Insulin Suppresses Endotoxin Induced Oxidative, Nitrosative and Inflammatory Stress in Humans

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Objective. To investigate whether insulin reduces the magnitude of oxidative, nitrosative and inflammatory stress and tissue damage responses induced by endotoxin (LPS).

Methods. Nine normal subjects were injected with 2ng/Kg of LPS prepared from E. coli intravenously. Ten others were infused with insulin (2U/h) for 6h in addition to the LPS injection along with 100ml/hr of 5% dextrose to maintain normoglycemia.

Results. LPS injection induced a rapid increase in plasma concentrations of nitric oxide metabolites, nitrite and nitrate (NOM), thiobarbituric acid reacting substances (TBARS), increase in reactive oxygen species (ROS) generation by polymorphonuclear leucocytes (PMNL) and marked increases in plasma free fatty acids, tumor necrosis factor α (TNFα), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), macrophage migration inhibition factor (MIF), C-reactive protein (CRP), resistin, visfatin, lipopolysaccharide binding protein (LBP), high mobility group-B1 (HMG-B1) and myoglobin concentrations. The co-infusion of insulin led to a total elimination of the increase in NOM, FFA, TBARS and a significant reduction in ROS generation by PMNL and plasma MIF, visfatin, myoglobin concentrations. Insulin did not affect TNFα, MCP-1, IL-6, LBP, resistin and HMGB-1 increases induced by the LPS.

Conclusions. Insulin reduces significantly several key mediators of oxidative, nitrosative and inflammatory stress and tissue damage induced by LPS. These effects of insulin require further investigation for its potential use as anti-inflammatory therapy for endotoxemia.

Endotoxin (lipopolysaccharide, LPS) induces inflammation by binding to its specific receptor, toll like receptor-4 (TLR-4) (1;2). This leads to the nuclear translocation and activation of NFKB, the major pro-inflammatory transcription factor and an increase in the transcription of pro-inflammatory genes with a corresponding increase in the concentration of the products of these genes in plasma. Endotoxemia in association with clinical Gram negative septicemia syndrome in the human leads to a high incidence of morbidity and mortality, especially in patients in intensive care units.

Insulin has been shown to suppress inflammatory changes, in vitro, and, in vivo. It suppresses intranuclear NFKB and Egr-1 binding and the expression of several pro-inflammatory mediators including intercellular adhesion molecule (ICAM)-1 and monocyte chemoattractant protein (MCP)-1, MMP-9, CRP and SAA (3;4). In addition, it suppresses the expression of several TLRs (5). It also suppresses reactive oxygen species (ROS) generation and p47phox expression.

Resistin and visfatin, two peptides originally discovered as adipokines (6;7), have now been shown to be products of pro-inflammatory myeloid cells (8;9) and to induce pro-inflammatory cytokines, chemokines and ROS. Both have been implicated in atherogenesis (10).

LPS induces an increase in the expression of inducible nitric oxide synthase and NO release (11) from macrophages. LPS is also known to stimulate the generation of ROS including superoxide.

There are recent data showing that LPS injection in experimental animals leads to a reduction in myocardial function. Insulin administration in LPS injected animals reduces LPS induced damage (12).

On the basis of the above, we hypothesized that (a) the injection of LPS in
normal human subjects will induce an increase in ROS generation, lipid peroxidation; nitrosative stress as reflected in plasma concentrations of NO metabolites, nitrite and nitrate; plasma free fatty acid concentration, plasma myoglobin concentrations; novel adipokines, resistin and visfatin, and LBP in parallel with pro-inflammatory cytokines like TNF-α, IL-6 and macrophage migration inhibitory factor (MIF); and high mobility group box-1 (HMGB-1) protein which acts like a pro-inflammatory cytokine when released into the circulation; and (b) insulin will suppress LPS induced increases in ROS generation, lipid peroxidation, plasma concentrations of NOM, free fatty acids, resistin, visfatin, markers of tissue damage including myoglobin, HMGB-1, TNFα and IL-6.

