Differential Effects of Cream, Glucose and Orange Juice on Inflammation, Endotoxin and the Expression of Toll Like Receptor-4 and Suppressor of Cytokine Signaling-3

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**Background:** We have recently shown that a high fat high carbohydrate (HFHC) meal induces an increase in plasma concentrations of endotoxin (LPS), and the expression of toll-like receptor-4 (TLR-4) and suppressor of cytokine signaling-3 (SOCS3) in mononuclear cells (MNC) in addition to oxidative stress and cellular inflammation.

**Hypothesis:** Saturated fat and carbohydrates, components of the HFHC meal, known to induce oxidative stress and inflammation, also induce an increase in LPS, TLR-4 and SOCS3.

**Methods:** Fasting normal subjects were given 300 Calorie drinks of either glucose, saturated fat as cream, orange juice or only water to ingest. Blood samples were obtained at 0, 1, 3 and 5 hours for analysis.

**Results:** Indices of inflammation including NFκB binding, and the expression of SOCS3, TNFα and IL-1β in MNC increased significantly following glucose and cream intake, but TLR-4 expression and plasma LPS concentrations increased only after cream intake. The intake of orange juice or water did not induce any change in any of the indices measured.

**Conclusions:** While both glucose and cream induce NFκB binding and an increase in the expression of SOCS3, TNFα and IL-1β in MNC, only cream caused an increase in LPS concentration and TLR-4 expression. Equicaloric amounts of orange juice or water did not induce a change in any of these indices. These changes are relevant to the pathogenesis of atherosclerosis and insulin resistance.
Our recent work has shown that a HFHC meal induces oxidative and inflammatory stress in addition to inducing an increase in plasma endotoxin (lipopolysaccharide, LPS) levels and the expression of toll like receptor-4 (TLR-4), the specific receptor for LPS (1). In contrast, a high fiber and fruit meal does not induce any of these changes. These data are of great interest since the content of LPS in these meals is not significantly different and thus, it would appear that the inflammatory nature of the meal may lead to a partial breakdown of the intestinal barrier which normally protects the body from invasion of bacteria and the entry of LPS from the gut. The concept of this immunological barrier of the gut has developed rapidly over the past few years and is vital to the protection from bacterial toxins and immunological responses to the commensal and pathogenic intestinal bacteria.

In this context, we wanted to analyze which macronutrient was responsible for the induction of oxidative stress and inflammation on the one hand and the increase in LPS concentrations and the expression of TLR-4 and SOCS3 on the other. In order to elucidate this, we investigated the effect of glucose, the most important carbohydrate, cream, a saturated fat and orange juice, a carbohydrate containing food product which does not induce either oxidative stress or inflammation.

Suppressor of cytokine signaling-3 (SOCS3) is a protein which has been shown to interfere with insulin and leptin signal transduction (2-5). Our recent work has shown that SOCS3 expression in the circulating mononuclear cells (MNC) of the obese human is markedly increased when compared to that in normal subjects (6). In addition, our work demonstrated that SOCS3 expression in MNC is inversely related to the tyrosine phosphorylation of the insulin receptor and directly related to BMI and insulin resistance (HOMA-IR), consistent with its role in the pathogenesis of insulin resistance. Leptin resistance in human obesity leads to the inability of leptin to cause satiety and weight loss while insulin resistance makes the obese vulnerable to diabetes. Human obesity is also a state of chronic inflammation characterized by an increase in inflammatory mediators in plasma, in adipose tissue and in circulating mononuclear cells (7;8). Since SOCS3 is induced in animal models by pro-inflammatory stimuli like the cytokines, TNFα, IL-6 and IL-1β (3;4;9), and since macronutrient intake causes oxidative stress (10;11) and inflammation (12;13), it is possible that the intake of glucose and saturated fat (cream) induces an increase in the expression of SOCS3 as a part of macronutrient induced inflammatory stress in parallel with increases in the activation of the pro-inflammatory transcription factor, nuclear factor κB (NFκB) and the expression of TNFα, IL-6 and IL-1β.

Recent work has shown that high fat diet induced insulin resistance is TLR-4 dependent such that TLR-4 deletion protects mice from NFκB mediated inflammation and the development of insulin resistance (14;15). In addition, it has also been shown that the plasma concentration of LPS is significantly increased in type 2 diabetic patients and that its concentration is significantly related to plasma insulin concentration and HOMA-IR, an index of insulin resistance, showing a link between LPS concentration and insulin resistance (16). However, there are no data available on the effect of specific macronutrients on TLR expression or on inflammatory processes triggered by TLR dependent mechanisms.

