Increase In Plasma Endotoxin Concentrations And The Expression Of Toll Like Receptors And Suppressor Of Cytokine Signaling-3 In Mononuclear Cells Following A High Fat High Carbohydrate Meal: Implications For Insulin Resistance

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Objective: To compare effect of a high fat high carbohydrate meal (HFHC) with that of a high fiber and fruit meal on the concentrations of endotoxin (LPS), lipopolysaccharide binding protein (LBP), the expression of toll like receptors (TLR) and the suppressor of cytokine signaling-3 (SOCS-3) in mononuclear cells (MNC).

Research Design and Methods: Healthy lean subjects were given 910 Calories of either a HFHC meal (n=10) or an American Heart Association (AHA) recommended meal rich in fiber and fruit (n=10) following an overnight fast. Blood was collected before and at 1h, 2h and 3h after the meal. Cellular indices of oxidative and inflammatory stress, the expression of SOCS-3, TLR2 and TLR4 in MNC and plasma concentrations of LPS and LBP were measured.

Results: HFHC meal intake induced an increase in plasma LPS concentration and the expression of SOCS-3, TLR2 and TLR4 protein, reactive oxygen species (ROS) generation and nuclear factor κ B (NFκB) binding activity ($P<0.05$, for all). These increases were totally absent following the AHA meal rich in fiber and fruit.

Conclusions: The novel changes described following HFHC meal elucidate further the mechanisms underlying post prandial inflammation and also provide the first evidence explaining the pathogenesis of insulin and leptin resistance mediated by SOCS-3 following such meals. In contrast an AHA meal does not induce these effects.
We have shown previously that macronutrients including glucose and cream and high fat-high carbohydrate (HFHC) meals induce inflammation and oxidative stress as reflected in increased reactive oxygen species (ROS) generation, increased expression of p47phox, an NADPH oxidase subunit, and Nuclear Factor Kappa B (NFκB) binding and other pro-inflammatory mediators in mononuclear cells (MNC) and plasma in normal subjects (1). In contrast, ROS generation by MNC and other indices of oxidative damage in the body fall with short-term caloric restriction (2) and weight loss in human obesity over a period of 4 weeks (3). We have also shown that a 48 hour fast in normal subjects reduces ROS generation by MNC, p47phox expression and oxidative damage of phenylalanine (3). Caloric restriction and weight loss over longer periods of time also result in a reduction in the concentrations of pro-inflammatory cytokines and CRP (3).

Toll like receptors (TLR) are a variety of pathogen pattern recognition receptors (PRR) which recognize pathogen associated molecular patterns (PAMPs) from bacterial and viral products and other pathogens (4). TLR4 recognizes endotoxin (lipopolysaccharide, LPS). On the basis of its interaction with LPS, TLR4 may be a mediator and a modulator of endotoxin induced inflammation and shock. TLR4 has also been shown to play an important role in the pathogenesis of atherosclerosis (5), diet-induced obesity and the related insulin resistance (6). TLR2, in a heterodimeric association with TLR1 or TLR6, recognizes certain lipopeptides, peptidoglycans and other lipid moieties derived from Gram positive bacteria (7). Both TLR4 and TLR2 are expressed in atherosclerotic plaques (8).

Recent work has also demonstrated that the expression of TLR2 and TLR4 is increased in patients with type 1 and type 2 diabetes (9,10). In addition, it has also been shown that the plasma concentration of LPS is significantly higher in these patients and that its concentration is related to plasma insulin concentration and insulin resistance (9). High fat diet induced insulin resistance and obesity are known to be TLR4 dependent such that TLR4 deletion protects mice from NFκB mediated inflammation and the development of insulin resistance (6). We have recently shown that a low dose insulin infusion in type 2 diabetics caused a significant suppression of TLR1, 2, 4, 7 and 9 mRNA expression and the suppression of PU.1, a major transcription factor which regulates the expression of many TLRs in addition to exerting its known anti-inflammatory action (11). However, the possibility that food intake may either increase TLR expression or increase plasma endotoxin concentration has not been investigated in the human.

