normal range until 2 days after LPS administration. Insulin increased hepatic IL-10 protein concentration on days 2 and 7 after LPS injection, $p<0.05$ (Figure 3d). Hepatic IL-4 protein concentration was decreased after LPS application until 5 days after the injection. Insulin significantly increased hepatic IL-4 concentration 7 days after LPS application, $p<0.05$ (Figure 3e). Hepatic IL-2 protein concentration was decreased in both groups LPS and LPS+insulin during the first 5 days after LPS administration, but normal levels at day 7 after LPS. There was no significant difference in hepatic IL-2 protein concentration between LPS compared to LPS+insulin.

Serum cytokine concentration

Serum IL-1β concentrations increased during the first day after induction of endotoxemia. Levels demonstrated a rapid decrease and reached normal range seven days after LPS induction. Insulin significantly decreased serum IL-1β concentration on days one, two and five after LPS injection when compared with endotoxemic animals receiving saline, $p<0.05$ (Figure 4a). LPS injection caused an increase in serum TNF-α concentration on days 1 through 7. Insulin attenuated the increase of serum TNF-α on days one, two, five and seven and significantly decreased serum TNF-α when compared with endotoxemic rats, $p<0.05$ (Fig. 4b). In addition, insulin significantly decreased serum IL-6 concentrations on day one, two and seven after LPS compared to controls, which were almost 300 times elevated above normal levels, $p<0.05$ (Fig. 4c). Macrophage inhibitory factor was elevated immediately after LPS injection. Insulin significantly decreased serum MIF two and seven days after endotoxemia, $p<0.05$ (Fig. 4d).

In order to determine the effect of insulin on the systemic homoeostasis, we measured anti-inflammatory cytokines, IL-2, IL-4 and IL-10. Similar to pro-inflammatory cytokines, anti-inflammatory

cytokines IL-2 and IL-10 were elevated after endotoxemia, while IL-4 was found to be decreased after LPS injection. The anti-inflammatory cytokine IL-10 increased immediately after LPS application in both groups. Insulin treated animals showed a significant increase of IL-10 immediately after endotoxemia induction, $p<0.05$ (Figure 4e). There were no significant difference between insulin and endotoxemia for serum levels of IL-2 and Il-4. Insulin increased dose and time dependent anti-inflammatory cytokines and decreased pro-inflammatory cytokines. Thus, ratios of pro-inflammatory to anti-inflammatory cytokines, which are predictors for organ function and systemic homoeostasis significantly improved towards normal in the insulin treatment group.

Serum HMG-1 protein concentration was found to be increased after LPS injection. On day one after LPS administration HMG-1 were higher in endotoxemic rats receiving insulin, but beginning day two, serum HMG-1 was 2-3 folds lower in the insulin treated group when compared to the endotoxemic group receiving saline (Figure 5).

Discussion

Sepsis and septic shock represent clinical pathophysiologic states for which a sufficient therapy is not existent. Therefore sepsis is associated with a high mortality. One major fragment of the fatal cascade is thought to be the inflammatory response syndrome. The systemic inflammatory response after trauma leads to protein degradation, catabolism and hypermetabolism. As a consequence the structure and function of essential organs, such as muscle, heart, immune system and liver are compromised and contribute to multi organ failure and mortality (21, 22). The magnitude and duration of the systemic inflammatory response syndrome (SIRS) determines the development and incidence of tissue damage, multi organ failure or even death (14, 15). Research approaches encompassed the attenuation of the inflammatory response using anti-inflammatory agents or antibodies against pro-inflammatory cytokines such as tumor necrosis (TNF), interleukin-1β (IL-1β), or their receptors (15, 23, 24). These approaches showed promising results *in vitro* and in animal models by increasing survival rates in the state of septicemia (15, 23, 24). However, when these approaches entered clinical trials the promising animal data couldn't be found in humans. Over the last two years two large clinical studies demonstrated success in the treatment of sepsis or even septic shock, the treatment with activated protein C and the adjustment of blood glucose levels with the administration of insulin (16, 25). Van den Berghe et al. showed that insulin administration at a dose that kept blood glucose below 110 mg/dl decreased early and late mortality in critically ill patients, mainly patients who underwent thoracic surgery (16). The authors showed further that insulin prevented the incidence of multi organ failure in patients with a septic focus. Our group found that insulin administration decreased pro-inflammatory cytokines and intracellular signals and increased anti-inflammatory cytokines and intracellular signals after a thermal injury (17). These data indicated that insulin may act as an anti-inflammatory agent. The effects of insulin during endotoxemia have not been defined. Therefore, the purpose of the present study was to investigate the effect of insulin on the systemic inflammatory response and hepatic signal transcription factors after the induction of an endotoxemic state with intraperitoneal LPS administration.