SUBJECTS & METHODS
Subjects. Nineteen normal weight (Body mass index (BMI): 20-25 Kg/m²) healthy male subjects aged between 20 to 33 years (mean age 26±3 years) were recruited for this study. Following an overnight fast, nine subjects were injected with 2ng/Kg of LPS prepared from E. coli intravenously along with saline at 100 ml/hr. The other ten were infused with insulin (2U/h) 1h prior to the LPS injection along with 100 ml/hr of 5% dextrose co-infused with insulin to maintain normoglycemia. Insulin/dextrose or saline infusions continued for 6 hrs following the LPS injection while in fasting state to avoid the potential pro-inflammatory effect of a meal (13). They were then provided with a 900 calorie meal at 6PM after which they ate nothing till the next morning. Subjects were monitored for vital signs (temperature, pulse, blood pressure, headaches, body aches and chills) for 24 hrs following the LPS injection. Blood samples were collected 1h before the LPS injection and at 0, 1, 2, 4, 6 and 24 hrs following the injection. The protocol was approved by the internal review board of the State University of New York at Buffalo and written consent was obtained from all subjects.

Polymorphonuclear Leukocytes (PMNL) isolation and ROS generation. Blood samples were collected and PMNL were isolated and ROS generation measured as previously described (14). The intra-assay coefficient of variation (CV) for ROS generation is 8%.

Measurement of glucose, insulin, FFA, NOM and thiobarbituric acid reacting substances (TBARS) concentrations and HOMA-IR calculation. Plasma concentrations of glucose were measured by an YSI 2300 STAT Plus glucose analyzer (Yellow Springs, Ohio). Insulin concentrations were measured from plasma samples using an enzyme-linked immunosorbent assay (ELISA) kit (Diagnostics Systems Laboratories, Inc., Webster, TX). Free fatty acids (FFA) concentrations were measured using the Half-Micro calorimetric kit from Roche Diagnostic (Indianapolis, IN). NOM (NO₂/NO₃) were assayed by Griess reaction (R&D systems, Minneapolis, MN) and TBARS was assayed by spectrofluorometry with a kit from Zeptometrix (Buffalo, NY). The CVs for these assays ranged from 2-7% and 4-11% for intra- and inter-assay variations, respectively. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated according to the formula: (fasting insulin (µU/ml) X fasting glucose (mmol/L))/22.5.

Measurements of Plasma cytokines, CRP, visfatin, resistin, myoglobin, HMGB-1 and LBP concentrations. Commercially available ELISA assays were used to measure concentrations of circulating cytokines concentrations (R&D systems, Minneapolis, MN), CRP (American Diagnostica Inc., Stamford, CT), visfatin (Phoenix Pharmaceuticals Inc, Belmont, CA), myoglobin (Life Diagnostics Inc., West Chester, PA), HMGB-1 (IBL Transatlantic Corp) and LBP (Cell Sciences, Canton, MA). The CVs for
these assays ranged from 3-8% and 6-11% for inter- and intra-assay variations, respectively.

**Statistical Analysis.** Statistical analysis was conducted using SigmaStat software (SPSS Inc., Chicago, IL). All data are represented as mean ± S.E. Statistical Analysis from baselines was carried out using Holm-Sidak one-way repeated measures analysis of variance (RMANOVA). Dunnett’s two-factor RMANOVA method was used for multiple comparisons between different groups. Paired t-test was used to compare changes from baseline at 24 h.

**RESULTS**

**Clinical Features.** LPS injection induced an increase in temperature from 97.7±0.5°F to a peak of 100.9±0.9°F at 4h, systolic blood pressure from 113±11 to a peak of 129±13 at 2h and pulse rate from 63±7/min to a peak of 97±9/min at 6h and was not affected by insulin (Table 1). Headaches, chills and body aches scores increased following LPS injection with peaks at 1h to 2h with insulin infusion reducing body aches score significantly (Table 1) without affecting headaches and chills.

**Blood Leukocyte Counts.** Following LPS, total leukocyte count increased from a baseline of 4,300±900 at -1h to a peak of 11,800±1,200 cells/mm³ at 6h and was still elevated (7,000±1,100 cells/mm³) at 24h, mainly attributable to polymorphonuclear leucocytosis (Table 1). Monocytes and lymphocytes fell rapidly from 6±0.4% to 0.5±0.1% and from 39±5% to 3±0.8%, respectively. Insulin infusion did not alter this pattern. The marked reduction in monocytes and lymphocytes prevented us from examining ROS generation and other cellular markers/mediators in MNC fraction.

