Insulin Treatment Improves Hepatic Morphology and Function Through Modulation of Hepatic Signals After Severe Trauma

Dagmar Klein, PhD, Thomas Schubert, MD,† Raymund E. Horch, MD, PhD,‡ Karl-Walter Jauch, MD, PhD,§ and Marc G. Jeschke, MD, PhD‡*

Objective: The purpose of the present study was to determine the effect of insulin therapy on hepatic function, structure, and hepatic mRNA and protein cytokine expression during the hypermetabolic cascade post burn.

Summary Background Data: Liver function and morphology are crucial for survival of patients suffering from trauma, operations, or infections. Insulin decreased mortality and prevented the incidence of multiorgan failure in critically ill patients.

Methods: Rats received a thermal injury and were randomly divided into the insulin or control group. Our outcome measures encompassed the effect of insulin on hepatic proteins, hepatic pro- and anti-inflammatory cytokines mRNA and proteins, hepatocyte proliferation, including Bcl-2 and hepatocyte apoptosis, with caspases-3 and caspases-9.

Results: Insulin significantly improved hepatic protein synthesis by increasing albumin and decreasing c-reactive protein and fat ($P < 0.05$). Insulin decreased the hepatic inflammatory response signal cascade by decreasing hepatic pro-inflammatory cytokines mRNA and proteins IL-1β and tumor necrosis factor at pretranslational levels. Insulin increased hepatic cytokine mRNA and protein expression of IL-2 and IL-10 at a pretranslational level when compared with controls ($P < 0.05$). Insulin increased hepatocyte proliferation along with Bcl-2 concentration, while decreasing hepatocyte apoptosis along with decreased caspases-3 and -9 concentration, thus improving liver morphology ($P < 0.05$).

Conclusions: Our data provide insight that insulin attenuates the inflammatory response by decreasing the pro-inflammatory and increasing the anti-inflammatory cascade, thus restoring hepatic homeostasis, which has been shown to be critical for organ function and survival of critically ill patients.

(*Ann Surg* 2004;240: 340–349)

The systemic inflammatory response after trauma leads to hypermetabolism and thus protein degradation and catabolism. As a consequence, the structure and function of essential organs, such as the muscle, skin, heart, immune system, and liver are compromised and contribute to multiorgan failure and mortality.[1,2] During the aftermath of these multiple reactions, the liver has been shown to play a crucial role. Under physiologic conditions, the liver synthesizes mainly constitutive hepatic proteins, such as albumin, prealbumin, or transferrin. After trauma the synthesis shifts from constitutive hepatic proteins to acute-phase proteins, such as haptoglobin, α_2-macroglobulin, α_1-acid glycoprotein, and C-reactive protein (CRP).[3] This reaction of the liver is called the hepatic acute-phase response. The goal of the hepatic acute-phase response is to restore homeostasis; however, a prolonged and exaggerated response leads to the enhancement of hypermetabolism and catabolism, thus to increased morbidity and mortality.[4–6] Mediators of the acute-phase response are pro-inflammatory cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), tumor-necrosis factor (TNF), or the anti-inflammatory cytokines interleukin-2 (IL-2), interleukin-4 (IL-4), and interleukin-10 (IL-10).[3] Several studies showed that increased pro-inflammatory cytokine synthesis also contributes to hypermetabolism and catabolism.[7,8]

Insulin at a dose that kept blood glucose below 110 mg/dL decreased mortality and prevented the incidence of multiorgan failure in critically ill patients.[9] In an animal model, insulin had anti-inflammatory effects by decreasing systemic pro-inflammatory mediators.[10] The aim of the present study was to determine the effect of insulin on liver function, structure, and the hepatic acute-phase response, including hepatic cytokine concentrations. Another aim was

From the *Klinik und Poliklinik für Chirurgie, Klinikum der Universität Regensburg, Germany; †Abteilung für Pathologie, Klinikum der Universität Regensburg, Germany; ‡Abteilung für Plastische und Handchirurgie, Klinikum der Friedrich-Alexander Universität Erlangen, Germany; and §Klinik und Poliklinik der Ludwig-Maximillian Universität München, Klinikum Grosshadern, München, Germany.

Reprints: Marc G. Jeschke, MD, PhD, Klinik und Poliklinik für Chirurgie, Abteilung für Plastische Chirurgie und Handchirurgie, Universität Erlangen-Nürnberg, Krankenhausstr. 12, 91054 Erlangen, Germany. E-mail: Mcjeschke@hotmail.com.

Dagmar Klein and Marc G. Jeschke contributed equally to this work.

ISSN: 0003-4932/04/24002-0340

DOI: 10.1097/01.sla.0000133353.57674.cd

to determine whether insulin affects cytokine expression through pretranscriptional or at post-translational levels in the liver.

MATERIALS 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. Each rat received a 30% total body surface area (TBSA) full-thickness scald burn under general anesthesia (pentobarbital 50 mg/kg body weight) and analgesia (buprenorphin 1 mg/kg body weight) following a modified procedure as previously described. Rats were anesthetized, shaved, and received a 30% TBSA scald burn (99°C hot water; water contact 10 seconds to the back). This model ensures that both groups are similar in metabolic rates. After the thermal injury, rats were immediately resuscitated by intraperitoneal injection of Ringer's Lactate (50 mL/kg body weight) and then randomly divided into two groups: burn plus 5 IU/kg insulin s.c. (n = 28) or burn plus NaCl s.c. (n = 28).

Prior to the main experiment, we performed a dose-response study, in which rats received a thermal injury plus 0.5, 1, 2.5, 5, or 10 units/kg insulin s.c. injected in an area of uninjured skin.[10] We found that insulin at a dose of 5 units/kg body weight resulted in the most substantial anti-inflammatory effect and also resulted in a decrease of blood glucose levels. Therefore, we used in the present study 5 IU/kg of insulin. 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. We injected 5 units insulin per kilogram body weight every day in the morning at the same time. The insulin administered was not driven by glucose levels but driven by the results we found in the dose-response study.