Insulin decreased pro-inflammatory mediators IL-1, IL-6, and TNF, as well as MIF. At the same time, insulin significantly increased anti-inflammatory cytokines IL-2, IL-4 and IL-10. It has to be mentioned, that IL-4 was only determined as a protein and not as hepatic mRNA as the IL-4 primer to

quantify mRNA did not result in valid and reproducible data. Clinical studies demonstrated that non-survivors with pancreatitis had increased interleukin-6 (IL-6) to interleukin-10 (IL-10) ratios when compared with survivors (26). Our group found that ratios of pro-inflammatory to anti-inflammatory cytokines correlate with organ function and can be used as predictors for mortality in severely pediatric burn patients (27). Hence, by decreasing pro-inflammatory and increasing anti-inflammatory cytokines insulin equilibrates the balance between pro- and anti-inflammatory cytokines and may improve organ function and mortality after trauma.

Insulin decreased serum IL-1, TNF-α, IL-6, MIF and increased IL-10 and IL-4. In the liver we found that insulin decreased IL-1, TNF-α, IL-6 and increased IL-10 and IL-4 at a protein level. However, insulin affects only the hepatic mRNA expression of IL-1, IL-6 and IL-10. Thus it appears that for these cytokines (IL-1, IL-6 and IL-10) insulin acts at a pre-transcriptional level and through intracellular signal transcription factors. As for hepatic TNF only the protein concentration was affected, and not the mRNA expression, it seems likely that either insulin modulates TNF at a post-transcriptional level, or insulin affects the cytokine synthesis and release in other organs, which lead to a decreased concentration in the circulation and subsequently in the liver. We did not determine cytokine concentration in other organs therefore we cannot answer this question.

High mobility group 1 protein (HMG-1) was named for its rapid migration properties on electrophorectic gels (3). HMG-1 is a member of the nonhistone chromatine-associated proteins (3). Intracellular HMG-1 has been studied for its function in binding DNA and stabilizing nucleosome formation, extracellular HMG-1 was recently shown as a late mediator of delayed endotoxin lethality (3, 14). During lethal endotoxemia in mice serum HMG-1 increased 16-36 hours after LPS administration. Lethality could be improved by administering anti-HMG-1 antibodies (3, 14). In critically ill patients HMG-1 levels were increased, but even more in non-survivors (14). Furthermore in animal studies it was shown that attenuating HMG-1 levels, e.g. with the administration of acetylcholine, improved survival (28). In the present study we showed that insulin decreased extracellular serum HMG-1 levels in endotoxemic rats starting from day 2 and therefore decreased HMG-1 levels indicate possible improved outcome with the administration of insulin.

In order to determine whether insulin exerts its effects on cytokines through changes in blood glucose or directly by modulating signal transcription factors, we measured hepatic signal transcription factor mRNA expression. We found that insulin alters the intracellular signal cascade in the liver. Insulin decreased the pro-inflammatory signal transcription factors STAT-3, STAT-5, and C/EBP-β. An up-regulation of these transcription factors has been associated with impaired organ function and protein synthesis, such as albumin (8, 9, 10). Therefore, it appears that insulin improves organ function and protein synthesis during the hypermetabolic response through these signal transcription factors. In addition to pro-inflammatory transcription factors we determined factors that were identified to either suppress cytokine signaling (SOCS) or regulate the T-cell function (RANTES). Members of the SOCS family of proteins play key roles in the negative regulation of cytokine signal transduction, by acting in a negative feedback loop and inhibiting the cytokine-activated Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway to modulate cellular responses (29). Direct interaction of SOCS SH2 domains with the JAK kinases or cytokine receptors allows their recruitment to the signaling complex, where they inhibit JAK catalytic activity or block access of the STAT's to receptor binding sites (29). As we have shown that insulin decreased STAT-5 and increased SOCS-3, major players during the aftermath of a thermal injury (11), it remains to be defined whether insulin decreases cytokines, STAT-3, STAT-5 and C/EBP-β in a direct fashion or indirectly through SOCS-3.

RANTES (regulated upon activation, normal T cell-expressed and secreted) is a member of a large super gene family of pro-inflammatory cytokines called CC chemokines that appear to play a fundamental role in inflammatory processes. Although expression of RANTES was first thought to be limited to activated T cells, recent data have shown that it is produced by a variety of tissue types in response to specific stimuli. Deletion analysis of the promoter region indicates that different transcriptional mechanisms control expression of RANTES in the various tissues studied. Post trauma the immune system plays a crucial role for survival and clinical outcome (13). Growth factors, such as growth hormone can affect the Th-1 and Th-2 by restoring the Th-1 response and improve the immune system after a thermal injury (29). We did not define the function of the immune system in the present study, however, it appears that insulin may have some beneficial effects by modulating the T-Cell

response as insulin significantly increased hepatic RANTES mRNA expression seven days after endotoxemia.