**Plasma Insulin, Glucose, Free Fatty Acid, Triglyceride, LDL-cholesterol and HDL-cholesterol Concentrations.** In the group receiving insulin, insulin concentrations increased by up to 4 fold ($P<0.001$, Table 1). In the control group, insulin concentrations did not change in the first 6h but were significantly higher than baseline at 24h ($P<0.05$, Table 1). There was no significant change in glucose concentrations. Consistent with that, HOMA-IR increased significantly at 24h from 1.22±0.24 vs. 2.36±0.39 in the control group (Table 1). Following LPS injection, there was a significant increase in plasma FFA concentration. Insulin infusion prevented this increase (Table 1). Serum triglyceride concentration fell significantly in both groups (Table 1). LDL-cholesterol, VLDL-cholesterol and HDL-cholesterol concentrations did not alter.

**ROS Generation by PMNL.** LPS injection induced an increase in ROS generation by PMNL by 200±42% over the basal with a peak at 1h and another peak at 4-6h. Insulin infusion reduced (Figure 1A) ROS generation throughout the infusion period ($P<0.05$, by two-factor RMANOVA).

**Plasma TBARS Concentrations.** LPS injection induced a rapid increase in TBARS concentration from 1.29±0.29 to 2.15±0.41μM ($p<0.01$) at 1h, with a return to baseline at 2h (Figure 1B). This pattern was observed in each of the LPS injected subjects. Insulin infusion totally prevented this increase.

**Plasma NOM Concentrations.** Plasma NOM concentration increased rapidly after the injection of LPS at 1h peaked to 75±24% over the baseline at 2h (from 29.4±2.6 to 47.7±5.4 μM) and declined to the baseline by 4h. There was a secondary rise in NOM concentration at 6h (Figure 1C). With insulin infusion, the LPS induced increase in NOM was totally prevented and in fact there was a small but significant decrease by 16±10% below the baseline in plasma NOM concentrations (from 31.7±2.8 to 26.9±2.6 μM).

**Plasma MIF, TNFα, IL-6 and CRP Concentrations.** Plasma concentrations of MIF increased significantly following LPS injection at 1h with a secondary increase at 4-
6h from 727±111 to 1345±145ng/ml at 6h (Figure 2A). Insulin infusion with LPS prevented the LPS induced increase of MIF during the initial increase and suppressed it significantly during the secondary increase (from 700±137 to 1080±87ng/ml at 6h). Plasma concentration of TNFα increased at 1h, peaked between 1h and 2h (P<0.001, Table 1) and declined thereafter, reaching near the baseline at 24h. IL-6 increased at 1h, peaked at between 2h and 4h (P<0.001, Table 1), and declined towards the baseline by 24h. CRP concentration increased at 6h and was still elevated at 24h (from 1.2±0.2 to 15.2±5.7 mg/l, P<0.001, Table 1). Insulin infusion did not alter the LPS induced increases in TNFα, IL-6 or CRP concentrations.

**Plasma resistin, visfatin and LBP concentrations.** Plasma visfatin concentrations increased significantly following LPS injection starting at 4h, peaking at 6h (87±37% above baseline, from 11.4±1.2 to 19.2±2.4ng/ml, P<0.001, Figure 2B) and maintained at that level for 24h. When insulin was infused prior to LPS injection, visfatin concentrations fell significantly from 11.9±1.5 to 7.7±1.10ng/ml (P<0.001, Figure 2B) at 4hr and were significantly different than that in the control group. LPS injection also caused a significant increase in resistin concentrations which started at 2h following the injection, peaked at 6h (223±25% above baseline, from 8.1±1.1 to 24.7±4.7 ng/ml P<0.001, Table 1) and continued to be higher than baseline at 24h. Plasma LBP concentrations increased gradually following LPS injection and was higher by 48±19% above the baseline at 24h (P<0.05, Table 1). Insulin did not cause any significant change in the LPS induced increases in resistin or LBP concentrations.

**Plasma myoglobin and HMGB-1 concentrations.** Plasma concentration of myoglobin also increased significantly from 22.3±4.1 to 32.8±5.3ng/ml at 4h and to 39±5.8ng/ml (p<0.05) at 24h following LPS injection (Figure 3B). Insulin prevented the increase in myoglobin concentrations. HMGB-1 concentrations in plasma increased following LPS injection starting at 1 hr and peaked at 6 h (Figure 3C) while insulin infusion had no effect on LPS induced increase in HMGB-1 concentrations.