Suppressor of cytokine signaling-3 (SOCS-3) has been shown to interfere with insulin and leptin signal transduction (12,13) in experimental animals. Pro-inflammatory cytokines, Tumor Necrosis Factor alpha (TNFα), Interleukin (IL-1β) and (IL-6), induce an increase in the expression of SOCS-3 in animal models (13). Our recent work has shown that SOCS-3 expression in the circulating mononuclear cells (MNC) of the obese human is markedly increased in parallel with other indices of inflammation and insulin resistance, when compared to that in normal subjects (14,15).
Since macronutrient intake causes inflammation and oxidative stress we have now hypothesized that the intake of a high fat high carbohydrate meal (HFHC), induces SOCS-3, TLR2 and TLR4 in parallel with the induction of cellular oxidative stress and inflammation and that this is associated with an increase in plasma endotoxin (lipopolysaccharide, LPS) and LPS binding protein (LBP) concentrations. In addition, we hypothesized that a meal based on fiber and fruit as recommended by the American Heart Association does not induce inflammation or an increase in the expression of TLRs and SOCS-3 and the plasma concentrations of LPS or LBP.

METHODS

Subjects: Two groups (10 each) of normal healthy lean (BMI<25 kg/m²) subjects 20-50 years old were included in the study. Subjects from both group had comparable mean age, BMI and gender distribution. After an overnight fast, a baseline blood sample was collected. Subjects from the first group (5 males, BMI=23.1±0.6 kg/m², mean age=32.4±1.3yrs) were asked to ingest a 910 Calories HFHC meal (egg muffin and sausage muffin sandwiches and two hash browns which contain 88g carbohydrates, 51g fat (33% saturated) and 34 g protein, carbohydrates 41%, protein17%, fat 42%)) while subjects from the 2nd group (6 males, BMI=22.8±0.6 kg/m², mean age=31.2±1.1yrs) were given an isocaloric meal rich in fruit and fiber consisting of oatmeal, milk, orange juice, raisins, and peanut butter, English muffin (carbohydrates 58%, protein15%, fat 27%) as recommended by the American Heart Association (AHA). This meal has been termed ‘AHA’ meal in the subsequent text of this paper. Blood samples were collected at 0, 1, 2 and 3 h. None of the subjects was a smoker and those who drank alcohol, had less than 30ml per day. The study was approved by the human research committee of the State University of New York at Buffalo. Each informed participant signed a consent form.

MNC isolation: Blood samples were collected in Na-EDTA as an anticoagulant. Three and a half mL of anticoagulated blood sample were carefully layered over 3.5 mL of PMN medium (Cedarlane Laboratories, Hornby, ON). Samples were centrifuged and at the end of the centrifugation, two bands separate out at the top of the RBC pellet. The MNC band was harvested and washed twice with Hank’s balanced salt solution (HBSS). This method provides yields greater than 95% pure PMN and MNC suspensions.

ROS Generation Assay. Five hundred µl of PMN or MNC (2 x 10⁵ cells) were delivered into a Chronolog Lum-Aggregometer plastic flat bottom cuvette. Fifteen µl of 10 mM luminol was then added, followed by 1.0 µl of 10 mM formylmethionyl leucinyl phenylalanine (fMLP). Chemiluminescence was recorded for 15 minutes. Our method, developed independently, is similar to that published by Tosi and Hamedani (16) correlated with that measured by the ferricytochrome C method (16). Intra and inter-assay variation in ROS generation is < 10% for readings obtained 1 to 2 weeks apart in normal healthy subjects.

