SEPTIC SHOCK is associated with a high mortality rate (2, 19, 49), and a driving hypothesis has been that excessive inflammation plays a causative role. Clinical trials have failed to show an effect of anti-TNF-α, IL-1 receptor antagonist, and anti-endotoxin (1, 2, 19, 28, 30, 35, 55), whereas activated protein C as well as low-dose corticosteroids have recently been demonstrated to reduce mortality in patients with severe sepsis (3, 5, 30). Hyperglycemia is common in critically ill patients (29, 31); lately it has been demonstrated that intensive insulin treatment to maintain blood glucose at or below 6.1 mM reduces mortality and morbidity of critically ill surgical patients. The effect on mortality was mainly obtained through a reduction in late deaths (after 5 days of admission) due to a septic focus (49).

The pathogenesis of sepsis-associated hyperglycemia is probably multifactorial (29, 31). Elevated levels of circulating hormones in the critically ill patients are thought to cause insulin resistance. Epinephrine infusion in humans results in hyperglycemia and insulin resistance (6, 20, 44), as does norepinephrine infusion in dogs (9), but the exact mechanism of action is not known. Glucocorticoids impair the insulin-mediated glucose uptake in skeletal muscle in rats, probably by inhibiting the glucose transporter GLUT4 (12), and growth hormone in high concentrations is associated with insulin resistance as well, although the precise molecular mechanism is unclear (13). The production and/or release of cytokines may also play a role in the development of hyperglycemia; in particular, TNF-α has been demonstrated to induce insulin resistance in animals (22). In addition, high levels of free fatty acids (FFA) are associated with insulin resistance (4, 7), and high levels of FFA are found in sepsis (25, 46). TNF infusion increases an increase in circulating levels of FFA (39, 40, 50). Although TNF has been shown to stimulate lipolysis directly in cultured fat cells (17), this increase could also be elicited by a TNF-induced elevation of IL-6, since IL-6 infusion alone increases the levels of circulating FFA (26, 51). Thus FFA levels may be mechanistically involved in sepsis-associated insulin resistance.

Patients with type 2 diabetes mellitus are characterized by low-grade inflammation with elevated circulating levels of neutrophils, TNF, and IL-6 (21). In addition, higher levels of FFA are found in these patients (8). The potential causal relationship between hyperglycemia/hyperinsulinemia and chronic inflammation in diabetic patients has not been established.

Studies of cytokine responses in septic humans are potentially confounded by the absence of a well-defined onset time of sepsis, as well as by substantial delays from the presumed initiation of infection until measurement; thus multiple interacting cascades are often activated at the time of study, making the interpretation of data difficult. Extrapolation of results from animal studies to humans is limited by the fact that a high difference exists among species with regard to their endotoxin sensitivity; this necessitates the use of rather large doses and of cumbersome experimental models for achieving comparable effects (36, 53). To overcome such problems, a human experimental model for sepsis has been developed by use of an intravenous bolus injection of purified Escherichia coli endotoxin (14). In a dose of 2–4 ng/kg, this substance triggers brief

Effect of hyperglycemia and hyperinsulinemia on the response of IL-6, TNF-α, and FFAs to low-dose endotoxemia in humans

Rikke Krogh-Madsen,1 Kirsten Møller,1 Flemming Dela,2,3 Gitte Kronborg,1 Sune Jauffred,1 and Bente Klarlund Pedersen1,2
1Department of Infectious Diseases, University Hospital Rigshospitalet, 2Copenhagen Muscle Research Centre, and 3Department of Medical Physiology, The Panum Institute, 2100 Copenhagen, Denmark

Krogh-Madsen, Rikke, Kirsten Møller, Flemming Dela, Gitte Kronborg, Sune Jauffred, and Bente Klarlund Pedersen. Effect of hyperglycemia and hyperinsulinemia on the response of IL-6, TNF-α, and FFAs to low-dose endotoxemia in humans. Am J Physiol Endocrinol Metab 286: E766–E772, 2004.—Insulin therapy to maintain eu-
glycemia increases survival in critically ill patients. To explore pos-
sible mechanisms of action, we investigated the effect of endotoxin on circulating cytokines, free fatty acids (FFA), and leukocytes during manipulated plasma glucose and insulin concentrations. Ten volun-
teers underwent three trials each, receiving an intravenous bolus of endotoxin (0.2 ng/kg) during normoglycemia (trial A, control), during a hyperglycemic clamp at 15 mM (trial B), and during a hyperinsu-
linemic euglycemic clamp (trial C). Endotoxin induced an increase in neutrophil count, a decrease in lymphocyte count, and an increase in serum levels of TNF-α, IL-6, and FFA. There was no difference in the TNF response between the three trials; the IL-6 levels were increased during the late phase of trials B and C compared with trial A. The endotoxin-induced elevation in FFA in trial A was suppressed during trials B and C. Clamping (trials B and C) caused a reduction in lymphocyte count that persisted after endotoxin injection. We con-
clude that low-dose endotoxemia triggers a subclinical inflammatory response and an elevation in FFA. The finding that high insulin serum concentrations induce a more prolonged increase in the anti-inflam-
matory cytokine IL-6 and suppress the levels of FFA suggests that insulin treatment of patients with sepsis may exert beneficial effects by inducing anti-inflammation and protection against FFA toxicity, and thereby inhibit FFA-induced insulin resistance.

