Euglycemic hyperinsulinemia augments the cytokine and endocrine responses to endotoxin in humans

MATTIAS SOOP,1 HELEN DUXBURY,2 ANSELM O AGWUNOBI,1 J. MARTIN GIBSON,3 STEPHEN J. HOPKINS,1 CHARMAINE CHILDS,2 ROBERT G. COOPER,4 PAULA MAYCOCK,2 RODERICK A. LITTLE,2 AND GORDON L. CARLSON1

1North West Injury Research Collaboration, 2Medical Research Council Trauma Group, and Departments of 3Endocrinology and 4Rheumatology, Hope Hospital, Salford, M6 8HD, United Kingdom

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Soop,Mattias, Helen Duxbury, Anselm O Agwunobi, J. Martin Gibson, Stephen J. Hopkins, Charmaine Childs, Robert G. Cooper, Paula Maycock, Roderick A. Little, and Gordon L. Carlson. Euglycemic hyperinsulinemia augments the cytokine and endocrine responses to endotoxin in humans. Am J Physiol Endocrinol Metab 282: E1276–E1285, 2002.—Type 2 diabetes is associated with biochemical evidence of low-grade inflammation, and experimental studies have suggested that both insulin and glucose affect inflammatory responses. To determine the effect of in vivo changes in glucose availability and plasma insulin concentrations in humans, we administered 20 U/kg Escherichia coli lipopolysaccharide (LPS) or saline (control) to 14 subjects during a euglycemic hyperinsulinemic clamp (n = 6) or an infusion of sterile saline (n = 8). Parallel in vitro studies on human whole blood were undertaken to determine whether there was a direct effect of glucose, insulin, and leptin on proinflammatory cytokine production. Infusion of glucose and insulin significantly amplified and/or prolonged the cardiovascular, plasma interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and counterregulatory hormone responses to LPS, whereas the effects on fever, plasma norepinephrine concentrations, and oxygen consumption were unaffected. In vitro studies showed no modulation of LPS-stimulated IL-6 or TNF-α production by glucose, insulin, or leptin at physiologically relevant concentrations. Hyperinsulinemia indirectly enhances key components of the systemic inflammatory and stress responses in this human model of infection.

diabetes; cytokines; lipopolysaccharide; inflammation; sepsis

THE EFFECT OF INFLAMMATION and injury on insulin sensitivity has been recognized for many years. Inflammation associated with infection and connective tissue disease is accompanied by profound reductions in insulin sensitivity. The fact that corticosteroid treatment, a potent cause of insulin resistance in itself, may actually reduce insulin resistance in patients with rheumatoid arthritis (36) indicates that the negative influence of inflammation on insulin sensitivity exceeds even that of pharmacological doses of steroids. It has also recently been suggested that low-grade, chronic inflammation may play a role in the pathogenesis of insulin resistance in obesity and type 2 diabetes. This suggestion is based on the demonstration of elevated plasma concentrations of proinflammatory cytokines, such as interleukin-6 (IL-6) (9, 28) and tumor necrosis factor-α (TNF-α) (14, 22), as well as acute-phase reactants (21) in diabetes. Increased expression of TNF-α mRNA in the adipose tissue (13) and skeletal muscles (32) of obese and diabetic patients, and protection from obesity-induced insulin resistance in mice lacking TNF receptor function (38). Raised plasma IL-6 concentrations, indicative of low-grade inflammation, have been suggested to represent the link between the insulin resistance and the vascular disease that characterize the metabolic syndrome X (29).

An alternative explanation for the demonstration of increased levels of proinflammatory cytokines in type 2 diabetes might be that increased plasma glucose and/or insulin concentrations enhance the inflammatory response. In this scenario, the hyperglycemia/hyperinsulinemia or other endocrine changes associated with insulin resistance might accentuate the inflammatory response, rather than inflammation leading directly to insulin resistance. This suggestion is supported by the finding that administration of glucose significantly augments the TNF-α and hemodynamic responses to endotoxin administration in rabbits (18), although the converse has been demonstrated in mice (33). Glucose (23, 27) and insulin-like growth factors (30) have also been shown to significantly augment secretion of IL-1, IL-6, and TNF-α from mononuclear cells in vitro. It is unclear whether glucose availability and/or hyperinsulinemia upregulates the inflammatory response in humans, although this could provide an alternative explanation for the demonstration of increased circulating and tissue levels of proinflammatory cytokines in patients with type 2 diabetes, in whom hyperglycemia and hyperinsulinemia are common.

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Address for reprint requests and other correspondence: G. L. Carlson, NWIRC, Clinical Sciences Bldg., Hope Hospital, Salford M6 8HD, UK (E-mail: gcarlson@fs1.ho.man.ac.uk).
The aim of this study was therefore to test the hypothesis that hyperinsulinemia and increased glucose availability would significantly augment the systemic response to an inflammatory stimulus in humans and, if so, to determine whether this might be attributable to a direct effect on cytokine-producing cells.