It was very surprising that insulin at the dose administered in the present study did not alter glucose levels. In previous experiments we used the same insulin dosage and we found that insulin significantly decreased blood glucose levels (17). This leads us to hypothesize that the glucose metabolism must be different during sepsis and the hypermetabolism post burn or the dose of insulin chosen in this study was not sufficient to significantly reduce insulin resistance during endotoxemia. It was furthermore surprising that glucose levels were decreased in the LPS and LPS plus insulin group on the first day after the induction of endotoxemia and not increased. One has to mention that we measured glucose with large time intervals and may miss slight and temporal changes that may occur during the early period after LPS and insulin administration. On the other hand during the stress response insulin levels are usually normal or decreased, despite the peripheral insulin resistance (30, 31). Changes in whole-body glucose uptake and glucose oxidation in sepsis are complex and dependent on the severity of illness and the stage of the disease. Whole-body glucose uptake and glucose oxidation are increased during the early stages of endotoxemia and sepsis (32, 33). This results from a cytokine-induced increase in non-insulin mediated glucose uptake by tissue that encompass high concentrations of mononuclear phagocytes, including the liver, lung, spleen and ileum (34, 35). This could explain why in our study glucose levels were decreased at an early time point. During the endotoxemic and septic phases the insulin resistance increases with a decrease of glucose utilization oxidation leading to hyperglycemia.

Stress related hyperglycemia and insulin resistance are almost universal findings in patients with sepsis (36). The pathophysiologic causes of this response are probably pro-inflammatory mediators and stress-related hormones (36). Hyperglycemia is per se pro-inflammatory however it appears also in light with the results presented in the present study that insulin exerts anti-inflammatory properties (37). We showed that insulin attenuated the inflammatory response by decreasing pro-inflammatory and increasing anti-inflammatory cytokines, thus, restoring systemic homeostasis. Based on our data we hypothesize that the anti-inflammatory effect of insulin is probably due to modulation of cellular signal transcription factors rather than through changes in blood glucose and metabolism. We suggest that insulin may represent an important and safe therapeutic option in the treatment of critically ill patients.

Acknowledgements

The authors like to thank Kevin J Tracey for the HMG-1 measurements and valuable suggestions for the manuscript.

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Figure legends

Figure 1: Hepatic mRNA expression of signal transcription factors quantified by light cycler analysis. (a) Hepatic C/EBP-β mRNA expression increased after induction of endotoxemia and remained elevated during the study period. Insulin decreased hepatic C/EBP-β mRNA expression on the first, second and fifth day after LPS injection, $p<0.05$. (b) Hepatic mRNA expression of STAT-3 increased after endotoxemia induction in both groups. Insulin administration decreased hepatic STAT-3 mRNA expression on days one, two and five compared to endotoxemic animals receiving saline, $p<0.05$. (c) Hepatic STAT-5 mRNA expression increased after LPS, however, insulin significantly decreased STAT-5 mRNA expression on days one, two and five when compared to controls, $p<0.05$. (d) Hepatic RANTES mRNA expression increased in the LPS group compared to normals. Insulin decreased hepatic RANTES mRNA at days 1, 2 and 5 compared to endotoxemic rats receiving saline, $p<0.05$. (e) Hepatic SOCS-3 mRNA was found to be increased with LPS. Insulin further increased hepatic mRNA expression of SOCS-3 five and seven days after the induction of endotoxemia, $p<0.05$.

* Significant difference between insulin and control, $p<0.05$. Data presented as means±SEM.

Figure 2 a-d: Hepatic cytokine mRNA expression quantified by light cycler. (a) LPS administration caused an increase in hepatic IL-1β mRNA expression in both groups LPS and LPS+insulin. Insulin administration, however, significantly decreased hepatic IL-1β at days one and two after LPS injection when compared to endotoxemic rats receiving no insulin, $p<0.05$. (b) Hepatic IL-6 mRNA expression increased after LPS injection 100 fold compared to normal values. Insulin treatment decreased hepatic IL-6 mRNA concentration on days 2 and 7 after LPS injection compared to endotoxemia, $p<0.05$. (c) Insulin administration increased hepatic IL-10 mRNA expression on day 1 after LPS injection compared to normals, $p<0.05$.

* Significant difference between insulin and control, $p<0.05$. Data presented as means±SEM.

Figure 3 a-e: Hepatic cytokine protein concentration. (a) LPS administration increased hepatic IL-1β protein concentration by 20 fold. Insulin administration significantly decreased hepatic IL-1β expression on days 2 and 5 after induction of endotoxemia compared to the endotoxemic animals, $p<0.05$. (b) TNF-α demonstrated an increase over the study period and reached its maximum in the

endotoxemia and insulin group seven days after LPS application. Insulin decreased hepatic TNF-α expression on day one after LPS application compared to endotoxemia, $p<0.05$. (c) Endotoxemia increased hepatic IL-6 protein concentration immediately after LPS injection. Insulin decreased hepatic IL-6 protein on days one and two after LPS injection compared to endotoxemia, $p<0.05$. (d) Hepatic IL-10 protein concentration remained in the normal range until 5 days after LPS administration. Insulin increased hepatic IL-10 protein concentration on days 2 and 7 after LPS injection, $p<0.05$. (e) Hepatic IL-4 protein concentration was decreased after LPS application until 5 days after the injection. Insulin significantly increased hepatic IL-4 concentration 7 days after LPS application, $p<0.05$.