Animals were killed at 1, 2, 5, and 7 days postburn. At each time point, 7 animals per group were therefore killed. Blood was collected into serum and plasma separators, spun at 2500g for 15 minutes, and the supernatant and pellet were separated and stored at −80°C. Samples of liver, muscle, and kidney were harvested, snap-frozen in liquid nitrogen, and stored at −80°C for analysis.

Nutrition

Rats were fed with a liquid diet, riche in vitamin, protein, and carbohydrate (Fresubin, Fresenius Medical Care) and had a caloric distribution of 24% protein, 21% fat, and 55% carbohydrate, resulting in an energy intake of 1.01 cal/mL. Both groups of rats were pair-fed according to the caloric intake. The feeding protocol was as follows: 25 calories on the day of burn, 51 calories on the first postburn day, 76 calories on the second, and 101 calories from the third day postburn on. It was ensured that the nutritional intake was the same in all groups.

Normal

Eight rats received no injury, no treatment, no anesthesia, and no analgesia. Rats received the same liquid diet (Fresubin, Fresenius Medical Care, Germany) and were pair-fed to the treatment animals. Animals were killed the same time points as treated animals 1, 2, 5, and 7 days. These rats served as unburned-untreated, time-matched sham rats to establish baseline levels in the present study.

Serum Glucose, Electrolytes, Fat, Constitutive and Acute Phase Protein Synthesis

Serum constitutive protein (albumin), acute-phase-protein (CRP), fat (cholesterol, triglycerides, HDL, LDL, and VLDL), glucose, and electrolytes levels were determined by standard laboratory techniques (Böhringer, Ingelheim, Germany).

Hepatic Cytokine mRNA and Hepatic Cytokine Protein Expression

Hepatic 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 (GibcoBRL, Gaithersburg, MD).[11] 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 (1.0 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 transcription (GibcoBRL). The following primers were used to determine mRNA concentrations:

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

IL-1 (305 bp) forward 5′ - CTTCCTTGTGCAAGTGTCTGAAGC - 3′, reverse 5′ - AAGAAGGTCCTTGGGTCCTCATCC - 3′.

IL-6 (559 bp) forward 5′ - AGCCCACCAGGAACGAAAGTC - 3′, reverse 5′ - TGGAAGTTGGGGTAGGAAGGA - 3′.

TNF (209 bp) forward 5′ – TGCCTCAGCCTCTTCTCATT – 3′, reverse 5′ – GCTTGGTGGTTTGCTACGAC – 3′.

MIF (470 bp) forward 5′ - CGGCCGTCGTTCGCAGTCTC - 3′, reverse 5′ - CCGGAAGGTGGCCATCATTACG - 3′.

IL-2 (190 bp) forward 5′ - CAGCGTGTGTTGGATTTGAC - 3′, reverse 5′ - TGATGCTTTGACAGATGGCTA - 3′.

IL-10 (417 bp) forward 5′ - GAACCACCCGGCGTC-TAC - 3′, reverse 5′ - AGGGATGAGGGCAAGTGAAA - 3′

IFN-γ (292 bp) forward 5′ - GGCAAAAGGACGG-TAACACGA - 3′, reverse 5′ - CGACTCCTTTTCCGCTTC-CTT - 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; 5 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 (LightCycler, Roche Diagnostics, Mannheim, Germany). Each gene-specific standard was prepared using the corresponding gel-purified amplicon followed 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 10 μL sample volume in glass capillaries using the experimental conditions as follows: a) 95°C 10 minutes preincubation, b) amplification 95°C 5 seconds, 55°C 10 seconds with fluorescence detection, 72°C 18 seconds, 45 cycles, c) melting curve: 94°C 10 seconds, 50°C 60 seconds, than 0.1°C/s 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 determining of each PCR product confirmed a 100% homology to the published rat sequences. As negative controls, water instead of RNA was always used.

Hepatic Cytokine Protein Expression

Pro-inflammatory cytokines IL-1, IL-6, MIF, and TNF, and anti-inflammatory cytokines IL-2, IL-4, and IL-10 were determined by enzyme linked immunosorbent assay (R&D Systems, Germany).

Liver was completely homogenized in a lysis buffer (HEPES, sucrose, CHAPS, DTT, PMSF, leupeptin, EDTA, pepstatin) or complete mini-protease inhibitor cocktail (Roche, Mannheim Germany) in a ratio 1:6. After homogenization samples were centrifuged at 4°C, 14,000 U/min for 10 minutes. The clean supernatant was then used to determine cytokine protein concentration.

Hepatic Morphology

Hepatocyte Proliferation and Apoptosis

We used the TUNEL (terminal deoxyuridine nick end labeling) immunohistochemical method (Apoptag, Oncogene, Baltimore, MD) for histologic identification of apoptotic cells in the liver. Formalin-fixed tissues were processed and embedded into paraffin. Sections of 4-μm, obtained at 40 to 50 μm intervals, were deparaffinized, rehydrated in graded alcohol, and washed in deionized water. After washing, the procedure according to the guidelines was performed. In each section, two blinded observers selected 5 fields in 4 different sections (approximately 6000 cells) for counting TUNEL-positive cells. Apoptotic cells were identified as those with a brown staining of the nucleus, or as apoptotic bodies, which are fragments of apoptotic cells engulfed by neighboring epithelial cells. All hepatocytes within the field were counted and apoptosis was expressed as a percentage of apoptotic cells per thousand hepatocytes. Values for all sections were averaged to calculate apoptosis for the liver of each animal.