On the basis of the above, we hypothesized that the intake of glucose and cream induces an increase in the expression of SOCS3 and TLR-4 and the plasma concentration of LPS as a part of post
prandial inflammation induced by a high fat, high carbohydrate meal (1). We hypothesized further that since orange juice does not induce oxidative stress or inflammation, its intake will not induce either an increase in the expression of SOCS-3 or TLR-4 or that of plasma LPS concentrations. We also examined the expression of other SOCS family members, SOCS1 and SOCS7 in these experiments for comparison with the effect on SOCS3 and since they are also thought to contribute to insulin resistance in experimental animal models.

SUBJECTS AND METHODS

**Subjects.** Four groups (12 each) of healthy normal weight (Body Mass Index (BMI) of 21.5-24.4 Kg/m², age 25-47yrs) subjects ingested either 75 g (=300 Calories) of glucose (Glucola drink, Fisher Scientific, Pittsburgh, PA), 33g (=300 Calories) of cream (gourmet heavy whipping cream, Land ‘O’ Lakes Inc., Arden Hills, MN), an equicaloric amount of orange juice or 300 ml water following an overnight fast. The fat content of the dairy cream used includes 70 % saturated fat; 28% unsaturated fat, a protein content of less than 2% and no carbohydrates. All subjects were given 10 minutes to finish their drinks. Blood samples were collected before and at 1, 3 and 5 hours following glucose, cream or water intake. The Human Research Committee of the State University of New York at Buffalo approved this protocol. Informed consent was obtained from all subjects.

In our previous work, we had used orange juice obtained from a local supermarket and had used portions of the juice from half or one gallon packages for multiple experiments. To minimize any potential for instability of orange juice constituents, we used packages of recently produced ‘Not from Concentrate’ Florida Orange Juice (provided by the Florida Department of Citrus). Each package, once opened, was discarded after a single experiment.

**MNC isolation and NFκB DNA binding activity.** Fasting blood samples were collected in Na EDTA as an anticoagulant. MNC isolated and NFκB DNA binding activity was measured as described previously (1).

**Total RNA isolation and Real Time RT-PCR.** Total RNA isolation and RT-PCR for SOCS3, TLRs inflammatory cytokines was performed as previously described (1:8). The specificity and the size of the PCR products were tested by adding a melt curve at the end of the amplifications and by running it on a 2% agarose gel. All values were normalized to expression of 3 housekeeping genes (β-actin, cyclophylin and ubiquitin).

**Western blotting:** MNC total cell lysates were prepared and electrophoresis and immunoblotting was carried as described before (12). Polyclonal or monoclonal antibodies against TLR2 (Imgenex, San Diego, CA) SOCS3 and TLR4 (Abcam Inc., Cambridge, MA) and actin (Santa Cruz Biotechnology, Santa Cruz, CA) were used and all values were corrected for loading to actin levles.

**Measurement of Plasma glucose, insulin, FFA, concentrations.** Insulin, glucose and FFA were measured as previously described (1).

**Plasma endotoxin and LBP concentrations:** Plasma endotoxin concentration was measured by a commercially available kit (Cambrex Limulus Amebocyte Lysate (LAL) kit, Lonza Inc. Walkersville, MD) as previously described (1). LBP was measured using an immunoassay kit from (Cell Sciences, Canton, MA).

**Determination of LPS content in food challenges:** Dilutions comparable to the amounts ingested from cream, glucose and orange juice were prepared in plasma or endotoxin free water. Plasma was used as a diluent to maintain similar testing medium as in the post challenge LPS measurements. LPS concentrations were then measured as
described using LAL assay and endotoxin was calculated (n=3 each).

**Statistical analysis.** Statistical analysis was carried out using SigmaStat software (Systat Software, Inc., San Jose, CA). All the figures are represented as mean±S.E. Percent change was calculated from baselines and represented as mean±S.E. Statistical Analysis of changes 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 all multiple comparisons between different groups.