Western blotting and NFκB binding activity: Total cell lysates and nuclear extracts were prepared from freshly isolated MNC. Consensus sequence for electromobility shift assay for NFκB binding activity (Santa Cruz Biotechnology, CA). Monoclonal antibodies against p47phox (BD Biosciences), TLR2, TLR4, CD14 and
SOCS-3 (Abcam, Cambridge, MA) and a polyclonal antibody against actin (Santa Cruz Biotechnology, CA) were used and the membranes were developed using super signal west-femto, chemiluminescence reagent (Pierce Chemical, IL). Densitometry was performed using molecular analyst software (Biorad, CA) and all values were corrected for loading with actin.

**LPS, LBP and Pro-inflammatory Mediators Measurements in plasma:** Plasma LPS concentration was measured by a commercially available kit (Cambrex Limulus Amebocyte Lysate (LAL) kit, Lonza Inc. Walkersville, MD). This assay has a sensitivity range of 0.1 EU/ml – 1.0 EU/ml. Normal values from lean subjects measured in our laboratory ranged from 0.15-0.35 EU/ml. **Inter and intra-assay variations for this test is <10%**. Plasma samples used for LPS determination were stored in LPS-free glass tubes to prevent loss of endotoxin to plastic tubes wall. All materials used for the assay were rendered LPS-free. Plasma was diluted 10 folds and heated to 75°C for 5 min prior to LPS measurement. LBP was measured using an immunoassay kit from (Cell Sciences, Canton, MA) **with an intra-assay variations of <5% calculated from our results**. Plasma MMP-9 and TNFα ELISA kits were purchased from R&D Systems (Minneapolis, MN) with intra and inter-assay variation of <5% and <10% respectively. **CRP measurement was done using immunoassay kits from Alpha Diagnostic International (San Antonio, TX) with coefficient of variation for intra-assay precision of <5% and inter-assay precision of <7%**.

**Measurement of Plasma insulin and free fatty acid (FFA) concentrations.** Insulin was measured from plasma samples using an enzyme-linked immunosorbent assay kit (Diagnostics Systems Laboratories, Inc., Webster, TX). FFA concentrations were measured using the Half-Micro calorimetric kit from Roche Diagnostic (Indianapolis, IN). **Inter and intra-assay variations for FFA test is <10%**.

**Statistical analysis.** Statistical analysis was carried out using SigmaStat software (Systat Software, Inc., San Jose, CA). Data is presented as mean±S.E. The percent change is calculated from the means of the groups. Percent change from baseline was calculated and statistical analysis was carried out using Holm-Sidak one-way repeated measures analysis of variance (RMANOVA). Dunnett’s two-factor RMANOVA method was used for comparisons between the two groups. Correlation analysis was performed using Spearman rank order correlation between changes in TLRs and SOCS-3 protein and markers of oxidative stress and inflammation.

**RESULTS**

**Effect of HFHC and AHA meals on glucose, insulin and lipids concentrations.** The intake of HFHC meal induced a significant increase in glucose concentration at 1h, 2h and 3h. However, AHA meal did not induce an increase at these times. In fact, there was a tendency for the glucose concentration to fall. However, there was
as a similar increase in insulin concentrations after both meals. (Table 1) Both the HFHC and AHA meals increased triglyceride concentrations. The increase following HFHC meal was significantly greater. FFA concentrations fell significantly following both meals.

**Effect of HFHC and AHA Meals on Oxidative stress.** ROS generation by MNC and PMN increased significantly by a peak of 78±20% and 65±23% over the baseline, respectively, 2h after the HFHC meal. (Table 1). NADPH oxidase subunit, p47\textsuperscript{phox}, also increased significantly at 1h by 32±18% over the baseline (P<0.05) after the HFHC meal and continued thereafter (Table 1). TBARS concentrations increased gradually and significantly following the HFHC meal and reached to 43±17% above the baseline at 3hr (Table 1). AHA meal intake was not associated with any significant change in ROS generation, p47\textsuperscript{phox} expression or TBARS.