The balance between proliferation and apoptosis is an important indicator for organ homoeostasis, in this case the liver. Hepatic cell proliferation was determined using antibodies against Ki-67. Ki-67 stains cells that underwent mitosis over the last 24 hours. Paraffin sections 3- to 5-μm thick were mounted on "Superfrost Plus" slides, heated for 20 minutes at 72°C, deparaffinized and rehydrated. Sections were placed in a microwave for 30 minutes at 240 W in a citrate buffer at pH 7.3, then cooled to room temperature. Following antigen retrieval, the slides were rinsed, endogenous peroxidase activity was blocked by using methanolic peroxide, the slides were rinsed again, and primary monoclonal antibody against Ki-67 (BD PharMingen, USA, Catalog no. 36521A) in a dilution of 1:100 was applied. All slides were incubated using a Ventana machine, and each antibody incubation was performed at 37°C, and labeled streptavidin-biotin-peroxidase method at 37°C was used to visualize positive reaction (Ventana Medical Systems basic DAB detection kit, Ventana Medical Systems Inc.). A dark brown nuclear precipitate was evaluated as positive reaction. Sections incubated without primary antibody had no detectable immunoreactivity. Positive cells (stained red-brown) were counted on 2 sections from each animal. In each section, 2 "blinded observers" selected 4 different sections for counting PCNA-positive cells. Proliferating cells were identified as those with a brown staining of the nucleus or cytoplasm. All hepatocytes within the field were counted and proliferation was expressed as a percentage of proliferating cells per hundred hepatocytes. Values for all sections were averaged to calculate apoptosis for the liver of each animal.

To determine net cell balance, which determines net cell turnover, the quotient between proliferation divided by apoptosis was calculated.

Hepatic Caspase-3, -9, and Bcl-2 Expression

Hepatic caspases-3, and -9 and Bcl-2 protein expression was determined by Western blotting. After homogenizing the liver in a lysis buffer the samples were centrifuged and a protein assay performed to determine protein concen-

tration. After assuring equal loading of the samples standard Western blotting techniques were used. Caspase-3, -9, and Bcl-2 antibodies are commercially available (Santa Cruz Biotechnology Inc, Santa Cruz, CA). The blots were stained using ECL and exposed to a membrane. Membranes were scanned in a Western blot reader and bands were traced, normalized and so concentration calculated.

Ethics and Statistics

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

RESULTS

No animal was excluded from the study. There were no significant differences in mortality rate between the two groups.

Serum Glucose, Electrolytes, Fat, Constitutive and Acute-Phase Protein Synthesis

Insulin at a dose of 5 units/kg body weight significantly decreased rat serum glucose levels of approximately 120 mg/dL at all days. Controls demonstrated blood glucose levels at a range of 150 to 170 mg/dL ($P < 0.05$; Table 1). Serum calcium, sodium, potassium, calcium, and phosphate levels, however, were similar in the control and the treatment group 1, 2, 5, and 7 days after the injury.

Serum cholesterol increased immediately after burn in the control and insulin group but decreased toward the end of the study period to normal levels. There were no significant differences between control and insulin (Table 1). Triglycerides also increased immediately after burn, then decreased and increased again at the end of the study period. Insulin administration prevented an increase of triglycerides on the first day after burn when compared with controls ($P < 0.05$; Table 1). Serum HDL levels did not show any change, neither compared with normal levels nor to insulin treatment. Serum LDL and VLDL demonstrated a significant increase after burn. Insulin significantly decreased serum LDL on days 1, 2, and 5 after burn, and serum VLDL on day one after burn when compared with controls ($P < 0.05$; Table 1).

Serum constitutive-hepatic protein albumin decreased immediately after burn trauma and levels remained decreased throughout the entire study period. Insulin increased serum albumin at days 1, 5, and 7 post-trauma when compared with controls ($P < 0.05$; Fig. 1a). In contrast to constitutive-hepatic proteins, acute-phase proteins are increased after trauma. Serum CRP increased after burn and remained elevated at 7 days after burn. Insulin administration significantly decreased serum CRP levels at days 1 and 7 when compared with animals receiving saline ($P < 0.05$; Fig. 1b).

Hepatic Cytokine mRNA and Hepatic Cytokine Protein Expression

Hepatic Cytokine mRNA Expression

Burn injury increased hepatic IL-1β mRNA expression beginning from day 1 on and concentration increased over the 7-day study period. Insulin significantly decreased IL-1β mRNA on the first, fifth, and seventh days post-trauma ($P < 0.05$; Fig. 2a). Hepatic TNF mRNA expression was also increased after burn. Insulin decreased TNF mRNA levels on 2 and 5 days after thermal trauma when compared with controls ($P < 0.05$; Fig. 2b). Insulin did not have any effect on hepatic mRNA levels of IL-6 and MIF.

Anti-inflammatory cytokine hepatic IL-2 mRNA decreased after burn injury and approached normal levels on the fifth day post-trauma. Insulin significantly increased hepatic IL-2 mRNA levels on days 1 and 2 when compared with controls ($P < 0.05$; Fig. 2c). Hepatic IL-10 mRNA expres-

TABLE 1. Serum Glucose, Cholesterol, Triglycerides, HDL, LDL, and VLDL in Insulin-Treated and Control Rats After Thermal Trauma

	Controls				Insulin			
	Day 1	Day 2	Day 5	Day 7	Day 1	Day 2	Day 5	Day 7
Glucose (mg/dL)	149 ± 3	148 ± 3	149 ± 3	150 ± 3	122 ± 5*	121 ± 5*	125 ± 7*	122 ± 5*
Cholesterol (mg/dL)	95 ± 20	96 ± 11	61 ± 8	61 ± 8	78 ± 14	89 ± 14	58 ± 5	54 ± 11
Triglycerides (mg/dL)	80 ± 16	47 ± 8	56 ± 19	120 ± 36	55 ± 17*	45 ± 16	41 ± 7	70 ± 26*
HDL (mg/dL)	59 ± 7	68 ± 6	42 ± 10	43 ± 6	61 ± 5	60 ± 18	52 ± 12	44 ± 10
LDL (mg/dL)	29 ± 7	25 ± 5	17 ± 5	10 ± 4	19 ± 9*	15 ± 5*	11 ± 3*	8 ± 3
VLDL	17 ± 5	9 ± 2	11 ± 10	24 ± 9	11 ± 3*	10 ± 5	8 ± 2	22 ± 5

Data presented as mean ± SD. Range of normal rat values: glucose, 133 ± 6 mg/dL; cholesterol, 60 ± 13 mg/dL; triglycerides, 50 ± 20 mg/dL; HDL, 62 ± 12 mg/dL; LDL, 5 ± 0.7 mg/dL; VLDL, 11 ± 5 mg/dL.

*Significant difference between controls and insulin ($P < 0.05$).