endotoxin; inflammation; insulin; free fatty acids
flu-like symptoms, such as headache, chills, malaise, and fever. We have recently applied only 0.06 ng/kg in an attempt to establish a model of low-grade subclinical inflammation (47), which is likely to be a common phenomenon in patients in the intensive care units, who are exposed to invasive procedures with catheters and the like. The low dose elicits a significant and reproducible cytokine response in the absence of subjective symptoms, facilitating the performance of repeated studies in the same subject.

Given that IL-6 is not only induced by TNF but also that TNF production is inhibited by IL-6 (47), evidence exists that IL-6 has strong anti-inflammatory effects. The beneficial effect of insulin treatment in clinical sepsis makes us suggest that insulin stimulates IL-6 production. The present study was performed to monitor the cytokine response and levels of FFA in healthy young men given endotoxin alone (control trial), during a hyperglycemic clamp, and during a hyperinsulinemic euglycemic clamp. We hypothesized that insulin clamps would induce an anti-inflammatory response and suppression of FFA during endotoxemia.

MATERIALS AND METHODS

Subjects

Ten healthy human males of mean age 24.5 yr (range 21–32 yr), body mass index (BMI) 23.2 kg/m² (range 21.1–25.2 kg/m²), and with an unremarkable medical past were included after oral and written informed consent. Before the study, all 10 subjects underwent a thorough clinical examination. Blood samples for renal, hepatic, and thyroid function, hemoglobin, white blood cell counts, electrolytes, and plasma glucose were analyzed as well. All tests were normal. The study was approved by the Scientific-Ethical Committee of Copenhagen and Frederiksberg Municipalities [jr. no. (KF) 11–032/02].

Study Design

All subjects received an intravenous injection of endotoxin during three repeated trials: trial A with endotoxin alone, trial B during a steady-state hyperglycemic clamp, with blood glucose clamped at 15 mM, and trial C during a hyperinsulinemic euglycemic clamp with blood glucose clamped at fasting level. The studies took place in the following order: A, B, C (n = 2); A, C, B (n = 2); C, B, A (n = 3); B, C, A (n = 3). For logistical reasons, trial A (control) was placed either before or after trials B and C. The interval between any two of three studies was 15 (range 8–26) days.

On the study day, the subject reported to the laboratory (at 8:00 AM) after an overnight fast. A peripheral catheter was placed in an antecubital vein for blood sampling and, during the clamp trials, another was placed in the contralateral antecubital vein for infusion of insulin and glucose. A peripheral catheter was placed in a dorsal hand vein; this hand was then wrapped in a heating blanket to obtain arterialized venous blood for measurement of glucose, insulin, C-peptide, and potassium. An ECG was continually monitored; heart rate, noninvasive blood pressure, and tympanic temperature were recorded, as we will indicate.

After catheterization, and after steady-state blood glucose levels had been achieved during the clamp trials, an intravenous bolus of endotoxin (Endotoxin Escherichia coli, Lot EC-6, United States Pharmacopoeia Convention, Rockville, MD) was administered at a dose of 0.2 ng/kg body wt. Every study lasted 6 h after injection of the endotoxin bolus.

Trial A (endotoxin alone). Isotonic saline (1,000 ml) was infused at maintenance rates to avoid dehydration. After baseline blood sampling, endotoxin was injected. Venous samples for measurement of cytokines, FFA, cortisol, C-reactive protein (CRP), and white blood cell and differential counts were drawn at baseline and 60, 120, 180, 240, 300, and 360 min after endotoxin injection. Arterialized venous blood was drawn at baseline and after 60, 120, 180, 240, 300, and 360 min after the endotoxin injection to measure insulin and C-peptide concentrations, as well as every 10 min for monitoring glucose and potassium levels.