**Effect of HFHC and AHA meals on NF\textsubscript{\kappa}B DNA binding in the MNC and Plasma MMP-9, CRP and IL-6 Concentrations.** DNA binding by NF\textsubscript{\kappa}B increased significantly by 72±24% over the baseline (P<0.05, Table 1) at 2h after HFHC meal intake while AHA did not cause any significant change in NF\textsubscript{\kappa}B. (Table 1) HFHC meal induced an increase in MMP-9 concentration while there was no significant increase in MMP-9 following the AHA meal. In addition, there was no change in plasma CRP and TNF-\alpha concentrations following either meal.

**Effect of HFHC and AHA meals intake on SOCS-3, TLR2 and TLR4.** SOCS-3, TLR2 and TLR4 expression increased significantly at 2 and 3 hr following HFHC meal (Figure 1). There was no significant change in these proteins following AHA meal intake. CD14 expression and PU.1 binding did not change after either meal.

**Effect of the HFHC and AHA meals on plasma LPS and LBP concentrations.** Plasma endotoxin (LPS) concentrations increased significantly following the intake of the HFHC meal from 0.39±0.07 to 0.58±0.10 EU/ml at 3h (47±14% over the baseline, \(P<0.05\), Figure 2A). This was associated with an increase in plasma levels of the LBP by 34±14% over the baseline at 2h (from 10.5±1.5 to 14.7±2.2 g/ml, \(P<0.05\), Figure 2B). The intake of the AHA meal did not induce any significant change in either LPS or LBP plasma concentrations. The total LPS content of HFHC and AHA meal measured from meal homogenates was 4,200EU and 5,700EU, respectively.

**Relationships between SOCS-3 and TLR expression and indices of oxidative stress and inflammation.** The maximum percent increase in TLR2 protein (at 3h) was significantly related to maximal changes in NF\textsubscript{\kappa}B and ROS generation in the MNC at 2h (\(r= 0.414, P<0.01\) and \(r= 0.488, P<0.01\), respectively). There was a trend towards a significant relationship between TLR4 protein and both ROS generation and NF\textsubscript{\kappa}B (\(r= 0.274, P=0.10\) and \(r= 0.321, P=0.10\), respectively). There was no relationship between SOCS-3 protein levels at 3 h and either NF\textsubscript{\kappa}B or ROS generation. The expression of SOCS-3 was significantly related to that of TLR4 (\(r= 0.612, P=0.003\)).

**DISCUSSION**

Our data show clearly for the first time that HFHC meal intake acutely induces an increase in SOCS-3, TLR4 and TLR2 protein expression in parallel with the increase in ROS generation, p47\textsuperscript{phox} expression, NF\textsubscript{\kappa}B binding activity and plasma MMP-9 concentrations. We have
also shown for the first time that the intake of such a meal induces an increase in plasma LPS and LBP concentrations. On the other hand, the intake of AHA meal, rich in fruit and fiber, did not cause any significant increase in any of these pro-inflammatory mediators including the expression of SOCS-3, TLR2 and TLR4. These observations are relevant to the pathogenesis of post-prandial oxidative and inflammatory stress, insulin resistance and atherosclerosis since the cumulative effects of such meals may manifest not only in chronic oxidative and inflammatory stress but also potentially insulin resistance and atherosclerosis. While chronic intake of high fat diets has been shown to lead to insulin resistance through an increase in TLR2 and TLR4 expression in experimental animals, there are no data on the effect of a single meal on TLR2, TLR4 or SOCS-3 expression in either humans or experimental animals.

It is relevant that insulin, the hormone secreted in response to macronutrient intake, exerts the opposite effect: it suppresses the expression of TLR4 and TLR2, and exerts a general anti-inflammatory effect and ROS suppressive effect (11;17). Clearly, the magnitude of insulin increase following a HFHC meal is not able to neutralize the pro-inflammatory effect of this meal including the induction of SOCS-3, TLR4 and TLR2. In contrast, a similar insulin response after the fruit and fiber AHA meal is associated with a total absence of post prandial inflammation.