**RESULTS**

**Plasma Glucose, Insulin and Lipid concentrations.** There was no significant difference in fasting plasma glucose, insulin or FFA between he groups. There was a significant increase in glucose (from 80±2 to 110±9mg/dl, \( P<0.01 \)) at 1h following glucose intake while there was no significant change in glucose concentrations following orange juice, cream or water. Insulin concentrations increased significantly by 10 and 6.6 folds \( (P<0.001) \) following glucose and orange juice intake, respectively, but did not change significantly following cream or water intake. Lipid concentrations did not change following glucose, orange juice or water intake while the intake of 33g (300 Calorie) cream caused a significant increase in the plasma concentration of free fatty acids (from 0.32±0.09 to 0.60±0.14mM), triglycerides (from 103±59 to 171±60mg/dl) and VLDL cholesterol (from 19.5±13 to 32.3±15 mg/dl at 3 hours \( P<0.01 \) for all). There was no significant change in the concentration of total cholesterol, LDL cholesterol and HDL cholesterol following cream.

**Effect of glucose, orange juice and cream intake on SOCS3 expression.** The intake of glucose caused a significant increase in the mRNA expression of SOCS3 following cream intake by 45±14%, 56±15% over the baseline at 3h and 5h respectively, \( (P=0.014, \text{ Fig. 1A}) \) while there was no significant change in SOCS3 mRNA following orange juice or water intake. SOCS1 and SOCS7 mRNA expression did not change significantly following any challenge (data not shown). SOCS3 protein in MNC also increased significantly after the intake of glucose and cream by 45±16% and 53±18% over the baseline, respectively, but not after orange juice or water \( (P<0.05, \text{ Fig. 1C&D}) \).

**Effect of glucose, orange juice and cream intake on TLR-4 expression.** The intake of glucose did not result in any change in TLR4 expression while cream intake induced a significant increase in TLR-4 mRNA expression by 37±11% over the baseline \( (P<0.05, \text{ Fig. 1B}) \). Orange juice or water did not induce any change in TLR-4. Similarly, there was a significant increase in TLR4 protein levels in MNC following cream intake by 38±18% over the baseline but not following glucose, orange juice or water intake \( (P<0.05, \text{ Fig. 1C&E}) \). There was no significant change in TLR2 mRNA or protein expression following any of the macronutrient challenges (data not shown).

**Effect of glucose, orange juice and cream intake on pro-inflammatory cytokine expression.** TNFα mRNA expression in MNC increased significantly by 53±16% at 1h and 51±10% at 3h, over the baseline following glucose and cream intake, respectively \( (P<0.05, \text{ Fig. 2A}) \). There was also a significant increase in IL-1β mRNA expression in MNC following glucose and cream intake by 96±25% at 5h and 168±32% at 3h over the baseline, respectively \( (P<0.01, \text{ Fig. 2B}) \). On the other hand, IL-6 expression did not alter significantly following glucose or cream intake (data not shown). There was no increase in the expression of any of these cytokines following orange juice or water.
Effect of glucose, orange juice and cream intake on NFκB DNA binding. DNA binding by NFκB increased significantly by 53±17% and 54±18% over the baseline (P<0.05, Fig. 3A) at 3h following glucose and cream intake, respectively. There was no significant change in NFκB binding activity following orange juice or water intake.

Effect of glucose, orange juice and cream intake on plasma LPS and LBP concentrations. Plasma endotoxin concentrations increased significantly following the intake of cream from 0.29±0.03 to 0.41±0.07 EU/ml at 3 hrs (45±17% over the baseline, P<0.05, Fig. 3B) but not after glucose, orange juice or water intake. The concentration of LPS in plasma following the intake of cream continued to be significantly higher than basal levels up to 5h. Endotoxin content of cream and orange juice drinks were 104±24 EU/ml and 85±21 EU/ml, respectively. This was equivalent to a total endotoxin load of 10,400EU for the cream taken and 55,250EU for the orange juice drink. Glucola drink and water did not contain measurable endotoxin. LBP concentration increased significantly after the intake of glucose by 16% over the baseline at 5h (from 9.4±0.8 to 11±1.2µg/ml, P<0.05) but not after cream or orange juice.

DISCUSSION

Our data show clearly for the first time that the intake of either 33g of cream or 75g glucose resulted in a significant increase in the expression of SOCS3 mRNA in the circulating MNC. With cream, this increase was observed at 1h and continued till at least 5h which time the experiment ended. Glucose caused an increase in SOCS3 which peaked at 1h (83% over baseline) and was still elevated at 5h. In parallel with the induction of SOCS3, there occurred an increase in NFκB binding, consistent with an acute inflammatory response in MNC. On the other hand, while the intake of cream resulted in an increase in both plasma LPS concentration and TLR-4 expression, glucose had no effect on either TLR-4 expression or plasma LPS concentration. Orange juice did not induce either SOCS3 or TLRs; nor did it induce an inflammatory response.