MATERIALS AND METHODS

In Vivo Study

Subjects. Fourteen subjects (12 male, 2 female), of mean age 32.7 (range 24–38) yr and mean body surface area (BSA) 1.9 (range 1.7–2.1) m², were admitted to the clinical investigation facility of Hope Hospital, Salford. Before study, all subjects were screened by history, physical examination, and electrocardiogram. No subject had a history of cardiorespiratory, metabolic, or inflammatory disease, and none was receiving any medication. The study was approved by the local research ethics committee of Salford and Trafford Health Authority, and informed consent was obtained from each subject before enrollment in the study.

Study protocol. Lipopolysaccharide (LPS) was administered intravenously to six (4 male, 2 female) subjects during conditions of euglycemic hyperinsulinemia and to eight male subjects during an infusion of sterile normal saline.

Each subject was studied on two occasions, 10–14 days apart. On the first occasion, subjects received either an intravenous bolus of LPS or an equivalent volume (6–8 ml) of sterile isotonic saline. On the second occasion, subjects received the alternative infusion, the order being determined randomly. There were thus four study arms, with each subject receiving either LPS or saline (control), during an infusion of insulin-glucose or saline.

All subjects were studied at 0830 after an overnight fast and having refrained from smoking or beverages containing caffeine for 24 h before the study. After voiding, subjects were weighed to the nearest 0.1 kg with an Avery beam balance and their tympanic membrane temperature was measured at 30-min intervals. Hand skin temperature was maintained at 42°C and was monitored throughout the 8-h period using a Beckman glucose analyzer (Beckman Instruments, Fullerton, CA). Pulse rate and MAP were measured automatically every 30 min. Tympanic membrane temperature was measured at 30-min intervals, and the mean of each series was used in subsequent calculations.

After 30-min rest period, an 8-h infusion of insulin-glucose (see Euglycemic hyperinsulinemic clamp) or saline (150 mmol/l, 50 ml/h) was commenced. One hundred twenty minutes after the start of these infusions, subjects received either an intravenous infusion of 20 U/kg National Reference Bacterial Endotoxin (Lot EC-6, prepared from E. coli 0113, USPC, Rockville, MD) over a 5-min period or an equivalent volume of sterile saline (150 mmol/l). Repeated measurements of mean arterial pressure (MAP), heart rate, oxygen consumption, and plasma hormone, cytokine, and leptin concentrations were performed over the following 6 h, as detailed in the following sections.

Indirect calorimetry. Oxygen consumption was measured for the last 30 min of each hour by use of open-circuit indirect calorimetry (Deltratrac, Datex, Helsinki, Finland). The calorimeter was calibrated before and after each measurement with the manufacturer’s recommended gases, having previously been validated by alcohol combustion and shown to deliver values within 98% of those predicted. All measurements were standardized to BSA.

Euglycemic hyperinsulinemic clamp. Insulin (Humulin S, Eli Lilly, Basingstoke, Hampshire, UK) was administered by primed-continuous infusion at 80 mU·m⁻²·min⁻¹ for 8 h, during which euglycemia (5 mmol/l) was maintained by a variable infusion of sterile aqueous glucose (20 g/100 ml, Baxter, Norfolk, UK). The concentration of glucose in arterialized venous plasma was monitored at 5-min intervals throughout the 8-h period using a Beckman glucose analyzer (Beckman Instruments, Fullerton, CA).

Sample collection. Arterialized venous blood samples were taken hourly for measurement of analyte concentrations. Samples for IL-6 and TNF-α assays were taken into EDTA. All other samples were taken into lithium heparin (10 U/ml).

Samples were centrifuged, and the plasma was separated and stored at −80°C pending analysis.

Biochemical analysis. Substrate and hormone concentrations. Plasma glucose concentrations were measured spectrophotometrically by use of a Cobas Bio Centrifugal analyzer (Roche Products, Welwyn, Garden City, Hertfordshire, UK). Commercially available radioimmunoassay (RIA) kits were used for measurement of plasma insulin (Pharmacia, Milton Keynes, UK) and plasma cortisol concentrations (Wallac Oy, Turku, Finland). Plasma growth hormone (GH) concentration was measured by fluor immunometric assay (Delfia, Wallac Oy). The maximum coefficient of variation (CV) for these assays was 5.5%. Plasma concentrations of norepinephrine were measured by reversed-phase HPLC and electrochemical detection, as described previously (20). Plasma IGF-1 concentration was determined by RIA as reported previously (11). The detection limit of the assay was 28 ng/ml, and inter- and intra-assay CVs were 5.2–7.4 and 4.0–5.7%, respectively. IGFBP-1 levels were also determined by RIA (42), utilizing human recombinant IGFBP-1 (donated by Dr. L. Fryklund, Pharmacia, Sweden) for standard and radiolabel and monoclonal antibody (MAb 6303, provided by Medix Biochemica, Kauniainen, Finland). MAb 6303 recognizes all isoforms of IGFBP-1, including the phosphoform characteristic of normal plasma. Detection limits for the assay were 3 μg/l, and inter- and intra-assay CVs were <8 and <6.8%, respectively. IGFBP-3 was measured by a commercially available RIA kit (Biocline Australia Pty, NSW, Australia). Quoted sensitivity was 3.5 ng/ml, with inter- and intra-assay CVs of <8.5 and <6%, respectively.