Study B (hyperglycemic clamp). The method for hyperglycemic clamping has been described previously (11). Briefly, glucose (200 g/1,000 ml) was infused intravenously. To maintain blood glucose levels of 15 mM, the rate of infusion was adjusted by a computer-controlled infusion pump, according to arterialized blood glucose levels. To maintain potassium at the baseline value, isotonic saline containing potassium (51 meq/l) was infused continuously. In addition, 1,000 ml of isotonic saline were infused during the study. Endotoxin was injected after steady-state hyperglycemia concentration was reached (after ~1 h). Measurement of glucose and potassium concentrations in arterialized blood was done every 10 min. Arterialized blood for measurements of insulin and C-peptide concentrations and venous blood for cytokines, FFA, cortisol, CRP, and white blood cell and differential counts were drawn before glucose infusion (time −1), after steady-state hyperglycemia was achieved (just before endotoxin infusion, baseline, time 0), and after endotoxin, as described for the baseline study.

Study C (euglycemic clamp). After a priming intravenous bolus containing 0.6 IU/m² insulin (Actrapid, Novo Nordisk Insulin, 100 IU/ml), insulin was infused continuously at an infusion rate at 0.08 IU·min⁻¹·m⁻². Glucose (200 g/1,000 ml) was infused by a computer-controlled infusion pump at rates adjusted to maintain blood glucose at baseline levels (fasting level), in a manner similar to that described for hyperglycemic clamping; isotonic saline, with potassium as well as 1,000 ml of isotonic saline, was infused continuously during the study. Arterialized blood was analyzed at intervals of 10 min. After steady-state blood glucose levels had been achieved (after ~1 h), an endotoxin bolus was injected. Arterialized blood for measurements of insulin and C-peptide concentrations and venous blood for cytokines, FFA, CRP, cortisol, and white blood cell and differential counts were drawn as mentioned in study B.

Measurements

Cytokines. Samples were drawn into tubes containing EDTA and immediately centrifuged. Plasma was stored at −80°C until analyzed. Plasma concentrations of TNF and IL-6 were measured by the enzyme-linked immunosorbent assay (ELISA) technique (R&D Systems, Minneapolis, MN). All cytokine determinations were measured in duplicate, and mean concentrations were calculated.

FFA. Samples were drawn into tubes containing EDTA and centrifuged. Plasma was stored at −80°C until analyzed. FFA were determined using an automatic analyzer (Cobas Fara, Roche).

Cortisol. Serum was stored at −80°C until analyzed. Serum concentrations of cortisol were measured by ELISA technique (DSL, Webster, TX).

Potassium and glucose concentrations. Arterialized blood samples were analyzed immediately using an ABL 700 (Radiometer).

CRP, white blood cells, and differential counts. Standard laboratory procedures were employed.

Insulin and C-peptide. Samples were drawn into tubes containing EDTA and aprotinin [Trasylol, 20,000 kallikrein inhibitor units (KIU)/ml Bayer] and immediately centrifuged. Plasma was stored at −80°C until analyzed with an ELISA technique (DAKO, Glostrup, Denmark).

Statistical Analysis

Data were analyzed using parametric methods, and \( P < 0.05 \) was considered statistically significant. For blood glucose and FFA concentrations, which were normally distributed as indicated by Kolmogorov-Smirnov analysis, reported values are means ± SE. Plasma
insulin, plasma C-peptide, and plasma cytokine (TNF and IL-6) concentrations, and serum cortisol, blood neutrophil, and lymphocyte counts were log-transformed before analysis, and reported values are geometric means [95% confidence interval (CI)]. Analysis was performed using SPSS Base and Advanced Models version 11.0 for Windows (SPSS, Chicago, IL). Within-subject variation over time and variation between groups were analyzed using a repeated-measures (two-way ANOVA, time-by-trial) approach followed by Bonferroni-corrected paired t-tests as appropriate to identify significant differences. Because the immune response might be influenced by repeated injections of endotoxin, we compared the change in IL-6 after the second and third trials with that observed during the first trial (two-way ANOVA, time-by-time).

RESULTS

Blood concentrations of glucose, as well as plasma concentrations of insulin and C-peptide, during the three trials are given in Table 1. The trial effect for blood glucose was significant (repeated measures, \( P < 0.001 \)) and restricted to trial B, i.e., blood glucose values did not differ between trials A and C. The marked hyperglycemia during trial B (hyperglycemic clamp) and the intravenous insulin infusion during trial C (hyperinsulinemic euglycemic clamp) were both associated with increases in plasma insulin; plasma insulin did not differ between trials B and C. Plasma C-peptide levels were significantly increased during trial B compared with trial A (control) and trial C (repeated measures, \( P = 0.0004 \)), reflecting that insulin was endogenously produced during trial B. There was no significant difference between trial A and trial C. There were no changes over time in concentrations of glucose, insulin, and C-peptide after endotoxin injection.