* Significant difference between insulin and control, $p<0.05$. Data presented as means±SEM.

Figure 4 a-e: Serum pro- and anti-inflammatory cytokines. (a) Serum IL-1β increased after LPS by almost 20 folds compared to normal. Insulin decreased serum IL-1β concentration on days 1, 2 and 5 after LPS when compared with animals receiving saline, $p<0.05$. (b) Serum TNF-α increased approximately 20 folds after LPS and decreased over time. Insulin prevented an increase of serum TNF-α on days 1, 2, 5 and 7 post endotoxemia when compared with endotoxemic rates receiving saline, $p<0.05$. (c) Serum IL-6 increased immediately after LPS injection. Insulin significantly decreased serum IL-6 concentrations at days 1, 2, and 7 when compared to endotoxemia, $p<0.05$. (d) Macrophage inhibitory factor was found to be increased immediately after LPS. Insulin significantly decreased serum MIF 2 and 7 days after endotoxemia, $p<0.05$. (e) Serum IL-10 was found to be increased after LPS injection. Insulin further increased IL-10 on the first day after LPS, $p<0.05$.

* Significant difference between insulin and endotoxemia, $p<0.05$. Data presented as means±SEM.

Figure 5: Serum HMG-1 protein level. Serum HMG-1 increased immediately after LPS injection. Insulin decreased HMG-1 2, 5 and 7 days after endotoxemia when compared to controls.

Table

Table 1: Serum glucose, sodium, potassium, calcium and phosphate in insulin treated and endotoxemic rats after LPS administration.

	LPS				**LPS+insulin**			
	Day1	**Day 2**	**Day 5**	**Day 7**	**Day1**	**Day 2**	**Day 5**	**Day 7**
Glucose (mg/dl)	130±29	145±5	199±16	179±14	124±78	157±19	198±27	189±53
Sodium(mg/dl)	155±11	150±4	184±35	174±28	156±10	145±2	173±27	175±25
Potassium(mg/dl)	5.9±1.1	8.7±2.9	7.3±1.9	6.5±1.2	7.0±1.1	5.5±0.5	7.4±1.7	7.4±1.9
Calcium(mg/dl)	2.9±0.3	3.4±0.4	3.3±0.6	3.3±0.6	3.1±0.3	3.0±0.3	3.2±0.5	3.4±0.5
Phosphate(mg/dl)	4.3±1.2	3.8±1.1	3.8±0.9	3.3±1.0	5.2±1.2	3.0±0.3	3.7±1.1	3.7±1.0

Data presented as means±SD.

Normal values: Glucose: 161±40 mg/dl, Sodium: 170±20 mg/dl, Potassium: 6.8±0.5 mg/dl, Calcium: 3.5±0.5 mg/dl, Phosphate: 3.5±0.5 mg/dl.