Cytokines and leptin. Plasma TNF-α and IL-6 concentrations were both measured by immunoenzymometric assay (Medgenix Europe, Pleurys, Belgium). Detection limits for TNF-α and IL-6 were 3 pg/ml and 2 pg/ml, respectively, with a mean CV of <10%. Plasma leptin concentration was measured using an RIA kit (Linco Research, St. Charles, MO), mean CV 3.4%. All samples were measured in triplicate, and the mean of each series was used in subsequent calculations.

Temperature, pulse, and blood pressure measurement. Tympanic membrane temperature was measured at 30-min intervals using an infrared probe (Thermoscan, San Diego, CA). Pulse rate and MAP were measured automatically every 30 min with an electronic monitor (BCI International, Waukesha, WI). In each case, the mean of three measurements taken over a 5-min period was used.
Hyperinsulinemia Augments the Response to Endotoxin

In Vitro Studies

Media and reagents. L-Glutamine and medium 199 (M199) were purchased from Life Technologies (Paisley, UK). Sterile sodium heparin and glucose infusate (20 g/dl) were obtained from C. P. Pharmaceuticals (Wrexham, UK) and Baxter-Clintec (Norfolk, UK), respectively. LPS (from E. coli 0128: B12), recombinant human insulin, and phenyldiamine dihydrochloride (OPD) were obtained from Sigma-Aldrich (Dorset, UK). Recombinant human leptin was purchased from R&D Systems (Minneapolis, MN). Hormone and LPS dilutions were prepared in M199 (+10% fetal calf serum and 2 mM glutamine).

Anti-human IL-6 monoclonal antibody was purchased from Eurogenetics (Middlesex, UK). Purified recombinant human IL-6 was obtained from Serono (Norwell, MA). Peroxidase-conjugated donkey anti-rabbit IgG was obtained from Jackson Immunoresearch Laboratories (Luton, UK). Rabbit anti-human IL-6 serum was produced after immunization of rabbits with recombinant human IL-6 (generously donated by Dr. L. A. Aarden, CLB, The Netherlands). Mouse anti-human TNF-α and biotinylated mouse anti-TNF-α monoclonal antibodies (clones 68B2B3/68B6A3 and 68B3C5, respectively) were purchased from Biosource International (Nivelles, Belgium). Streptavidin-horseradish peroxidase conjugate (streptavidin-HRP) was obtained from Zymed Laboratories (San Francisco, CA).

IL-6 and TNF-α ELISA. IL-6 concentration was measured in supernatants with a sandwich ELISA. Briefly, 96-well microtiter plates (Nalge NUNC, Roskilde, Denmark) were coated overnight at 4°C with either human anti-IL-6 or human anti-TNF-α MAb, at a concentration of 1 μg/ml in phosphate buffer (pH 7.2–7.4). After washing and blocking stages, freshly thawed samples and standards (recombinant human IL-6 at 0–1,000 pg/ml or human TNF-α at 0–1,600 pg/ml) were incubated in duplicate overnight at 4°C. Plates were washed before incubation at room temperature with rabbit anti-IL-6 polyclonal antibody for 2 h or with biotinylated anti-TNF-α for 1 h. Plates were then incubated at room temperature with peroxidase-conjugated donkey anti-rabbit IgG polyclonal antibody (1 in 2,500 dilution) for 1 h, or with 0.625 μg/ml streptavidin-HRP for 30 min. After washing, enzyme substrate (OPD 0.4 mg/ml in the presence of hydrogen peroxide) was added to each plate. IL-6 and TNF-α concentrations were measured using an automated plate reader (EL340 Microplate reader, Bio-tek Instruments, Winooski, Vermont) at 490 nm. The minimum threshold for detection in the assay was 10.2 pg/ml for IL-6 and 25 pg/ml for TNF-α. The intra-assay CVs for IL-6 and TNF-α were <10% at 125–500 pg/ml and 10–20% at 150–900 pg/ml, respectively.

Methods: effect of glucose, insulin, and leptin on cytokine production. Six healthy male volunteers (age range 28–45 yr, body mass index range 18.5–22.4), free of metabolic or inflammatory disease and not receiving any form of medication, were studied at 0800 after a 10-h fast. A 40-ml sample of venous blood was taken from each subject, and sterile sodium heparin was added to a final concentration of 10 U/ml.

Aliquots of heparinized venous blood (1 ml) were dispensed in 24-well microtiter plates (Nalge NUNC International, Forskilde, Denmark) in the presence or absence of supplementary glucose and/or insulin (to provide final well glucose and insulin concentrations, after supplementation, of 5, 12, or 30 mmol/l glucose and 1 mmol/l insulin). Samples were then incubated for 2 h at 37°C, after which LPS (100 ng/ml) or M199 (control) was added, and the samples were then incubated for a further 6 h. Cell-free supernatants were obtained by centrifugation and stored at −70°C pending measurement of cytokine concentrations. An identical procedure was followed for leptin studies, performed on samples from four of the subjects, in which leptin (0, 10, and 100 ng/ml) replaced the supplementary insulin in the incubation medium. Studies were performed in triplicate, and the mean was used in subsequent calculations.