It would thus appear that the oxidative stress and inflammation inducing actions of the HFHC meal are due to the combination of saturated fat and the carbohydrate (glucose) at least. On the other hand, only cream induced an increase in plasma LPS concentration and an increase in the expression of TLR-4, the receptor for LPS. Thus, saturated fats may have a more profound role in the pathogenesis of post prandial inflammation, as they may also perpetuate inflammation through the increases in LPS and TLR-4. By implication, saturated fats also appear to increase the permeability of intestinal epithelium and contribute to the breakdown of the intestinal barrier. Both carbohydrate and saturated fat induced an increase in the expression of SOCS3, the mediator of interference in insulin signal transduction. In contrast, orange juice does not induce any of the changes of oxidative and inflammatory stress or an increase of either LPS or TLR-4 or SOCS3. By implication, it may not affect the intestinal barrier either, as there is no increase in plasma LPS concentration after orange juice, in spite of it containing similar concentrations of endotoxin as cream. It is possible that observed effect of orange juice is attributable to its flavonoids, naringenin and hesperidin, since they exert ROS suppressive (17) and anti-inflammatory effects. These potent effects have been demonstrated in experimental animal models in relation to endotoxin induced inflammation and in cells, in vitro (18;19). In this context, it is important to state that our recent data demonstrate that the consumption of orange juice with a HFHC meal prevents not only oxidative stress and inflammation but also the increase in LPS concentrations and the increase in the
expression of TLR-4 and SOCS3, observed after ingesting the HFHC meal alone (unpublished data).

It is also relevant that LBP increased after glucose intake but not after cream. Thus, the increase in LBP following an HFHC meal observed by us was probably due to the carbohydrate component of the meal. LBP did not alter after the intake of orange juice or water either.

SOCS3 has previously been shown to be induced by TNFα, IL-6 and IL-1β and is thus a product of inflammation. Thus, it is of interest that mRNA for TNFα and IL-1β were induced by glucose and cream intake in parallel with the increase in NFκB binding. However, it was surprising that IL-6 was not induced by either glucose or cream. SOCS3, as its name suggests, was initially discovered as a molecule which interferes with cytokine signaling. It has since been shown to also interfere with both insulin and leptin signal transduction (4;20).

The induction of SOCS3 by cream and glucose intake in combination with the fact that its expression is increased in the obese suggests the possibility that chronic excessive fat and carbohydrate intake may result in a chronic increase in SOCS3 expression. It is also of interest that the intake of such macronutrients may result in resistance to leptin, one of the major signals which promotes satiety and thus potentially reduces food intake. Similarly, it is intriguing that the intake of a macronutrient should cause the induction of a molecule (SOCS3) which would interfere with the signal transduction of insulin, a hormone which causes the assimilation of nutrients following a meal including the distribution and storage of fat, carbohydrates and proteins. Since the intake of a modest amount of pro-inflammatory macronutrients led to the induction of SOCS3 which induces concomitant insulin and leptin resistance, our observation raises the issue about the search for foods which are not likely to induce SOCS3 or inflammation.

Although SOCS1 and SOCS7 genes are also induced by inflammatory cytokines and have been reported to modulate insulin signaling in vitro and in experimental animal models (2;21), there are no data about their relevance to human insulin resistance. Indeed, their expression did not change following glucose and cream intake.

SOCS3 may interfere with insulin signal transduction at various levels. Firstly, it can bind to the β subunit of the insulin receptor (IR-β), reduce tyrosine phosphorylation of IR-β and prevent the docking of IRS-1 to the receptor (22). Secondly, it may bind to IRS-1; and by so doing it may facilitate its ubiquitination and proteosomal degradation (2). By binding to IRS-1, it may also prevent the binding of IRS-1 to p85 subunit of PI3-kinase and thus prevent insulin signaling. We have recently shown that SOCS3 expression is increased in the obese and is inversely related to the tyrosine phosphorylation of the IR-β, that it is directly related to insulin resistance (HOMA-IR), BMI and other indices of inflammation (6). Our current observations are consistent with its potential role as a mediator of insulin resistance in the obese.

SOCS3 also interferes with leptin signal transduction by reducing the phosphorylation of the leptin receptor and Janus Kinase (23) attached to the leptin receptor. This results in a reduction of phosphorylation of STAT and thus the dimerization of STAT. This in turn prevents the nuclear translocation of STAT. Thus, the necessary gene transcription in response to the leptin signal cannot occur (5). The increase in SOCS3 expression and the presence of leptin resistance in the obese and their potential reversal with macronutrient restriction has important implications.