**DISCUSSION**

Our data has several novel observations on the effects of LPS and insulin. They demonstrate for the first time, the increase in ROS generation, TBARS, NOM, resistin, visfatin, myoglobin and HMGB-B1 concentrations in humans, in vivo, following an LPS injection. They also show for the first time that insulin infusion reduces or totally prevents the LPS induced increases in ROS generation and the concentrations of TBARS, NOM, MIF, visfatin and myoglobin. The relevance of each of these novel effects is discussed below.

LPS induced increase in ROS generation by PMNL and TBARS concentration is evidence of marked oxidative stress. Insulin suppressed the increase in ROS generation significantly while eliminating the increase in TBARS altogether. Following LPS, NOM peaked at 2h following and returned to the baseline by 4h. Insulin infusion eliminated the increase in NOM concentration. These actions of insulin were independent of any change in glucose concentrations.

Elevated plasma NOM concentrations and iNOS expression in the liver have previously been shown to be suppressed by insulin infusions in patients in intensive care (15). In this study, the plasma concentrations of NOM in the highest quartile were associated with seven times greater mortality than those in the lowest quartile. Thus, NOM concentration could be an important predictor of morbidity and mortality in the ICU setting (16). Whether this effect on mortality is directly related to an excess of NO generation or whether the increased NOM levels are merely
markers of the intensity of systemic inflammation is not clear. Either way, the rapid induction of an increase in NOM by LPS and its total prevention by insulin are important and relevant observations.

The biphasic increase in MIF following LPS was reduced by insulin infusion. On the other hand, insulin infusion did not prevent LPS induced increases in plasma TNFα, IL-6, ICAM-1 and MCP-1 concentrations. This is in contrast to our previous observation in patients with obesity and type 2 diabetes in whom insulin suppresses these mediators. CRP concentrations began to increase at 6h after the LPS injection at which time the infusion of insulin ended. Clearly, further studies with higher doses and for longer periods of insulin infusion are required.

LPS also induced an increase in plasma FFA concentrations within 1h which continued for 24h, consistent with a potent lipolytic effect of LPS. This increase was totally inhibited by insulin. The suppressive effect of insulin FFA is important since FFAs may induce oxidative and inflammatory stress (17).

Our data also show for the first time that LPS injection in the human induced an increase in plasma concentrations of resistin and visfatin. Insulin infusion resulted in the suppression of the increase in visfatin but not resistin. The LPS induced increase in visfatin and the prevention of this increase with insulin is of interest in terms not only of the acute LPS induced inflammation but also in terms of the chronic inflammation in atherosclerotic plaques since such plaques are known to contain LPS and TLR-4 expressing macrophages (18). Such plaques also express visfatin which may be secreted locally in response to the LPS-TLR-4 interaction (10). It is, therefore, of interest that insulin suppresses LPS induced increase in visfatin and has previously been shown to suppress TLR-4 expression (5). Resistin is also known to stimulate the secretion of pro-inflammatory cytokines and the evidence that its concentration increases after LPS in the human, in vivo, establishes it as a pro-inflammatory mediator (19).

Plasma concentrations of LBP also increased after LPS injection, demonstrated for the first time. The increase started late, at 6h, like that of CRP and the previously described increase in procalcitonin (20) and continued overnight at 24h. The increase in the concentration of LBP following LPS is important since LBP facilitates the binding of LPS to its receptor, TLR-4. As with CRP the increase in LBP was not affected by insulin, possibly because both increased at 6h and the insulin infusion was stopped at that time.

It is of interest that while plasma glucose concentrations did not alter significantly, insulin concentrations and HOMA-IR increased significantly 24h after LPS injection in concert with the induction of profound inflammation. This is consistent with the recent observation that the injection of LPS (3 ng/Kg) in normal subjects induced insulin resistance as measured by frequently sampled intravenous glucose tolerance (FSIGT) and HOMA-IR (21).