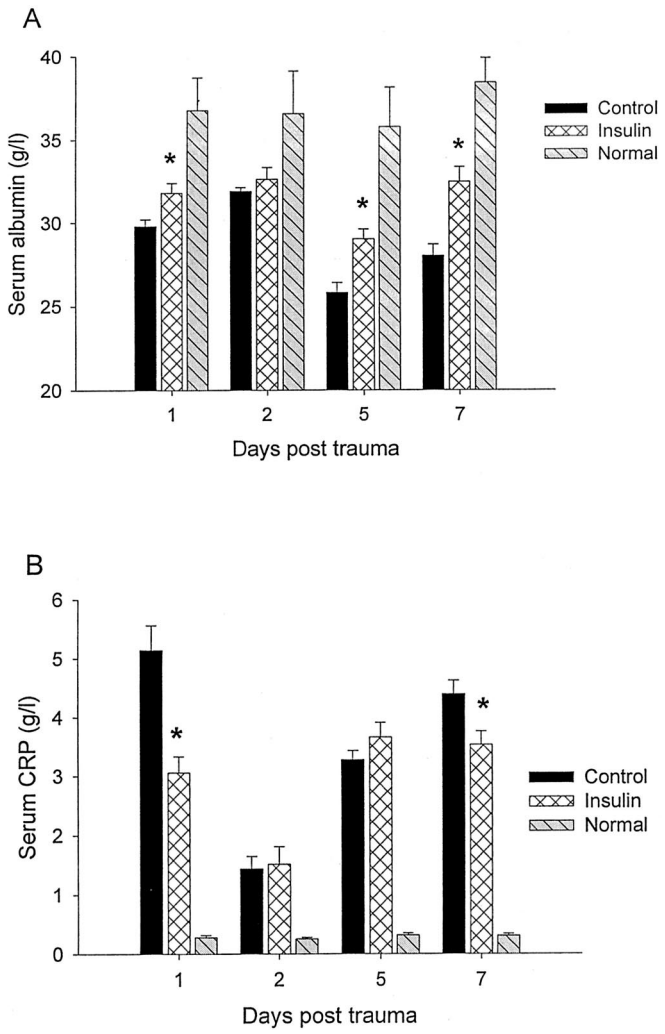

FIGURE 1. Serum constitutive hepatic protein albumin decreased immediately after burn trauma and levels remained decreased throughout the entire study period. Insulin increased serum albumin at days 1, 5, and 7 post-trauma when compared with controls, $P < 0.05$ (**A**). In contrast to constitutive hepatic proteins, acute-phase proteins are increased after trauma. Serum CRP increased after burn and remained elevated at 7 days after burn. Insulin administration significantly decreased serum CRP levels at days 1 and 7 when compared with animals receiving saline, $P < 0.05$ (**B**). Data are mean ± SEM.

sion remained in the normal range after injury. Insulin increased hepatic IL-10 mRNA expression 2 days postburn compared with controls ($P < 0.05$; Fig. 2d). Insulin did not have an effect on IL-4 and IFN-γ when compared with controls.

Hepatic Cytokine Protein Expression

Immediately after burn, hepatic IL-1β concentration increased and showed an increase over the 7-day study period in both groups. Insulin prevented an increase of IL-1β on day 1 after burn when compared with controls ($P < 0.05$; Fig. 3a). There were no significant differences between insulin and control at later time points. Hepatic TNF also increased immediately after burn and peaked at day 5 after burn. Insulin decreased hepatic TNF 5 and 7 days after burn when compared with controls ($P < 0.05$; Fig. 3b). There were no significant differences in IL-6 and MIF between the two groups.

Hepatic IL-2 demonstrated a slight increase after trauma in both groups. Insulin, however, significantly increased hepatic IL-2 concentrations on days 1 and 2 after trauma when compared with controls ($P < 0.05$; Fig. 3c). IL-4 levels decreased in the liver compared with normal values on days 1 and 2 after burn. Insulin significantly increased hepatic IL-4 on days 5 and 7 after trauma and elevated levels to normal range ($P < 0.05$; data not shown). Hepatic IL-10 demonstrated a drastic decrease compared with normal in both groups after thermal trauma. Insulin administration increased hepatic IL-10 concentrations at 5 and 7 days when compared with control ($P < 0.05$; Fig. 3d).

Hepatic Morphology

Hepatocyte Proliferation and Apoptosis

Thermal injury caused a significant increase in hepatocyte apoptosis by the biologic factor 4 compared with normal. Insulin decreased apoptosis on all study days by 50% compared with controls ($P < 0.05$; Fig. 4a). Hepatocyte proliferation increased in the control and the insulin immediately after burn and remained elevated throughout the entire study period. Insulin had a mitotic effect on hepatocytes and significantly increased proliferation compared with controls on days 1, 2, and 7 after thermal trauma ($P < 0.05$; Fig. 4b).

To determine net cell balance, proliferation was divided by apoptosis. As depicted in Figure 4c, insulin significantly improved cellular net balance by stimulating cell survival compared with controls on all study days ($P < 0.05$).

Hepatic Caspase-3, -9, and Bcl-2 Expression

To determine possible mediators for hepatocyte apoptosis and proliferation, hepatic caspases-3, -9, and Bcl-2 protein expression was measured. Hepatic caspase-3 increased immediately after burn by 100% compared with normal. Insulin prevented the increase on the first day after burn when compared with saline treated controls ($P < 0.05$; Fig. 5a). Similarly, caspases-9 was increased after burn in both groups. Insulin also decreased caspases-9 protein concentration in the liver on the first day after burn when compared with controls ($P < 0.05$; Fig. 5b). In contrast to the pro-apoptotic signals, Bcl-2 and pro-mitogenic signal was nearly 10-fold decreased after trauma. However, insulin significantly increased Bcl-2 protein levels in the liver 5 and 7 days after burn ($P < 0.05$; Fig. 5c).