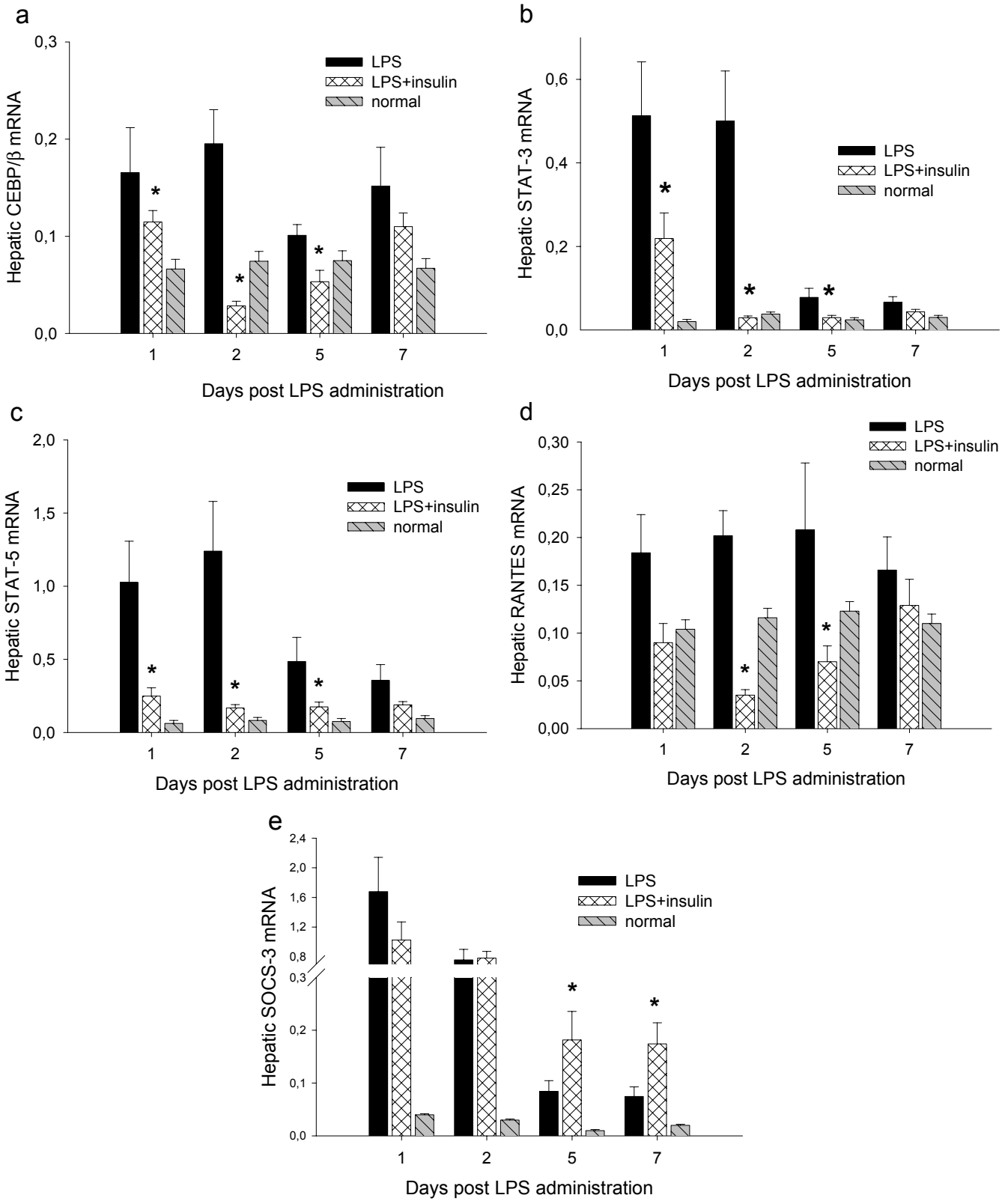
a
Hepatic CEBP/β mRNA
0,3
0,2
0,1
0,0
LPS
LPS+insulin
normal
*
*
*
1
2
5
7
Days post LPS administration
b
Hepatic STAT-3 mRNA
0,6
0,4
0,2
0,0
LPS
LPS+insulin
normal
*
*
*
1
2
5
7
Days post LPS administration
c
Hepatic STAT-5 mRNA
2,0
1,5
1,0
0,5
0,0
LPS
LPS+insulin
normal
*
*
*
1
2
5
7
Days post LPS administration
d
Hepatic RANTES mRNA
0,30
0,25
0,20
0,15
0,10
0,05
0,00
LPS
LPS+insulin
normal
*
*
1
2
5
7
Days post LPS administration
e
Hepatic SOCS-3 mRNA
2,4
2,0
1,6
1,2
0,8
0,3
0,2
0,1
0,0
LPS
LPS+insulin
normal
*
*
1
2
5
7
Days post LPS administration

Figure 1 a-e

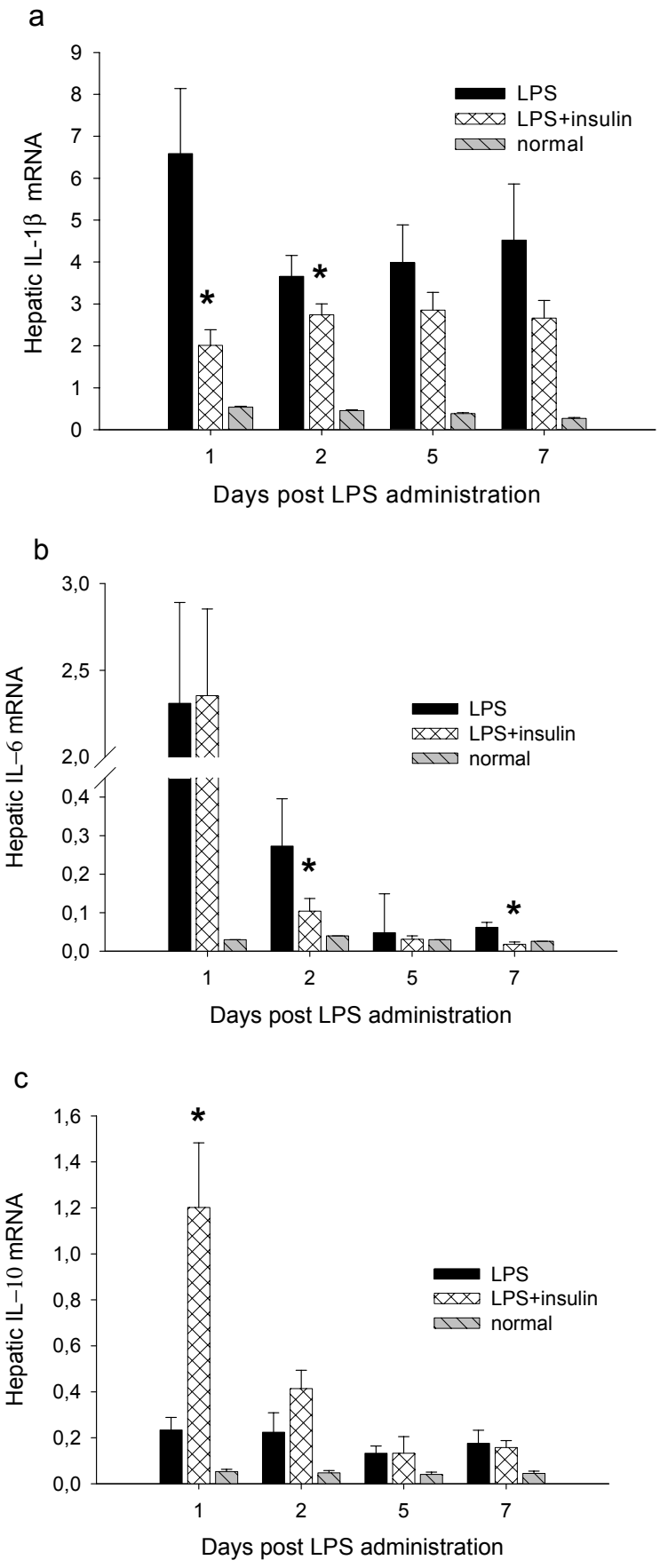
a
Hepatic IL-1β mRNA
LPS
LPS+insulin
normal
Days post LPS administration
b
Hepatic IL–6 mRNA
LPS
LPS+insulin
normal
Days post LPS administration
c
Hepatic IL–10 mRNA
LPS
LPS+insulin
normal
Days post LPS administration