Monocyte counts were determined for all blood samples, and supernatant cytokine concentrations were standardized to monocyte number to correct for interindividual differences. Blood leukocyte numbers were estimated, after dilution in white blood cell stain, by use of a modified Fuchs-Rosenthal hemocytometer. Differential white blood cell counts were obtained by counting 200 cells in blood smears stained with Romanowsky stains.

Statistical Analysis

Analysis of variance for repeated measures (MANOVA) was used to assess time effects and treatment-time interactions (clamp vs. saline). When MANOVA revealed a significant treatment-time interaction, post hoc unpaired comparisons between the groups were made with the Bonferroni test, with correction for multiple comparisons (24). For in vitro studies, one-way ANOVA was performed with post hoc analysis by the Neuman-Keuls test for intergroup comparisons and Dunnett’s test for intragroup comparison with control study arms. All calculations were performed using Graphpad Prism software (Graphpad Prism, San Diego, CA).

All data are expressed as means ± SD unless otherwise stated, and P < 0.05 was taken as the level of statistical significance.

RESULTS

In vivo studies

Subject characteristics. Six subjects (4 male and 2 female), mean age 33.7 ± 4.2 yr, mean weight 72.5 ± 12.6 kg, and BSA 1.8 ± 0.1 m², received LPS or saline during a euglycemic hyperinsulinemic clamp. Eight subjects (all male), mean age 32.0 ± 4.4 yr, mean weight 74.1 ± 7.8 kg, and BSA 1.9 ± 0.1 m², received LPS or saline during saline infusion. There were no significant differences between groups with respect to age, weight, or BSA. No variable changed significantly between the two study arms within each group.

Temperature, pulse, MAP, and oxygen consumption. Administration of LPS led to a significant pyrexia, irrespective of the presence of euglycemic hyperinsulinemia (P < 0.001, Table 1). Although the febrile response was, overall, significantly greater when LPS was administered under conditions of euglycemic hyperinsulinemia (F = 4.7, P < 0.05), there was no significant difference between the groups at any given time point, and peak pyrexia was similar in the two groups (38.1 vs. 37.8°C, Clamp/LPS vs. Saline/LPS, respectively). In parallel with the pyrexia, administration of LPS led to a significant tachycardia in both Clamp/LPS and Saline/LPS groups (Table 1). Administration of LPS during conditions of euglycemic hyperinsulinemia led to a significant prolongation of the tachycardia, however (F = 600, P < 0.001), with heart rate remaining at a plateau until 360 min, whereas...
Table 1. Physiological effects of endotoxemia in the presence or absence of euglycemic hyperinsulinemia