Effects of Clamp Before Endotoxin Challenge

Steady-state glucose levels were obtained 1 h after clamps were initiated. The concentrations of TNF, IL-6, neutrophils, lymphocytes, and FFA were similar in the three groups before clamps (data not shown). There was no effect of clamping alone on TNF, IL-6, and neutrophil counts. In contrast, lymphocyte counts decreased significantly during trials B (hyperglycemic clamp) and C (hyperinsulinemic euglycemic clamp) compared with trial A (control). The concentration of FFA also decreased during trials B and C compared with trial A (Fig. 1A; levels before clamp not shown).

Endotoxin Effects

Heart rate, blood pressure, and temperature remained unchanged after endotoxin injection, and no symptoms evolved in the subjects.

Bolus administration of endotoxin was associated with highly significant changes in plasma IL-6 (\( P < 0.001 \); Fig. 1B), plasma TNF (\( P < 0.001 \); Fig. 1C), blood neutrophil counts (\( P < 0.001 \); Fig. 1D), and lymphocyte counts (\( P = 0.001 \); Fig. 1E) in all three trials. Endotoxin increased the levels of FFA in trial A (control; Fig. 1A; \( P = 0.001 \)).

In contrast to the marked increase observed during trial A, FFA concentrations were unchanged after endotoxin injection in trials B and C (Fig. 1A).

The peak response of TNF to endotoxin occurred at 120–180 min. The overall response of TNF did not vary among trials (Fig. 1C).

The overall response of IL-6 peaked at 180 min in all trials. There was no significant overall difference between levels of IL-6 in the three trials for the entire study duration, as indicated by repeated-measures analysis. There was a borderline trial-by-time interaction, indicating different time courses among the three trials (\( P = 0.11 \)); visual analysis indicated that this effect was present during the late phase of the trial. This prompted us to perform a subset analysis of the early phase (0–120 min) and the late phase (180–360 min) of the trial. Whereas the early phase was similar between trials (\( P = 0.28 \)), the late phase was significantly different (\( P = 0.02 \)); thus, compared with trial A, the IL-6 levels were increased in the late phase both in trial B (\( P = 0.002 \)) and in trial C (\( P = 0.03 \)). For individual time points, IL-6 concentrations were significantly higher during trial B compared with trial A at 240, 300, and 360 min but not at 180 min (Bonferroni-corrected paired \( t \)-test); plasma IL-6 concentrations were not significantly different for any individual time point during the late phase of trial C compared with trial A. Values for IL-6 are given in Table 2. CRP values remained unchanged after endotoxin injection. Serum concentrations of cortisol decreased over time (\( P < 0.04 \) for all three trials), with no significant difference between trials (data not shown).

Endotoxin induced a biphasic response in the blood neutrophil count; thus a slight decrease at 120 min was followed by a marked increase, which peaked at 180 min without differences between trials (Fig. 1D). Blood lymphocytes decreased after endotoxin injection to reach a nadir at 180 min (repeated-measures \( P = 0.002 \); Fig. 1E). The effect of clamping on the blood lymphocyte count appeared to persist after endotoxin injection, as the counts were significantly higher during the control trial than during either the hyperglycemic or hyperinsulinemic euglycemic trial (repeated measures for effect of trial, \( P < 0.001 \); \( P < 0.001 \) for comparison between trials A and B; \( P = 0.02 \) for comparison between trials A and C). Furthermore, counts during hyperinsulinemic euglycemia were significantly higher than those obtained during hyperglycemia (\( P = 0.03 \)).

We considered the possibility that repeated injections of endotoxin might change the immune response (create resistance in the individual). Therefore, the subjects were randomized as described in MATERIALS AND METHODS. In addition, we compared the changes in IL-6 after endotoxin between the first, second, and third trial for the subjects over time; the IL-6