Figure 2 a-c

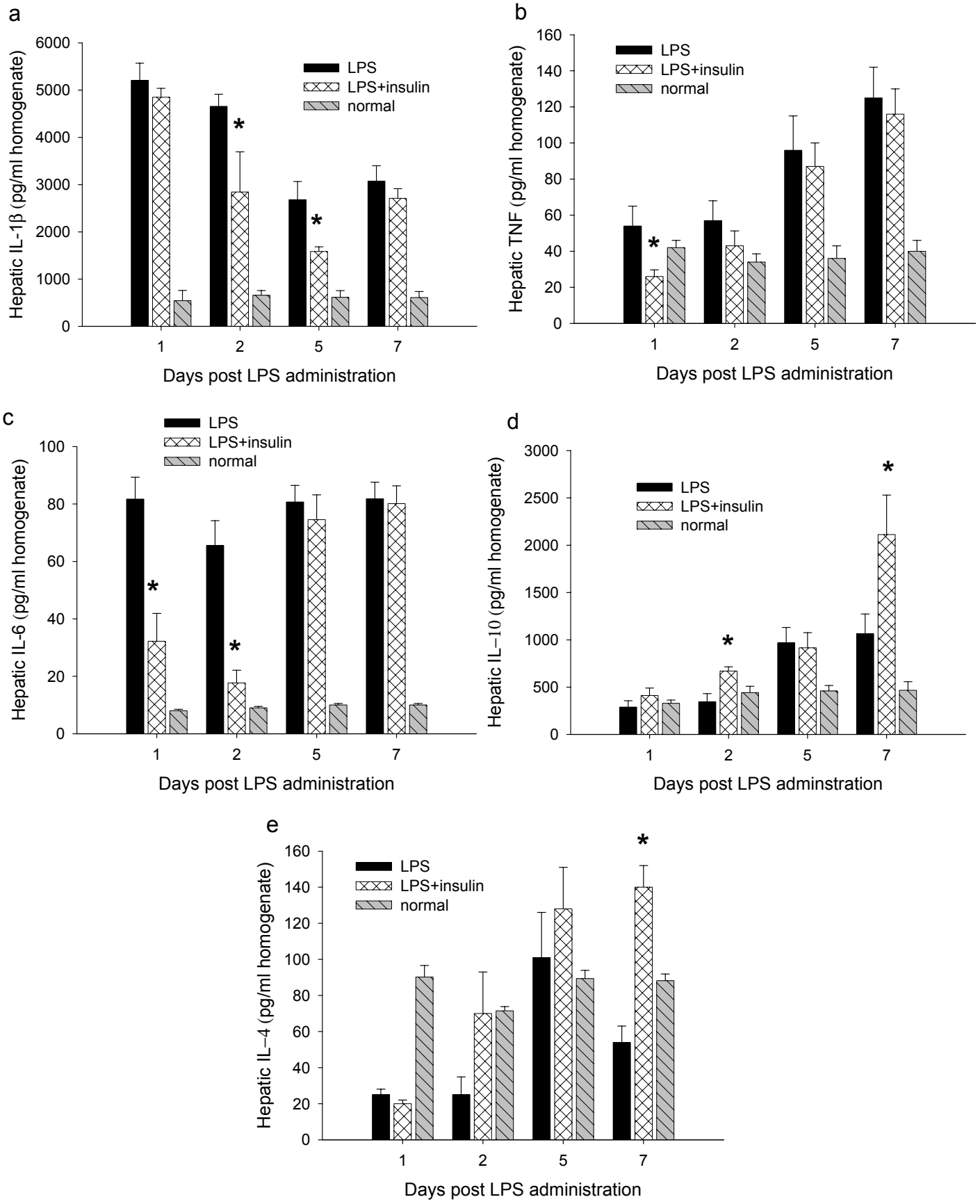
a
Hepatic IL-1β (pg/ml homogenate)
6000
5000
4000
3000
2000
1000
0
LPS
LPS+insulin
normal
*
*
1
2
5
7
Days post LPS administration
b
Hepatic TNF (pg/ml homogenate)
160
140
120
100
80
60
40
20
0
LPS
LPS+insulin
normal
*
1
2
5
7
Days post LPS administration
c
Hepatic IL-6 (pg/ml homogenate)
100
80
60
40
20
0
LPS
LPS+insulin
normal
*
*
1
2
5
7
Days post LPS administration
d
Hepatic IL−10 (pg/ml homogenate)
3000
2500
2000
1500
1000
500
0
LPS
LPS+insulin
normal
*
*
1
2
5
7
Days post LPS adminstration
e
Hepatic IL−4 (pg/ml homogenate)
160
140
120
100
80
60
40
20
0
LPS
LPS+insulin
normal
*
1
2
5
7
Days post LPS administration