Our data also show for the first time that LPS induces an increase in HMG-B1 concentration in humans. Insulin did not alter this increase. HMGB-1 is a nuclear protein which binds to histones to promote pro-inflammatory gene transcription. It can be released from damaged, necrotic tissues. Circulating HMG-B1 acts like a pro-inflammatory cytokine through its binding to the receptor for advanced glycation end products (RAGE) (22).

The increase in plasma myoglobin concentrations following LPS and its inhibition by insulin is important since it signifies damage to the skeletal muscle and possibly the myocardium. Consistent with this, we have previously shown a reduction in the increase in myoglobin concentrations in
patients with myocardial infarction treated with intravenous insulin infusions. This is suggestive of a cytoprotective effect of insulin.

The mechanisms underlying the effects insulin observed in this report are probably related to several of our previous observations. Insulin suppresses the expression of TLR-4, the receptor for LPS, and to reduce the activity of the major pro-inflammatory transcription factor, NFκB (5, 3). Insulin has also been shown to suppress ROS generation and the expression of the p47 subunit of NADPH oxidase. Insulin has been shown previously to suppress iNOS expression and plasma NOM concentration (15).

Consistent with our observations are those of Jeschke and colleagues (23) who have demonstrated that compared to controls, patients with severe burns given insulin had lower MIF and other pro-inflammatory cytokines and CRP concentrations with a tendency towards higher IL-10 concentrations. Insulin infusion with maintenance of normoglycemia has been shown to reduce mortality and morbidity in patients in a surgical ICU (24) and in patients in a medical ICU (25) whose stay in the ICU is for longer than 3 days.

In conclusion, the injection of LPS in the human induces an immediate increase in ROS generation by PMNL and plasma concentrations of TBARS, NOM, FFA, MIF, resistin, visfatin, LBP and myoglobin. The concomitant infusion of insulin induces a significant reduction in ROS generation and the total prevention of the increase in TBARS, NOM and FFA concentrations. These actions were associated with a significant reduction in the magnitude of increase in MIF, myoglobin and visfatin concentrations independently of any change in plasma glucose concentrations. On the other hand, insulin was not able to prevent or reduce the magnitude of increase in plasma concentrations of pro-inflammatory cytokines like TNFα, IL-6 or MCP-1. Clearly, the effect of more prolonged infusions and higher doses of insulin needs to be investigated.


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REFERENCES


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in murine and human lipid-rich atherosclerotic plaques and upregulated by oxidized LDL. 
*Circulation* 104:3103-3108, 2001


Table 1: Changes in clinical, oxidative and inflammatory endpoints following 2ng/Kg injection of LPS alone or LPS and 2U/hr insulin for 6 hr in normal healthy subjects. Data are presented as mean±SE. *: P<0.05 with One-way RMANOVA; #:Two-way ANOVA. $: P<0.05 with Paired t-test at 24hr compared to -1hr, NS=not significant