<table>
<thead>
<tr>
<th>Time, min</th>
<th>-60</th>
<th>0</th>
<th>60</th>
<th>120</th>
<th>180</th>
<th>240</th>
<th>300</th>
<th>360</th>
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<tr>
<td>T, °C</td>
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<tr>
<td>Clamp/LPS</td>
<td>36.8 ± 0.3</td>
<td>36.8 ± 0.3</td>
<td>36.9 ± 0.3</td>
<td>37.6 ± 0.4</td>
<td>38.1 ± 0.4</td>
<td>38.0 ± 0.4</td>
<td>37.8 ± 0.4</td>
<td>37.6 ± 0.4</td>
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<td>37.3 ± 0.5</td>
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<td>36.6 ± 0.3</td>
<td>36.6 ± 0.3</td>
<td>36.6 ± 0.3</td>
<td>36.7 ± 0.3</td>
<td>36.7 ± 0.3</td>
<td>36.5 ± 0.3</td>
<td>36.7 ± 0.3</td>
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<td>Clamp/Sal</td>
<td>36.4 ± 0.3</td>
<td>36.8 ± 0.1</td>
<td>36.7 ± 0.2</td>
<td>36.8 ± 0.2</td>
<td>36.8 ± 0.3</td>
<td>36.6 ± 0.3</td>
<td>36.8 ± 0.2</td>
<td>36.7 ± 0.2</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>Clamp/LPS</td>
<td>67.8 ± 2.7</td>
<td>69.0 ± 3.1</td>
<td>74.8 ± 4.1</td>
<td>83.4 ± 4.8</td>
<td>90.6 ± 11.1</td>
<td>95.7 ± 6.2*</td>
<td>96.5 ± 10.5*</td>
</tr>
<tr>
<td>Sal/LPS</td>
<td>65.2 ± 5.9</td>
<td>62.7 ± 7.5</td>
<td>67.9 ± 11.4</td>
<td>77.5 ± 9.2</td>
<td>90.3 ± 7.8</td>
<td>90.6 ± 10.3</td>
<td>87.0 ± 12.0</td>
<td>86.1 ± 13.4</td>
</tr>
<tr>
<td>Sal/Sal</td>
<td>66.3 ± 8.6</td>
<td>66.6 ± 9.0</td>
<td>64.2 ± 9.0</td>
<td>63.3 ± 9.3</td>
<td>65.1 ± 6.6</td>
<td>64.4 ± 8.8</td>
<td>62.9 ± 9.1</td>
<td>63.3 ± 6.7</td>
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<tr>
<td>Clamp/Sal</td>
<td>65.7 ± 3.0</td>
<td>65.4 ± 4.6</td>
<td>62.7 ± 5.1</td>
<td>66.5 ± 4.4</td>
<td>67.7 ± 4.8</td>
<td>66.3 ± 4.4</td>
<td>65.7 ± 3.8</td>
<td>67.6 ± 5.0</td>
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<tr>
<td>MAP, mmHg</td>
<td>Clamp/LPS</td>
<td>74.7 ± 4.9</td>
<td>77.3 ± 4.1</td>
<td>79.0 ± 2.6</td>
<td>84.9 ± 8.6</td>
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<td>Sal/LPS</td>
<td>76.0 ± 6.8</td>
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<td>82.7 ± 12.4</td>
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<td>79.7 ± 5.2</td>
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<td>73.4 ± 6.5</td>
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<tr>
<td>Clamp/Sal</td>
<td>75.1 ± 9.3</td>
<td>75.0 ± 3.8</td>
<td>76.9 ± 3.3</td>
<td>76.8 ± 6.4</td>
<td>75.0 ± 5.9</td>
<td>75.0 ± 3.5</td>
<td>76.0 ± 8.1</td>
<td>74.7 ± 4.6</td>
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<tr>
<td>VO2, ml·min⁻¹·m⁻²</td>
<td>Clamp/LPS</td>
<td>115.6 ± 9.3</td>
<td>118.5 ± 11.5</td>
<td>122.3 ± 13.1</td>
<td>136.8 ± 19.1</td>
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<td>Sal/LPS</td>
<td>119.8 ± 6.4</td>
<td>117.2 ± 6.9</td>
<td>119.0 ± 5.0</td>
<td>144.0 ± 26.5</td>
<td>144.3 ± 19.7</td>
<td>145.9 ± 18.7</td>
<td>145.4 ± 17.7</td>
<td>140.2 ± 10.6</td>
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<td>Sal/Sal</td>
<td>118.8 ± 7.0</td>
<td>119.4 ± 6.9</td>
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<td>129.1 ± 6.4</td>
<td>120.2 ± 8.3</td>
<td>120.5 ± 7.2</td>
<td>124.6 ± 5.3</td>
</tr>
<tr>
<td>Clamp/Sal</td>
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<td>121.4 ± 14.6</td>
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<td>120.2 ± 8.7</td>
<td>119.5 ± 7.7</td>
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</table>

Values are means ± SD. T, temperature; HR, heart rate; MAP, mean arterial pressure; VO2, oxygen consumption; LPS, lipopolysaccharide. See Study protocol in In Vivo Studies for description of groups. *P < 0.01, Clamp/LPS vs. Sal/Sal.

Table 2. Metabolic effects of endotoxemia in the presence or absence of euglycemic hyperinsulinemia

<table>
<thead>
<tr>
<th>Time, min</th>
<th>-60</th>
<th>0</th>
<th>60</th>
<th>120</th>
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<td>Glucose, mmol/l</td>
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<td></td>
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</tr>
<tr>
<td>Clamp/LPS</td>
<td>5.2 ± 0.3</td>
<td>5.0 ± 0.3</td>
<td>4.7 ± 0.2</td>
<td>5.1 ± 0.4</td>
<td>4.9 ± 0.6</td>
<td>5.3 ± 0.8</td>
<td>5.3 ± 0.4</td>
<td>4.9 ± 0.3</td>
</tr>
<tr>
<td>Sal/LPS</td>
<td>5.1 ± 0.2</td>
<td>5.0 ± 0.2</td>
<td>4.8 ± 0.3</td>
<td>4.3 ± 0.2*</td>
<td>5.0 ± 0.3</td>
<td>4.9 ± 0.2</td>
<td>4.7 ± 0.3</td>
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<tr>
<td>Sal/Sal</td>
<td>5.1 ± 0.3</td>
<td>4.8 ± 0.3</td>
<td>4.8 ± 0.2</td>
<td>4.8 ± 0.2</td>
<td>4.8 ± 0.2</td>
<td>4.7 ± 0.2</td>
<td>4.5 ± 0.2</td>
<td>4.5 ± 0.2</td>
</tr>
<tr>
<td>Clamp/Sal</td>
<td>5.3 ± 0.9</td>
<td>5.1 ± 0.5</td>
<td>5.2 ± 0.4</td>
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<td>5.1 ± 0.5</td>
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</tr>
</tbody>
</table>

Values are means ± SD. NE, norepinephrine. *P < 0.01, Saline/LPS vs. Saline/Saline; †P < 0.01, Clamp/LPS vs. Clamp/Saline; ‡P < 0.01, Saline/LPS vs. Saline/Saline.
Plasma insulin concentration was unaffected by the administration of LPS.