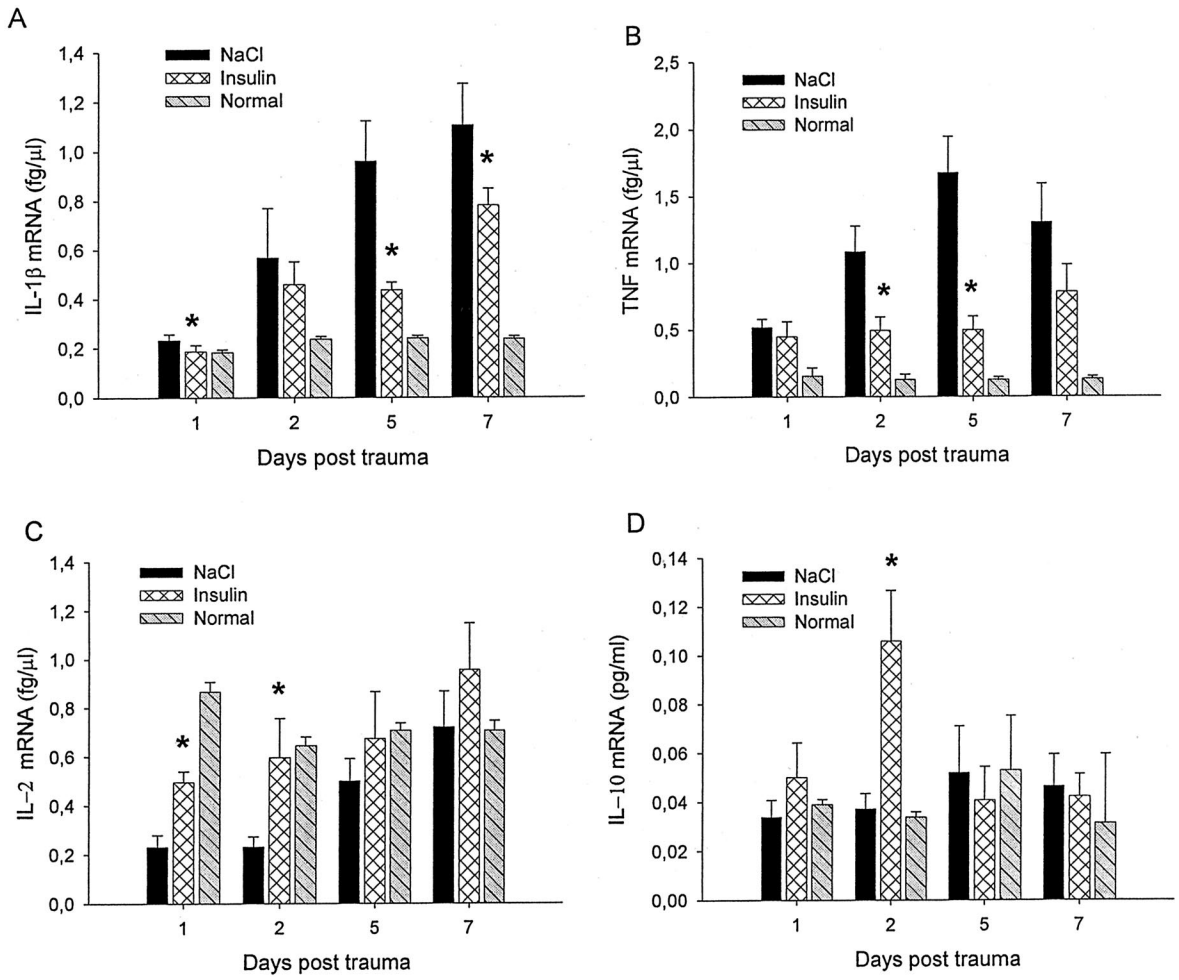

FIGURE 2. Burn injury increased hepatic IL-1β mRNA expression beginning from day 1 on and concentration increased over the 7-day study period. Insulin significantly decreased IL-1β mRNA on the first, fifth, and seventh days post-trauma, $P < 0.05$ (**A**). Hepatic TNF mRNA expression was also increased after burn. Insulin decreased TNF mRNA levels on 2 and 5 days after thermal trauma when compared with controls, $P < 0.05$ (**B**). Hepatic IL-2 mRNA levels decreased after burn injury and approached normal levels on the fifth day post-trauma. Insulin significantly increased hepatic IL-2 mRNA levels on days 1 and 2 when compared with controls, $P < 0.05$ (**C**). Hepatic IL-10 mRNA expression remained in the normal range after injury. Insulin increased hepatic IL-10 mRNA expression 2 days postburn compared with controls, $P < 0.05$ (**D**).

DISCUSSION

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 the muscle, heart, immune system, and liver, are compromised and contribute to multiorgan failure and mortality.[1,12] In several studies, it was shown that the liver–gut axis plays a crucial role during hypermetabolism. The gut was called the "fuel" and the liver was named the "engine" for the hypermetabolic cascade.[3] To restore systemic homeostasis, the liver reprioritizes its synthesis from constitutive hepatic proteins toward acute-phase proteins.[3] A prolonged increase in the acute-phase response, however, has been shown to increase morbidity and mortality.[4–6] One possible cause for the increase in morbidity and mortality is that pro-inflammatory cytokines, such as IL-1β and TNF-α, increase hypermetabolism and multiorgan failure.[6,7] A decrease in pro-inflammatory cytokine expression has been suggested to be beneficial after trauma.[6] Multiple clinical trials using anti-inflammatory agents to attenuate the overexpression of pro-inflammatory cytokines have been described.[13,14] These agents, however, have failed to control the exaggerated synthesis of pro-inflammatory cytokines and acute-phase proteins because they focused on only one pathway or mediator in the inflammatory cascade leading to compensation through other pathways. In the present study,