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<td>1.21±0.23</td>
<td>1.13±0.21</td>
<td>1.46±0.36</td>
<td>3.07±0.52</td>
<td>15.0±3.1</td>
<td>0.001</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>LPS+Ins</td>
<td>1.27±0.37</td>
<td>1.27±0.39</td>
<td>1.28±0.29</td>
<td>1.21±0.34</td>
<td>1.56±0.35</td>
<td>3.39±0.61</td>
<td>14.25±1.3</td>
<td>0.001</td>
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</tr>
<tr>
<td>TNFα (pg/ml)</td>
<td>LPS</td>
<td>1.2±0.7</td>
<td>1.2±0.7</td>
<td>123±29</td>
<td>136±40</td>
<td>18±6</td>
<td>8.3±2.2</td>
<td>2.6±1.1</td>
<td>0.002</td>
<td>NS</td>
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<td></td>
<td>LPS+Ins</td>
<td>0.9±0.6</td>
<td>0.9±0.6</td>
<td>138±34</td>
<td>134±43</td>
<td>21±8</td>
<td>8.1±2.1</td>
<td>2.1±0.8</td>
<td>0.001</td>
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<tr>
<td>IL-6 (pg/ml)</td>
<td>LPS</td>
<td>2.49±0.8</td>
<td>2.49±0.8</td>
<td>25.7±8</td>
<td>48.9±4.5</td>
<td>48.3±6.1</td>
<td>22.8±5.3</td>
<td>4.8±1.1</td>
<td>0.002</td>
<td>NS</td>
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<td></td>
<td>LPS+Ins</td>
<td>1.9±0.8</td>
<td>1.9±0.8</td>
<td>26.9±7</td>
<td>50.7±4.3</td>
<td>49.2±18</td>
<td>24.3±4.3</td>
<td>4.4±2.8</td>
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<td>MCP-1 (ng/ml)</td>
<td>LPS</td>
<td>727±111</td>
<td>727±111</td>
<td>1056±133</td>
<td>1003±117</td>
<td>1081±140</td>
<td>1345±145</td>
<td>828±138</td>
<td>0.008</td>
<td>0.028</td>
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<td>LPS+Ins</td>
<td>713±102</td>
<td>713±102</td>
<td>700±137</td>
<td>863±127</td>
<td>1093±121</td>
<td>1080±87</td>
<td>885±139</td>
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<td>LBP (μg/ml)</td>
<td>LPS</td>
<td>13.2±2.6</td>
<td>13.3±2.8</td>
<td>15.02±2.8</td>
<td>15.7±3.0</td>
<td>15.8±3.0</td>
<td>17.6±5.3</td>
<td>21.2±6.8</td>
<td>0.021</td>
<td>NS</td>
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<td>LPS+Ins</td>
<td>11.5±2.0</td>
<td>11.1±1.7</td>
<td>12.0±2.2</td>
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<td>14.8±2.8</td>
<td>20.2±3.8</td>
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<td>Resistin (ng/ml)</td>
<td>LPS</td>
<td>7.72±0.9</td>
<td>7.78±0.7</td>
<td>9.57±0.8</td>
<td>17.69±1.3</td>
<td>23.8±2.2</td>
<td>24.7±2.4</td>
<td>11.69±1.5</td>
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<td>NS</td>
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<td>LPS+Ins</td>
<td>8.15±1.1</td>
<td>8.04±0.9</td>
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<td>24.95±2.6</td>
<td>11.35±1.6</td>
<td>0.001</td>
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Figure Legends:

Figure 1: Change in ROS generation by PMNL (A) and plasma concentrations of TBARS (B) and NOM (C) following a 2ng/kg LPS injection (at 0h) in normal subjects with and without a 2U/h infusion of insulin with 5% Dextrose for 6 hours. N=10 & 9 each. Data are presented as mean±SE. * and **: P<0.05 by RMANOVA from baseline, # P<0.05 by 2-way RMANOVA between the groups.

Figure 2: Change in plasma concentrations of MIF (A) and visfatin (B) following a 2ng/kg LPS injection (at 0h) in normal subjects with and without a 2U/h infusion of insulin with 5% Dextrose for 6 hours. N=10 & 9 each. Data are presented as mean±SE. * and **: P<0.05 by RMANOVA from baseline, # P<0.05 by 2-way RMANOVA between the groups.

Figure 3: Change in plasma concentrations of myoglobin (A) and HMGB-1 (B) following a 2ng/kg LPS injection (at 0h) in normal subjects with and without a 2U/h infusion of insulin with 5% Dextrose for 6 hours. N=10 & 9 each. Data are presented as mean±SE. * and **: P<0.05 by RMANOVA from baseline, # P<0.05 by 2-way RMANOVA between the groups.

Figure 1

A

![Graph showing change in ROS generation by PMNL](image-url)
B.  

![Graph showing % Change in Plasma TBARS](image)  

**Time (Hours)**  

-1 0 1 2 4 6 24  

- % Change in Plasma TBARS  

Endotoxin  
Endotoxin + Insulin  

C.  

![Graph showing Plasma NO2/NO3 Concentrations (μM)](image)  

**Time (Hours)**  

-1 0 1 2 4 6 24  

- Plasma NO2/NO3 Concentrations (μM)  

LPS + Saline  
LPS + Insulin  

*#  
**  
**  
**
Figure 2

A

![Graph of Plasma MIF Concentrations](image)

B

![Graph of Plasma Visfatin Concentrations](image)
Figure 3

A

% Change in Troponin-i Concentrations

B

Plasma Myoglobin Concentrations (ng/ml)
Hours following Endotoxin

HMGB-1 (ng/ml)

- Endotoxin
- Endotoxin+Insulin

*  **