Plasma norepinephrine (Table 2) increased abruptly in both Clamp/LPS and Saline/LPS groups at 120 min after administration of LPS ($F = 5.5, P < 0.001$), returning rapidly to normal. There was, however, no significant effect of euglycemic hyperinsulinemia on the plasma norepinephrine response to LPS ($F = 1.1, P = 0.3$).

Plasma cortisol concentration (Fig. 1A) increased significantly in response to LPS administration, to a similar peak, at 240 min, irrespective of whether LPS was administered in conditions of euglycemic hyperinsulinemia or during saline infusion. When LPS was administered with saline alone, plasma cortisol levels declined rapidly after 240 min and returned to normal by 360 min. In contrast, when LPS was administered during euglycemic hyperinsulinemia, the rise in plasma cortisol was significantly prolonged ($F = 26.5, P < 0.001$), and plasma cortisol concentrations showed no sign of falling, even by the end of the study at 360 min after LPS administration.

Plasma GH concentration also increased significantly (Fig. 1B), reaching a peak 120 min after LPS administration ($F = 60.1, P < 0.001$). When LPS was administered during euglycemic hyperinsulinemia, however, the initial peak in plasma GH concentration was significantly higher than the plasma GH peak observed when LPS was administered during saline infusion ($48.4 \pm 11.8$ vs. $33.5 \pm 9.7$ mU/l, Clamp/LPS vs. Saline/LPS, $P < 0.001$, respectively). In addition, whereas plasma GH concentration returned rapidly to normal during saline infusion, GH concentrations remained significantly elevated for the duration of the study when LPS was administered during euglycemic hyperinsulinemia ($F = 130.6, P < 0.001$). Although infusion of saline alone had no effect on plasma GH concentration, prolonged euglycemic hyperinsulinemia resulted in a small but statistically significant increase in GH during the last 2 h of the study ($F = 23.4, P < 0.001$, Clamp/Saline vs. Saline/Saline).

The IL-6 response to LPS (Fig. 1C) was also significantly enhanced by prior infusion of glucose and insulin ($F = 10.4, P = 0.002$). The peak plasma IL-6 was almost doubled ($2,147.6 \pm 1,177.7$ pg/ml) when LPS was administered to subjects during euglycemic hyperinsulinemia, as opposed to the IL-6 response to LPS administration during infusion of saline ($1,074.5 \pm 745.0$ pg/ml). Plasma IL-6 concentrations were significantly greater at 120 ($t = 3.6, P < 0.05$) and 180 min ($t = 4.3, P < 0.001$) in subjects given LPS during euglycemic hyperinsulinemia than those in whom LPS was given during saline infusion. Infusion of saline, or euglycemic hyperinsulinemia alone, had no effect on plasma IL-6 concentration.

Plasma TNF-α concentration rose rapidly in response to LPS administration in both groups (Fig. 2A). Euglycemic hyperinsulinemia also increased the overall magnitude of the TNF-α response to LPS ($F = 4.5, P < 0.05$), although there was no significant difference between Clamp/LPS and Saline/LPS at any individual time point. Infusion of saline or glucose and insulin alone had no significant effect on plasma TNF-α concentration.

Plasma leptin concentration (Fig. 2B) increased significantly ($P < 0.01$) in response to euglycemic hyperinsulinemia, although significant increases were only observed 4 h after the start of the glucose-insulin infusion. No significant change in leptin concentration was observed after saline infusion, and administration of LPS had no effect on leptin concentration, irrespective of whether LPS was administered during euglycemic hyperinsulinemia or during saline infusion. No significant changes in the plasma concentration of IGF-I or IGFBP-3 were observed in response to any of the infusions. Plasma IGFBP-1 concentrations declined progressively during euglycemic hyperinsulinemia but were unaffected by LPS administration. The
The present data indicate, for the first time in humans, that key elements of the systemic inflammatory response to endotoxemia are significantly augmented by glucose-insulin infusion. Specifically, euglycemic hyperinsulinemic clamping significantly augmented the IL-6, GH, and cortisol responses to LPS administration, and these effects were associated with enhancement of LPS-induced tachycardia. In contrast, the fever, increase in oxygen consumption, and TNF-α and catecholamine responses to LPS were unaffected by euglycemic hyperinsulinemia. Although the increased subjective malaise associated with LPS administration during euglycemic hyperinsulinemia must be interpreted with caution, given the practical and ethical constraints that prevented blinding of the subjects in the study arms, it nevertheless suggests (when taken with the other findings) that the observed augmentation of the LPS response is an integrated pathophysiological effect and not simply a collection of unrelated biochemical changes.