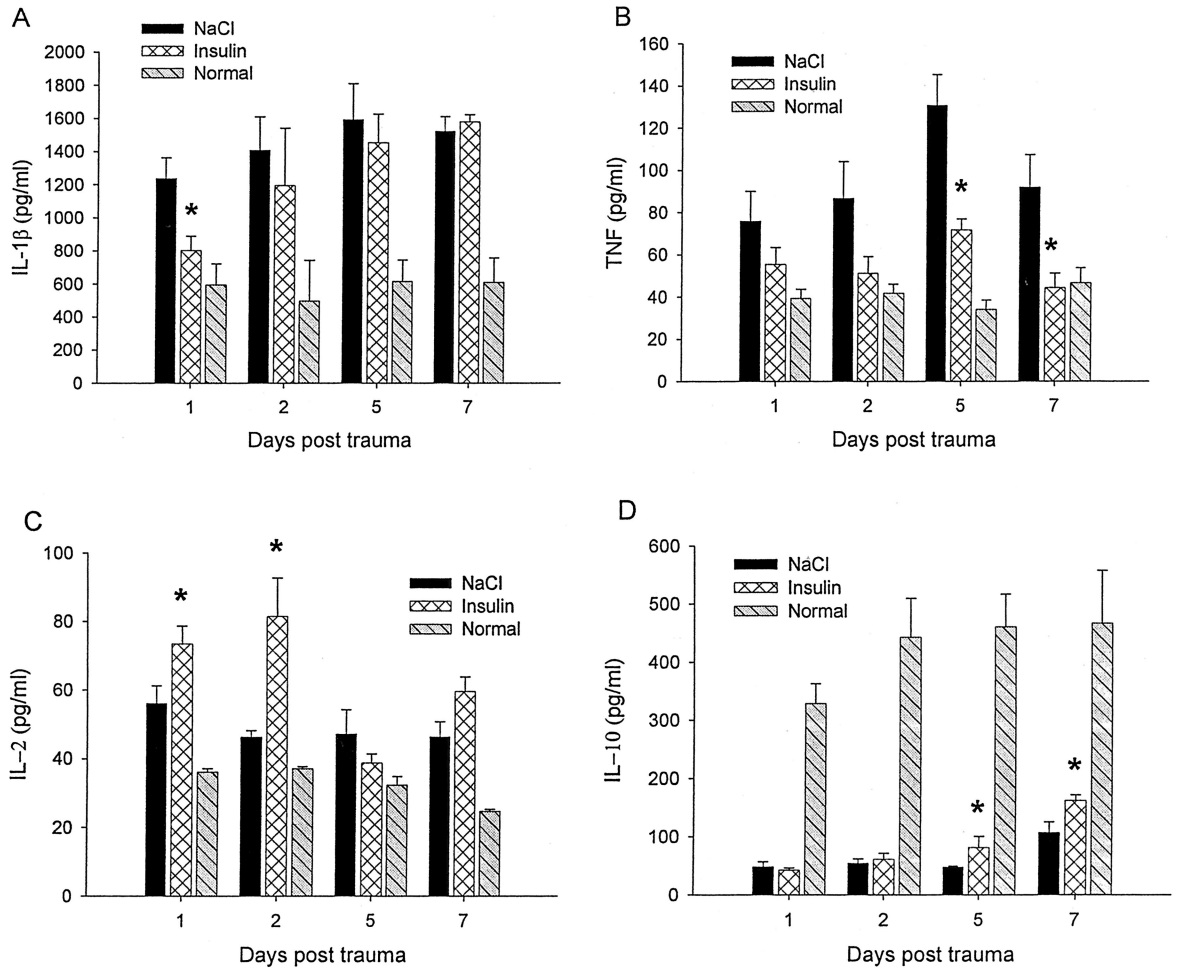

FIGURE 3. Hepatic pro- and anti-inflammatory cytokines after a 30% TBSA thermal injury. **A**: Immediately after burn, hepatic IL-1β concentration increased and showed an increase over the 7-day study period in both groups. Insulin prevented an increase of IL-1β on day 1 after burn when compared with controls, $P < 0.05$. **B**: Hepatic TNF also increased immediately after burn and peaked at day 5 after burn. Insulin decreased hepatic TNF 5 and 7 days after burn when compared with controls, $P < 0.05$. **C**: Hepatic IL-2 demonstrated a slight increase after trauma in both groups. Insulin, however, significantly increased hepatic IL-2 concentrations on days 1 and 2 after trauma when compared with controls, $P < 0.05$. **D**: Hepatic IL-10 demonstrated a drastic decrease compared with normal in both groups after thermal trauma. Insulin administration increased hepatic IL-10 concentrations at 5 and 7 days when compared with control. *Significant difference between insulin and control, $P < 0.05$. Data are mean $\pm$ SEM.

we demonstrated that insulin decreased pro-inflammatory cytokines with subsequent decreases of acute-phase proteins, which all were up-regulated after a thermal injury and increases of constitutive-hepatic proteins, such as albumin, which serves important physiologic functions.[3,15–17]

Preservation of organ homeostasis depends on a balance between cell proliferation and cell death.[18] Apoptosis, or programmed cell death, is a genetically determined energy-dependent process by which senescent or dysfunctional cells are removed without extrusion of the intracellular contents or subsequent inflammation.[18–20] This is in direct contrast to necrosis, another mode of cell death, which is a passive process initiated by direct injury to the cell. Alterations in the balance between apoptosis and proliferation may lead to changes in organ function, integrity, and homeostasis, and it would be therefore beneficial to either increase proliferation or decrease apoptosis.[21] In the present study, we demonstrated that insulin improved liver structure and integrity by increasing hepatocyte proliferation and decreasing hepatocyte apoptosis, thus improving hepatocyte net balance toward survival. The mitogenic/apoptotic cascade encompasses multiple signals that keep the apoptotic-proliferative process in homeostasis.[22] Signals that control apoptosis to a large degree through different pathways are the Fas-ligand and the Bcl-2