Table 1. Blood glucose, plasma insulin, and plasma C-peptide after endotoxin

<table>
<thead>
<tr>
<th>Trial</th>
<th>Blood Glucose, mmol/l</th>
<th>Plasma Insulin, pmol/l</th>
<th>Plasma C-Peptide, pmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.9±0.1</td>
<td>16</td>
<td>288</td>
</tr>
<tr>
<td></td>
<td>(8–30)</td>
<td>(182–457)</td>
<td></td>
</tr>
<tr>
<td>Trial B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperglycemic clamp</td>
<td>14.8±0.03</td>
<td>821</td>
<td>3,188</td>
</tr>
<tr>
<td></td>
<td>(499–1,361)</td>
<td>(2,660–5,492)</td>
<td></td>
</tr>
<tr>
<td>Trial C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperinsulinemic</td>
<td>5.1±0.07</td>
<td>819</td>
<td>294</td>
</tr>
<tr>
<td>euglycemic clamp</td>
<td>(661–1,048)</td>
<td>(183–475)</td>
<td></td>
</tr>
</tbody>
</table>

Values are given as an average over time (from time 0 to 360 min) and are presented as means ± SE for glucose and geometric means with 95% confidence intervals (CI) for insulin and C-peptide.
response to endotoxin was unchanged during the second and third trials compared with the first trial (data not shown).

**DISCUSSION**

The main finding in this low-dose endotoxin study was a robust increase in the circulating levels of inflammatory mediators despite the absence of clinical signs or symptoms of inflammation. In the face of quantitatively similar increases in TNF, the response of plasma IL-6 appeared to be prolonged during both the hyperglycemic and the hyperinsulinemic euglycemic trials compared with control. Finally, FFA failed to increase after endotoxin injection during either the hypergly-
induced an overall decrease in the lymphocyte number. The inflammatory response in this study mimicked the response of a subacute bacterial infection. Endotoxin produced a uniform and highly significant increase in TNF and IL-6, peaking at 120 and 180 min after endotoxin injection, respectively. The neutrophil number increased, peaking at 180 min, whereas the lymphocytes decreased in accord with previous studies (23, 52). The dose of endotoxin in the present study was 0.2 ng/kg, which is ~10 times lower than that used in other studies (14, 42, 45). The advantage of using this lower dose is that the subjects do not develop any symptoms during the studies.

The IL-6 response appeared to be similar in the early phases of the three trials, but it increased in the late phases of trials B and C compared with trial A. These findings were a result of a post hoc analysis and should be interpreted with caution. Because TNF levels were similar between trials, this prolonged IL-6 response during the two clamps compared with the control situation was not caused by differences in the TNF-induced production of IL-6; in contrast, the similarity in IL-6 levels between trial B (hyperglycemic clamp) and trial C (hyperinsulinemic euglycemic clamp) may indicate that the IL-6 response was induced by elevated plasma concentrations of insulin rather than by elevated blood glucose levels. In agreement, we have recently demonstrated that insulin stimulates the IL-6 production from adipose tissue and elevates circulating levels of IL-6 (24). The prolonged increase in IL-6 concentration during trials B and C compared with trial A (control) did not result in different levels in CRP or serum cortisol between trials. However, the CRP response to IL-6 is a late phenomenon, occurring 6 h after IL-6 infusion (48), and the cortisol response is known to rise in an endotoxin dose-dependent manner with no increase when 0.2 ng endotoxin/kg is used, with a minor increase when 0.4 ng/kg is used, and with the largest increase when 0.8 ng/kg is used (34). The decrease in serum cortisol levels observed in this study over time could be due to circadian rhythm (38) or stress-induced elevated serum levels at the beginning of the trials.

Animal experiments demonstrate that TNF is mechanistically involved in the development of insulin resistance (22); therefore, chronically elevated levels of TNF may contribute to the development of insulin resistance in patients with infectious diseases (10, 33). IL-6 may inhibit TNF production; this may explain this observation. FFA have potentially deleterious biological effects, including generation of free oxygen radicals, inhibition of mitochondrial function, and depletion of glutathione (16). Moreover, FFA appear to be implicated in the generation of insulin resistance, as reduction of FFA levels increase insulin sensitivity in insulin-resistant subjects (37, 41, 43). Thus the beneficial effect of insulin administration in critically ill patients (49) may be caused partially or totally by a reduction in FFA.

In conclusion, low-dose endotoxemia triggers a subclinical inflammatory response and elevation in FFA in healthy volunteers. The finding that insulin clamps suppress the levels of FFA and induce a more prolonged increase in the anti-inflammatory cytokine IL-6 suggests that insulin treatment of patients with sepsis may exert beneficial effects by inducing anti-inflammation and protection against FFA toxicity, thereby inhibiting TNF-induced insulin resistance.

ACKNOWLEDGMENTS

We thank Hanne Willumsen, Ruth Rousing, and David Benne Olsen for technical assistance.

GRANTS

We acknowledge financial support from The Danish National Research Foundation (504-14), The Foundation of 1870, The Novo Nordisk Foundation, and The A. P. Møller Foundation.

REFERENCES