It is of interest that while the intake of cream induced an increase in the expression
of TLR4 and in plasma LPS concentrations, glucose had no effect. Since TLR4 is the specific receptor for LPS, the concomitant increase in both is a recipe for an amplified inflammatory signal. It would be of interest to examine the effects of repeated intake of cream as reflected in these indices and the overall magnitude of the inflammatory process. Previously, Dasu et al reported that high glucose concentrations upregulated TLR4 expression and its downstream signaling in a monocytic cell line (24). However, the excursions of glucose concentrations in normal subjects and even the obese do not achieve those concentrations post prandially. Thus, the observations of Dasu et al are relevant to diabetics. Our investigation deals with post prandial changes in normal subjects. As far as the effects of cream intake are concerned, the amount taken is modest and the increase in triglycerides is consistent with previous studies. It should, therefore, be noted that although the amounts of cream and glucose taken induced similar increases in ROS generation and NFkB binding, glucose was not able to induce an increase in the expression of TLR-4.

In conclusion, the intake of a modest amount of glucose or cream results in a significant induction of SOCS3 mRNA and protein in parallel with the induction of an inflammatory response characterized by an increase in NFκB binding in MNC and the induction of two of the cytokines, TNFα and IL-1β which are known to induce SOCS3 in experimental animals. In addition, the intake of cream but not glucose also induces an increase in the expression of TLR4 at mRNA and protein level while also inducing an increase in plasma LPS concentrations. Both SOCS3 and TLR4 are putative mediators of inflammation, SOCS3, TLR4 or an increase in plasma LPS concentrations.

ACKNOWLEDGMENTS
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Figure Legends
**Figure 1:** Change in SOCS-3 (A,C&D) and TLR4 (B,C&E) mRNA and protein expression in MNC from normal subjects following a 300 Calorie drink of cream (○), glucose (▲), orange juice (OJ, △) or water (●). Data are present as mean±SE. * and + = P<0.05 with RMANOVA comparing changes in relation to baseline following cream and glucose challenges; # and $=P<0.05$ with 2-way RMANOVA for comparisons of cream and glucose changes, respectively, to water; (n=12).

**Figure 2:** Change in TNFα (A) and IL-1β (B) mRNA expression in MNC from normal subjects following a 300 Calorie drink of cream (○), glucose (▲), orange juice (OJ, △) or water (●). Data are present as mean±SE. * and + = P<0.05 with RMANOVA comparing changes in relation to baseline following cream and glucose challenges; # and $=P<0.05$ with 2-way RMANOVA for comparisons of cream and glucose changes, respectively, to water; (n=12).

**Figure 3:** Change in NFkB binding activity in MNC (A) and plasma endotoxin concentrations (B) in normal subjects following a 300 Calorie drink of cream (○), glucose (▲), orange juice (OJ, △) or water (●). Data are present as mean±SE. * and + = P<0.05 with RMANOVA comparing changes in relation to baseline following cream and glucose challenges; # and $=P<0.05$ with 2-way RMANOVA for comparisons of cream and glucose changes, respectively, to water; (n=12).
REFERENCES


1A

% Change in SOCS-3 mRNA Expression

Time (hours)

1B

% Change in TLR4 mRNA Expression

Time (hours)
### Table 1C

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### Graph

**% Change in SOCS-3 Protein Expression**

- **Water**
- **Cream**
- **Glucose**
- **Orange Juice (OJ)**

**Legend:**
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**X-axis:** Time (hours)

**Y-axis:** % Change in SOCS-3 Protein Expression

0 1 3 5
Figure 1: Change in SOCS-3 (A,C&D) and TLR4 (B,C&E) mRNA and protein expression in MNC from normal subjects following a 300 Calorie drink of cream (○), glucose (▲), orange juice (OJ, △) or water (●). Data are present as mean±SE. * and += P<0.05 with RMANOVA comparing changes in relation to baseline following cream and glucose challenges; # and $=#P<0.05 with 2-way RMANOVA for comparisons of cream and glucose changes, respectively, to water; (n=12).
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Figure 3: Change in NF-κB binding activity in MNC (A) and plasma endotoxin concentrations (B) in normal subjects following a 300 Calorie drink of cream (○), glucose (▲), orange juice (OJ, Δ) or water (●). Data are present as mean±SE. * and + = P<0.05 with RMANOVA comparing changes in relation to baseline following cream and glucose challenges; # and $= P<0.05$ with 2-way RMANOVA for comparisons of cream and glucose changes, respectively, to water; (n=12).