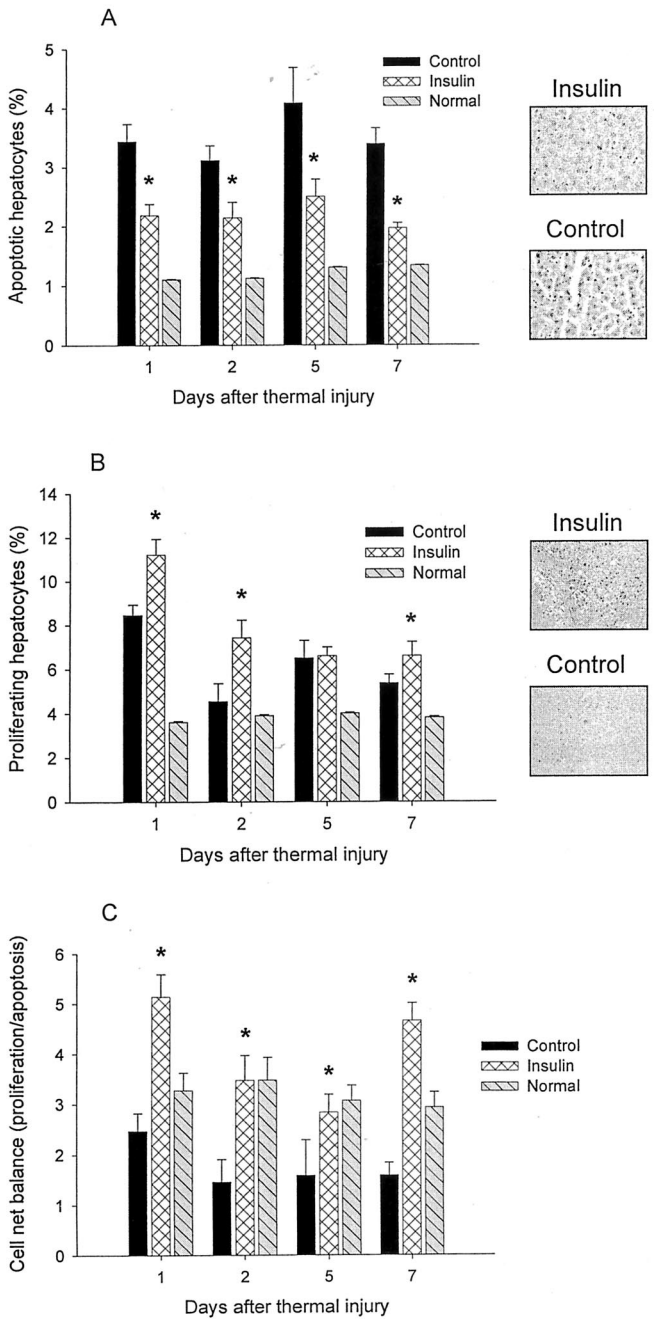

FIGURE 4. Hepatic morphology determined by measuring hepatocyte proliferation, apoptosis, and resulting hepatocyte net balance. **A**: Thermal injury caused a significant increase in hepatocyte apoptosis by the biologic factor 4 compared with normal. Insulin decreased apoptosis on all study days by 50% compared with controls, $P < 0.05$. Beside the figure, two representative histologic sections stained for apoptosis. While in control sections many apoptotic hepatocytes could be identified, only few where found positive in the insulin sections. **B**: Hepatocyte proliferation increased in the control and the insulin immediately after burn and remained elevated throughout the entire study period. Insulin had a mitotic effect on hepatocytes and significantly increased proliferation compared with controls on days 1, 2, and 7 after thermal trauma. Beside the figure, two representative histologic sections stained with Ki-67 for proliferation. While in control sections few proliferating hepatocytes could be identified, many hepatocytes were found positive in the insulin sections. **C**: To determine net cell balance, proliferation was divided by apoptosis. *Significant difference between insulin and control, $P < 0.05$. Data are mean ± SEM.

gene.[22] The Fas ligand has been shown to be pro-apoptotic and promotes cell death by binding to Fas, a cell membrane receptor, initiating a series of intracellular events leading to apoptosis, including the stimulation of caspases, such as upstream activators caspases 2, 8, and 10 and downstream activators caspases 3, 4, 7, and 9 and Bax expression.[22–24] The Fas ligand can be induced and up-regulated by the presence of interleukins.[24] The factors that have been shown to be protective against apoptosis are the Bcl-2 and Bcl-xL gene.[23,25] Protein expressed by Bcl-2 inhibits the apoptotic process and/or block it in the early phases by preventing the release of cytochrome c from the mitochondrial membrane into the cytosolic space.[25] In the present study, we showed that caspases-3 and -9 are increased and that Bcl-2 is strongly decreased after major trauma when compared with normal levels. Insulin modulated hepatic proliferation and apoptosis cascade by decreasing hepatic caspase-3 and -9 concentration at an early time after trauma. In contrast, insulin increased hepatic Bcl-2 concentration, which was found remarkably decreased after trauma, on days 5 and 7 after trauma. The effects determined in the present study are the modulation of the apoptotic process upstream, which results in decreased caspase and increased Bcl-2 activity downstream, leading to decreased apoptosis and increased proliferation and in addition to improved cell balance.

Mediator of hypermetabolism, hepatic acute-phase response, and the proliferative/apoptotic cascade are cytokines. After trauma, pro- and anti-inflammatory cytokines are synthesized and released to restore homeostasis.[26] Clinical studies have shown that a sustained or increased action of pro-inflammatory cytokines can contribute to multiorgan failure, hypermetabolism, morbidity, and mortality.[4–6] Multiple clinical trials have been undertaken in an attempt to attenuate the overexpression of pro-inflammatory cytokines or their receptors but utterly failed.[4,13,14,27] In the present study, we demonstrated that insulin, an exogenous anabolic agent, interfered with the hepatic cytokine cascade. We demonstrated that insulin affected the hepatic mRNA expression of IL-1, TNF, IL-2, and IL-10. In accordance with mRNA levels, insulin modulated hepatic protein levels. Insulin significantly decreased IL-1 early after burn, while it decreased TNF at later time points, 5 and 7 days after burn. In contrast to pro-