The increased counterregulatory hormone response to LPS in the clamp group was a particularly surprising finding, because the plasma glucose concentration was maintained at ~5 mmol/l, whereas a brisk hypoglycemia developed in subjects given LPS alone, as described previously (10). Preventing the hypoglycemic response to LPS therefore appears to have been associated with an augmentation of the counterregulatory hormone response. One would have anticipated the opposite to have occurred on theoretical grounds, and
these findings therefore indicate that, in humans, hypoglycemia does not play a significant role in the counterregulatory hormone response to LPS. These human findings contrast with data from mice, in which it has been shown that glucose-induced increases in plasma insulin concentration significantly attenuate the TNF-α response to LPS (33), and with data from rabbits in which the response was significantly augmented by intraportal glucose infusion (18). In neither of these studies were counterregulatory hormone or other cytokine responses to LPS studied, although the cardiovascular response to LPS was also augmented in rabbits by glucose administration (18). Insulin has been shown to be essential for normal

Fig. 4. Effect of glucose, insulin, and leptin on IL-6 production by whole blood. Whole blood was coincubated with (open bars, n = 6) or without (solid bars, n = 6) insulin, 1 nmol/l (A, B) and with (open bars, 10 ng/ml, n = 4; gray bars, 100 ng/ml, n = 4) or without (solid bars, n = 4) leptin (C, D), at varying glucose concentrations, in the presence (B, D) or absence (A, C) of 100 ng/ml LPS. All data are means ± SE; ‡P < 0.01 vs. control (no added leptin). IL-6 in the supernatant was determined by ELISA.

Fig. 5. Effect of glucose, insulin, and leptin on TNF-α production by whole blood. Whole blood was coincubated with (open bars, n = 6) or without (solid bars, n = 6) insulin 1 nmol/l (A, B) and with (open bars, 10 ng/ml, n = 4; gray bars, 100 ng/ml, n = 4) or without (solid bars, n = 4) leptin (C, D), at varying glucose concentrations, in the presence (B, D) or absence (A, C) of 100 ng/ml LPS. All data are means ± SE. TNF-α in the supernatant was determined by ELISA.
cytokine production in bronchoalveolar macrophages after LPS inhalation in rodents (3), although it is unclear whether, as suggested in the current study, a further increase in the plasma insulin concentration would have led to amplification of the inflammatory response.

The primary metabolic and endocrine changes induced by glucose-insulin infusion in the present study concern glucose availability, plasma insulin and leptin concentrations, and the IGF axis. Although fasting plasma glucose concentrations were maintained during conditions of euglycemic hyperinsulinemia, glucose availability at the tissue level would undoubtedly have been substantially increased, despite the modest insulin resistance induced by LPS (2). Increased glucose availability has been shown to increase basal TNF-α and IL-6 (23) and LPS-stimulated IL-1 production (27) by human monocytes in vitro, suggesting increased glucose availability as a possible mechanism for potentiation of LPS-mediated effects in vivo. The findings of the in vitro studies, however, do not support this suggestion, because we were unable to demonstrate any potentiation of LPS-induced cytokine production by increasing glucose availability in vitro. It is unclear why we were unable to confirm these previous findings, but it should be noted that, unlike the previous studies, our experiments were conducted over shorter incubation periods and in whole blood as opposed to isolated monocytes. These circumstances thus more closely reflect the physiological environment of the in vivo studies. In addition, the glucose solution employed in the present study was designed for clinical use and was free of endotoxin (manufacturer's data), whereas the earlier studies employed glucose solutions prepared in the laboratory that could conceivably have been contaminated with endotoxin. This possibility is supported by the findings of additional studies in this laboratory (H. Duxbury, S. J. Hopkins, and G. L. Carlson, unpublished observations). Similarly, although pharmacological insulin concentrations have been shown to increase basal TNF-α production by murine monocytes in vitro (30), the insulin concentrations employed were far higher than those observed in vivo in the present study. The findings of our in vitro studies are in keeping with previous reports, which have also failed to demonstrate increased basal TNF-α and IL-6 production after incubation with insulin at concentrations similar to those employed in vivo (26) and, in fact, suggest modest reductions in LPS-induced cytokine production.

The in vivo studies confirm earlier reports of increased plasma leptin concentrations after glucose-insulin infusion in humans (19). In view of the demonstration that leptin might upregulate proinflammatory cytokine production from macrophages (17), the in vitro studies were undertaken to test the hypothesis that the enhanced in vivo systemic inflammatory and counterregulatory hormone response to LPS after glucose-insulin infusion might have been mediated by a direct action of leptin on cytokine-producing cells. The increase in plasma leptin after glucose-insulin infusion was, however, only statistically significant beyond 120 min (i.e., after 4 h of glucose-insulin infusion) and not at the time point at which LPS was administered. Although a modest dose-related increase in basal IL-6 production was observed after incubation of whole blood with leptin, the effect was observed only at supraphysiological leptin concentrations, and there was no apparent influence of leptin on LPS-stimulated cytokine production. These data contrast with those previously reported (17), but it should be noted that, in our study, the in vitro protocol matched as closely as possible the conditions of the in vivo experiment, and the lower dose of leptin used replicated the plasma leptin concentrations in vivo, for a similar time period before and after LPS exposure. In contrast, the study reported by Loffreda et al. (17) involved leptin concentrations that are unlikely to have been achieved under any physiological conditions, and a 24-h incubation period, so the physiological relevance of these data is unclear.