Figure 3 a-e

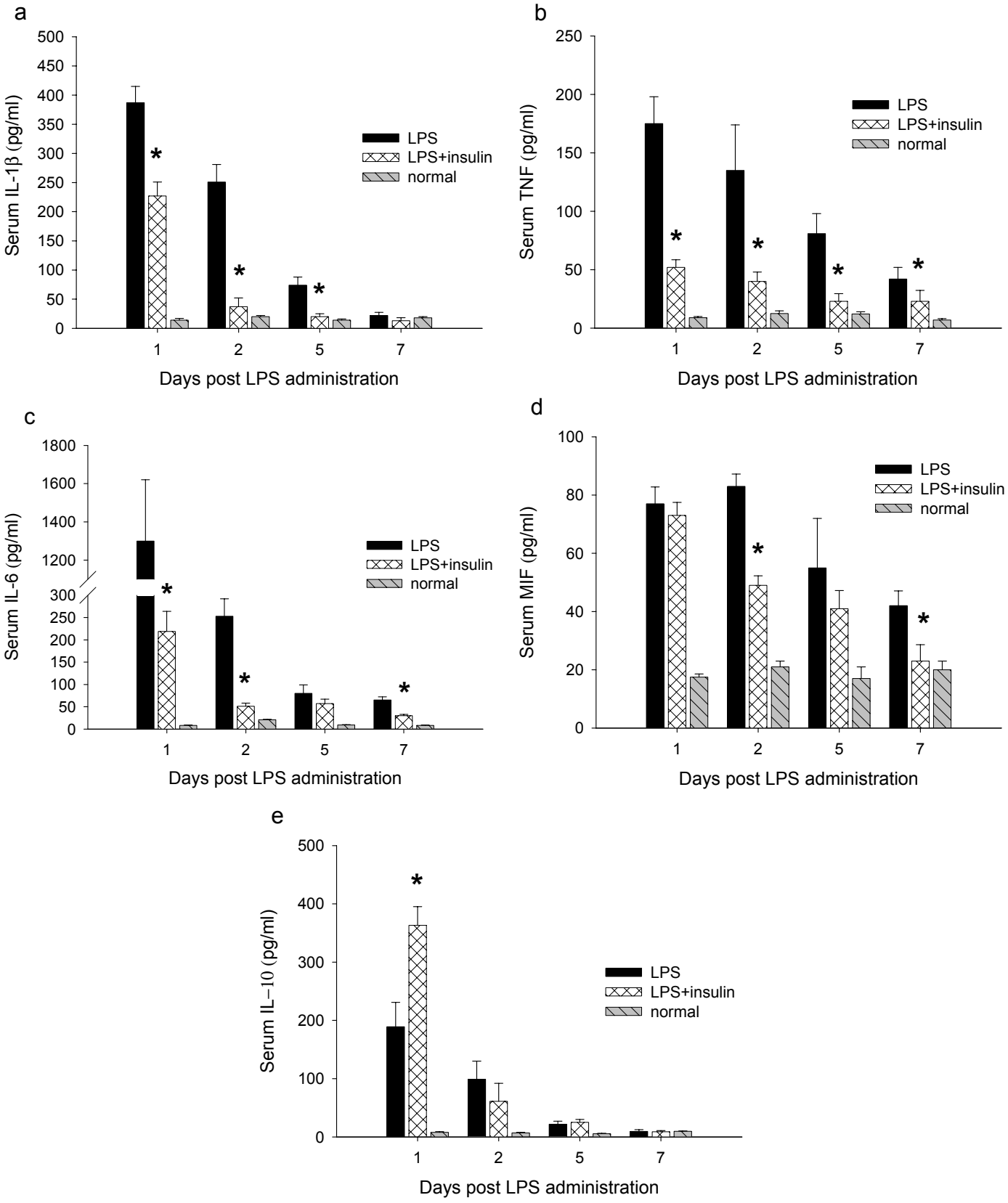
a
Serum IL-1β (pg/ml)
500
450
400
350
300
250
200
150
100
50
0
LPS
LPS+insulin
normal
*
*
*
1
2
5
7
Days post LPS administration
b
Serum TNF (pg/ml)
250
200
150
100
50
0
LPS
LPS+insulin
normal
*
*
*
*
1
2
5
7
Days post LPS administration
c
Serum IL-6 (pg/ml)
1800
1600
1400
1200
300
250
200
150
100
50
0
LPS
LPS+insulin
normal
*
*
*
1
2
5
7
Days post LPS administration
d
Serum MIF (pg/ml)
100
80
60
40
20
0
LPS
LPS+insulin
normal
*
*
1
2
5
7
Days post LPS administration
e
Serum IL−10 (pg/ml)
500
400
300
200
100
0
LPS
LPS+insulin
normal
*
1
2
5
7
Days post LPS administration