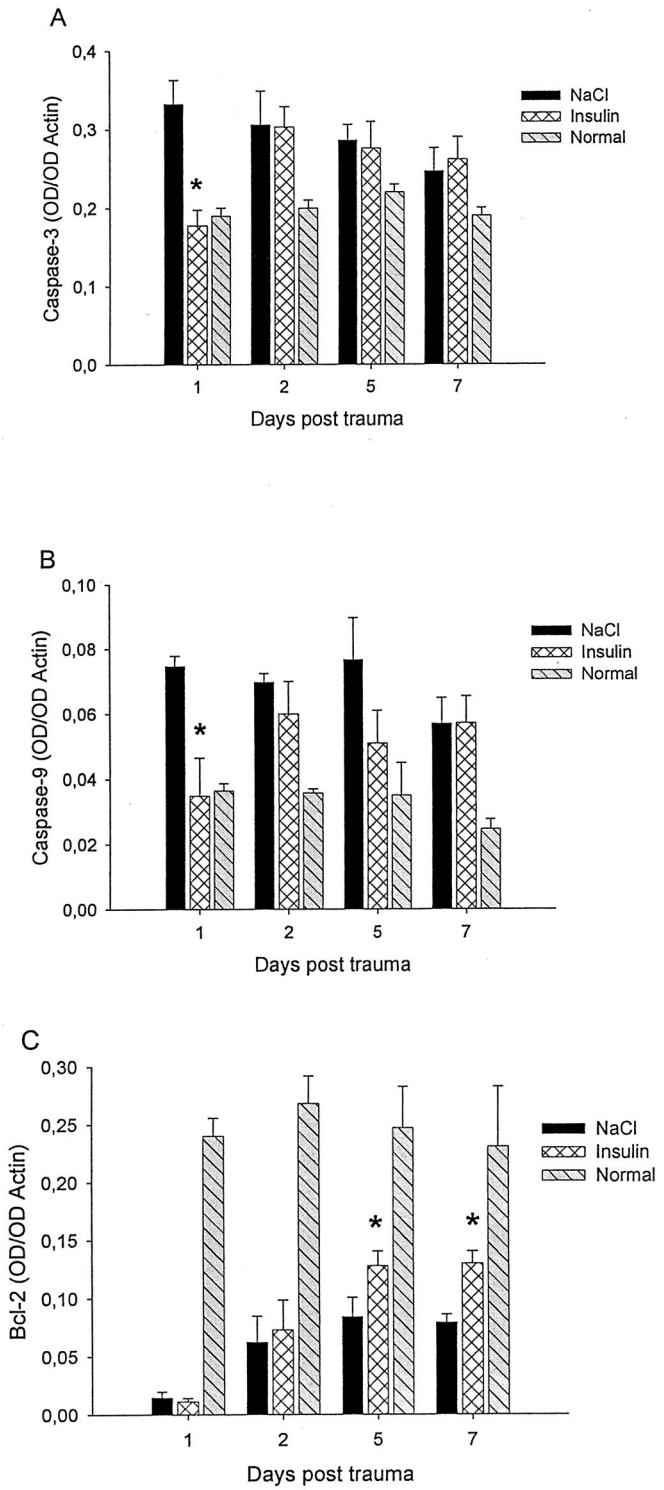

FIGURE 5. To determine possible mediators for hepatocyte apoptosis and proliferation, hepatic caspases-3, -9, and Bcl-2 protein expression was measured. **A**: Hepatic caspase-3 increased immediately after burn by 100% compared with normal. Insulin prevented the increase on the first day after burn when compared with saline treated controls. **B**: Caspase-9 was increased after burn in both groups. Insulin also decreased caspase-9 protein concentration in the liver on the first day after burn when compared with controls. **C**: In contrast to the pro-apoptotic signals, Bcl-2 an pro-mitogenic signal was nearly 10-fold decreased after trauma. However, insulin significantly increased Bcl-2 protein levels in the liver 5 and 7 days after burn. *Significant difference between insulin and control, $P < 0.05$. Data are mean ± SEM.

inflammatory cytokines, insulin increased hepatic IL-2 as well as anti-inflammatory cytokines IL-4 and IL-10 after trauma. An important finding was also that hepatic mRNA and protein demonstrate a different pattern as serum cytokine levels. In the liver, cytokines increased over the study and had their peak 5 or 7 days after trauma, while in the blood the peak is mostly at 1 or 2 days after trauma. These findings indicate the profound hypermetabolic reaction of the liver, which persists obviously longer in the liver then in serum. However, clinical studies demonstrated that ratios of pro- to anti-inflammatory cytokines correlate with organ function and can be used as predictors for mortality.[28,29] 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.

As we found differences in the hepatic mRNA expression of these cytokines, we hypothesize that insulin affects cytokine synthesis by pretranslational mechanisms. In another study, it was shown that insulin affects cellular signaling. Insulin decreased the pro-inflammatory signal transcription factors STAT-5 and C/EBP-β in the liver.[11] An upregulation of both transcription factors leads to impaired organ function and protein synthesis, such as albumin.[30,31] 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, insulin affected anti-inflammatory signal transcription factors that were identified to either suppress cytokine signaling (SOCS) or regulate the T-cell function (RANTES). Insulin increased SOCS-3 and RANTES, both of which are major players for survival and clinical outcome during the post-traumatic cascade.[11,32–34]

Insulin is known to have vasodilatory properties and increase glucose uptake in ischemic tissues for anaerobic ATP production following augmented glycolysis.[35,36] Insulin may also stimulate pyruvate dehydrogenase during ischemia and thereby exerts a beneficial effect associated with an improved energy state and less lactate production.[35,36] Furthermore, it was speculated that insulin may inhibit free fatty acids.[35] Increased levels of free fatty acids lead to increased hepatic triglyceride accumulation and consecutively to hepatic failure, which is associated with increased incidence of

sepsis and mortality.[37,38] In the present study, we have shown that insulin administration decreases serum triglycerides, LDL, and VLDL after thermal injury. This metabolic action of insulin represents an advantage as liver failure and death due to increased fat accumulation could be prevented.

Insulin therapy to maintain blood glucose below 110 mg/dL attenuated the pro-inflammatory response to trauma, prevented the incidence of multiorgan failure, and consecutively reduced mortality in critically ill patients.[9] The results presented in the present study provide insight by which mechanisms insulin may improve the clinical outcome. We showed that insulin given at doses to maintain blood glucose around 110 mg/dL attenuated the inflammatory response by decreasing pro-inflammatory and increasing anti-inflammatory cytokines, thus restoring systemic homeostasis. We further showed that the effect of insulin is probably due through direct binding of insulin on signal transcription factors, rather than indirectly through changes in blood glucose and metabolism. Based on our data and the data of van den Berghe,[9] we suggest that insulin may represent an important and safe therapeutic option in the treatment of critically ill patients.

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