There was also a significantly greater increase in glucose and triglyceride concentrations after the HFHC meal than that after the AHA meal. Glucose concentrations following HFHC meal were still elevated at 1h and 2h while those after the AHA meal were not significantly different from the baseline. This occurred in spite of the greater amount of carbohydrate in the AHA meal. This phenomenon is intriguing but we have previously observed it when comparing the effect of glucose with orange juice (18). Insulin increase following the AHA meal was similar to that after HFHC meal in spite of the fact that the excursion of glucose was significantly greater after the latter meal. Both meals resulted in similar and rapid reductions in FFA concentrations, probably due to insulin release.

It is of interest that PU.1, the major transcription factor regulating TLR gene expression, did not alter following HFHC intake in spite of the increase in the expression of TLR expression. It is thus likely that the increased protein expression of TLRs 4 and 2 following the HFHC meal is mediated by other transcription factors which need further exploration. It is, nevertheless, relevant that the suppression of TLRs by insulin is associated with a reduction in PU.1 activity (11).

Since the onset of oxidative stress and inflammation occur early after the intake of the HFHC meal as observed in our data, it is unlikely that the induction of TLRs contributes to the early inflammatory response following their intake. It is, however, possible TLRs may modulate the latter part of the response or modify the response to a subsequent pro-inflammatory challenge. It would be of interest to determine the duration of the increase in TLR expression in response to macronutrient challenge including repeated challenge and also determine the possible increased expression of TLRs in human obesity, the metabolic syndrome and type 2 diabetes. We have previously shown that the magnitude of oxidative and inflammatory stress after a
HFHC meal is greater and more prolonged in the obese when compared to normal subjects (19).

We also observed an increase in the plasma concentration of LPS and LBP following the HFHC meal intake but not after the AHA meal. While the LPS content of the meal probably contributes substantially to the increase in plasma concentrations of LPS, it is possible that some contribution also comes from LPS in the gastrointestinal tract since LPS is fat soluble. In addition, it has recently been shown that fat intake leads to increased intestinal permeability for LPS (20). However, it was remarkable that the AHA meal did not alter plasma LPS concentrations in spite of having LPS content similar to that of the HFHC meal.

LBP serves the function of binding and carrying LPS in plasma to CD14 which initially binds to LPS and then presents it to TLR4 which triggers the LPS induced signal transduction cascade. This result in NFκB activation and the transcription of pro-inflammatory genes. The concomitant increase in plasma LPS, LBP and TLR4 expression is the perfect combination to aggravate inflammation induced by Gram negative organisms. The increase in LBP concentrations following HFHC meal is of interest since its concentrations have previously been shown to be inversely related to insulin sensitivity (21).

Our observations are relevant to the pathogenesis of diet induced obesity and insulin resistance since the deletion of TLR4 and TLR2 in mice leads to the prevention of high fat diet induced insulin resistance (6;22). Thus, the acute induction of increased TLR2 and TLR4 expression by a single HFHC meal is intriguing and may contribute to insulin resistance after chronic and repetitive intake of such a meal. Our data are consistent with those of Dasu et al who demonstrated that high concentrations of glucose induce an increase in TLR expression, in vitro (23).

Our data show clearly for the first time that the intake of a HFHC fast food meal results in a significant increase in the expression of SOCS-3 protein in the circulating MNC. In contrast, there was no change in SOCS-3 expression following the AHA meal. The induction of SOCS-3 following a HFHC meal, in combination with the fact that its expression is increased in the obese suggests the possibility that repeated and chronic excessive fat and carbohydrate intake may result in a chronic increase in its expression. We have recently shown that SOCS-3 expression is increased in the obese and is inversely related to the phosphorylation of the insulin receptor, and that it is directly related to insulin resistance (HOMA-IR), BMI and other indices of inflammation (24).