Although the in vitro studies were designed to match as closely as possible the conditions and time course of the in vivo studies, caution should be exercised when comparisons are drawn. The endotoxin exposure is likely to have been substantially different, and the cytokine response observed in vivo may reflect stimulation of cells such as Kupffer cells, as opposed to blood monocytes (10). Although it is unclear whether cytokine production in these cell populations is equally sensitive to endotoxin and subjected to the same regulatory factors, the data presented in the present study cast doubt on a direct role for increased glucose availability, hyperinsulinemia, or leptin in the augmentation of the cytokine response observed after LPS administration in humans and suggest that other, related factors might be of importance.

In vitro studies indicate that IGF-I may directly augment both basal and LPS-induced TNF-α and IL-6 production in vitro (30, 37). In vitro incubations with IGF-I were not undertaken in the present study, because the aim of the in vitro experiments was to mimic, as far as possible, the in vivo conditions under which mononuclear cells would be subjected to the hormone and substrate environment induced by hyperinsulinemia. Because the biological availability of IGF-I is influenced by the plasma concentration of binding proteins (and especially IGFBP-1), the in vivo conditions of this experiment would have been impossible to reproduce accurately in vitro by the simple addition of IGF-I. Nevertheless, the demonstration that glucose-insulin infusion in vivo significantly increases the molar ratio of IGF-I to IGFBP-1 and that IGF-I concentrations are unaffected by LPS administration confirms the findings of previous studies (15, 34). The biological actions of IGF-I are thought to be modulated by IGFBPs (5); in particular, the IGF-I-to-IGFBP-1 (IGF-I/IGFBP-1) molar ratio has been suggested as a surrogate marker of bioavailability (16). The absence of an increase in the IGF-I/IGFBP-3 molar ratio after euglycemic hyperinsulinemia implies that more IGF-I would have been available at the time when LPS was administered. It is therefore possible that at least some
of the augmented IL-6 production observed in vivo is related to the increased biological availability of IGF-I.

The augmentation of TNF-α release in the present study was modest compared with that reported in previous animal studies (18), and it seems unlikely that the enhanced IL-6 response could be attributed to this alone. Although infusion of TNF-α largely reproduces the systemic inflammatory response to LPS, suggesting a pivotal role of TNF-α as a proximal mediator, blockade of endogenous TNF-α has been shown to attenuate, but not to abolish, the systemic inflammatory response to LPS in primates (41) and humans (35, 40). In addition, parenteral nutrition and hyperinsulinemia have been shown to modulate expression of cell-associated TNF-α receptors, providing a potential mechanism for upregulation of TNF-α sensitivity (4, 12). Although this suggests that glucose-insulin infusion might act via increased sensitivity to TNF-α-mediated events, other sites of action or other cytokine-mediated pathways might be equally or more important, and further studies will be required to clarify the precise mechanisms of action of glucose and insulin.

The plasma insulin concentrations achieved during the present study are generally higher than those observed in clinical disease. Nevertheless, these findings raise important questions about the relationship between hyperinsulinemia associated with type 2 diabetes and increased plasma concentrations of biochemical markers of inflammation (21, 22, 28) and monocyte proinflammatory cytokine production after in vitro stimulation with LPS (7) in such patients. It has been suggested that chronic low-grade inflammation may induce the insulin resistance, hyperinsulinemia, and atherosclerosis characteristic of type 2 diabetes mellitus (29), although exogenous insulin infusion has also recently been reported to cause an acute reduction in markers of inflammation in obese humans (6). However, the inhibition of inflammation was related primarily to changes in monocyte intranuclear nuclear factor κB and circulating plasma concentrations of intercellular adhesion molecule-1, rather than to plasma cytokines or counterregulatory hormones. Furthermore, these changes occurred under basal (as opposed to endotoxin-stimulated) conditions, at circulating insulin concentrations significantly lower than those employed in the present study. The suggestion that insulin acutely downregulates some features of basal inflammatory activity is not, therefore, incompatible with our demonstration of increased responsiveness to an inflammatory stimulus in healthy subjects.

The present findings may also be of relevance to nutritional support in sepsis. Glucose-based nutrition in this patient population may cause marked hyperinsulinemia due to peripheral insulin resistance (31). The findings of the present study suggest that hyperinsulinemia associated with glucose-based nutrition in sepsis might augment proinflammatory cytokine production and the stress response in septic patients. It is unclear whether these effects occur during the clinically relevant conditions of glucose-based total parenteral nutrition as opposed to euglycemic clamping and whether they are of clinical significance, but a recent randomized controlled trial has shown that administration of insulin to critically ill patients substantially reduces mortality and severe infection rates (39). These beneficial effects of insulin therapy were observed primarily in patients who remained in the intensive care unit for >5 days. Although no measurements of plasma cytokine or counterregulatory hormone concentrations were presented, prolonged duration of critical illness is associated with a state of immune anergy, in which anti-inflammatory cytokines predominate, predisposing the long-stay critically ill patient to infective morbidity (25). The findings of the present study suggest a potential mechanism for the beneficial effects of insulin therapy, attributable to modulation of the immune anergy associated with critical illness.

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REFERENCES


HYPERINSULINEMIA AUGMENTS THE RESPONSE TO ENDOTOXIN