Figure 4 a-e

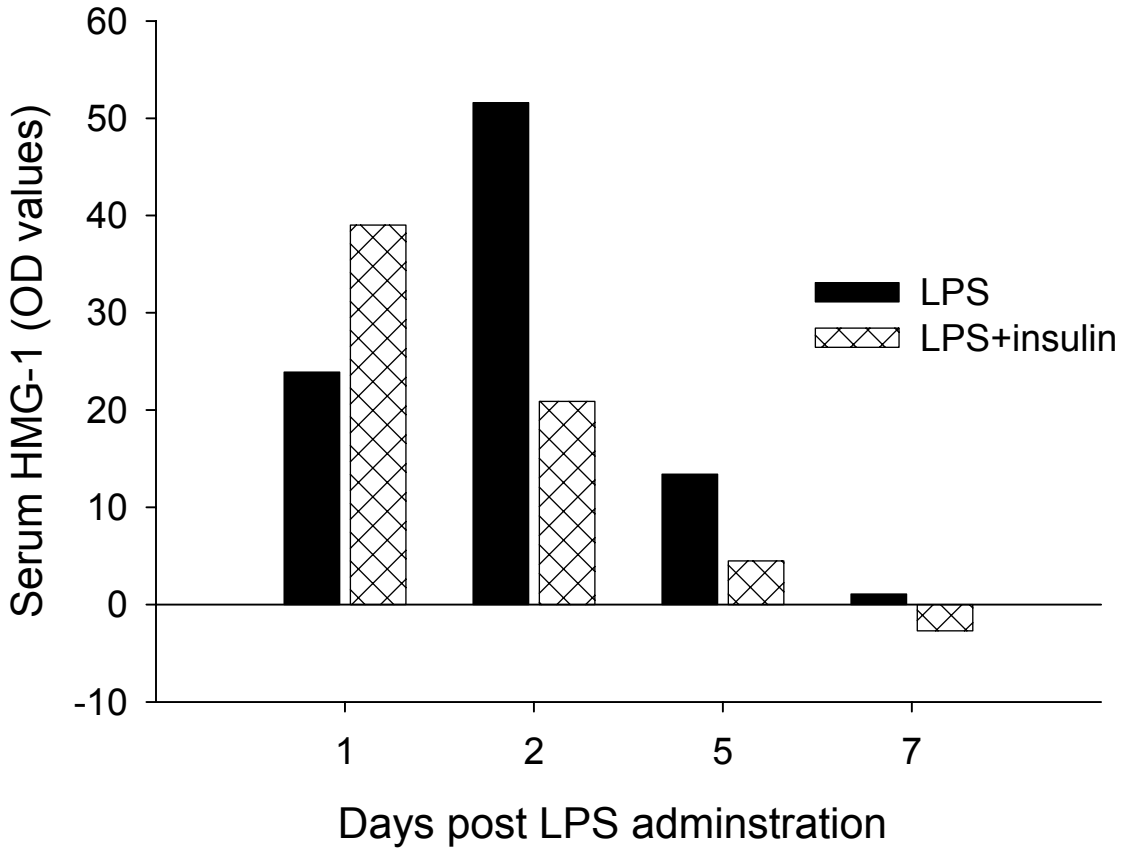
60
50
40
30
20
10
0
-10
Serum HMG-1 (OD values)
LPS
LPS+insulin
1
2
5
7
Days post LPS adminstration

Figure 5