Our observations are to some extent limited by the fact that we do not have detailed data on their dietary and exercise habits prior to their participation in this study. Just as the state of obesity can affect the oxidative and inflammatory stress responses to macronutrient challenge, eating habits and exercise may also do so. However, this fact should not affect the validity of our data since the responses with the HFHC meal and the AHA meal were consistently observed in each participant and the two groups tested were similar.

In conclusion, the intake of HFHC meal results in an increase in SOCS-3, TLR4 and TLR2 mRNA and protein expression in parallel with an increase in LPS and LBP concentrations and the induction of a comprehensive oxidative and inflammatory stress response characterized by an increase in ROS
generation and NFκB binding in MNC. The intake of the HFHC meal, therefore, potentially results in the induction of molecules which interfere with insulin and leptin signal transduction, a characteristic of human obesity. In contrast, an equicaloric fruit and fiber meal does not induce these changes. These observations have important implications for the understanding of the pathogenic mechanisms involved in post-prandial inflammation, diet induced obesity, insulin resistance, type 2 diabetes and atherosclerosis.

Figures Legends:

**Figure 1**: SOCS-3 (A&B), TLR2 (C&D) and TLR4 (E&F) protein levels by western blotting following HFHC or AHA meal challenges in normal healthy subjects (n=10 each). A) Representative SOCS-3 immunoblot in total cell lysate from MNC. B) SOCS-3 protein densitometry (n=10 each). C) Representative TLR2 immunoblot in total cell lysate from MNC. D) TLR2 protein densitometry (n=10 each). E) Representative TLR4 immunoblot in total cell lysate from MNC. F) TLR4 protein densitometry (n=10 each).*= \( P<0.05 \) by RMANOVA compared to baseline and #= \( P<0.05 \) by two-way RMANOVA comparing groups.

**Figure 2**: Plasma A) endotoxin and B) LBP concentrations following HFHC or AHA meal challenges in normal healthy subjects (n=10 each). Plasma endotoxin levels were measured by LAL assay. Basal endotoxin concentrations in HFHC group was 0.39±0.07 EU/ml and in AHA was 0.29±0.07 EU/ml. *= \( P<0.05 \), RMANOVA compared to baselines and #= \( P<0.05 \) by two-way RMANOVA comparing groups.
REFERENCES:
Table 1: Change in metabolic profile following 910 Calories AHA or HFHC meal in normal subjects (n-10 each); Data is represented as mean±SE*: P<0.05 compared to baseline with One-way RMANOVA, # P<0.05 between groups with two-way RMANOVA.

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<td></td>
</tr>
<tr>
<td>HFHC</td>
<td>2.09±0.32</td>
<td>1.93±0.27</td>
<td>2.00±0.30</td>
<td>1.96±0.27</td>
<td></td>
</tr>
<tr>
<td><strong>CRP (µg/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AHA</td>
<td>1.40±0.3</td>
<td>1.45±0.2</td>
<td>1.47±0.3</td>
<td>1.50±0.3</td>
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<tr>
<td>HFHC</td>
<td>1.43±0.2</td>
<td>1.43±0.2</td>
<td>1.52±0.2</td>
<td>1.54±0.2</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1

1A  SOCS-3

Hours following challenge

0 1 2 3

HFHC Meal

AHA Meal

1B

% Change in SOCS-3 Protein

0 1 2 3

Hours After Challenge

1C  TLR2

Hours following challenge

0 1 2 3

HFHC Meal

AHA Meal
1D

### TLR2

**% Change in TLR2 Protein in MNC**

- **AHA meal**
- **HFHC Meal**

1E

#### TLR4

**Hours following challenge**

- **HFHC Meal**
- **AHA Meal**

1F

### TLR4

**% Change in TLR4 Protein in MNC**

- **AHA meal**
- **HFHC Meal**

* * #
Figure 2

**2A**

Graph showing % Change in Plasma Endotoxin Concentrations over Hours After Meal.

**2B**

Graph showing Change in Plasma LRP Concentrations (μg/ml) over Hours After Meal.