Orange juice neutralizes the proinflammatory effect of a high-fat, high-carbohydrate meal and prevents endotoxin increase and Toll-like receptor expression[1–3]

Husam Ghanim, Chang Ling Sia, Mannish Upadhyay, Kelly Korzeniewski, Prabhakar Viswanathan, Sanaa Abuaysheh, Priya Mohanty, and Paresh Dandona

ABSTRACT

Background: The intake of glucose or a high-fat, high-carbohydrate (HFHC) meal, but not orange juice, induces an increase in inflammation and oxidative stress in circulating mononuclear cells (MNCs) of normal-weight subjects.

Objective: We investigated the effect of orange juice on HFHC meal–induced inflammation and oxidative stress and the expression of plasma endotoxin and Toll-like receptors (TLRs).

Design: Three groups (10 subjects in each group) of normal, healthy subjects were asked to drink water or 300 kcal glucose or orange juice in combination with a 900-kcal HFHC meal. Blood samples were obtained before and 1, 3, and 5 h after the drinks and meal combinations were consumed.

Results: Protein expression of the NADPH oxidase subunit $p47^{phox}$, phosphorylated and total p38 mitogen-activated protein kinase, and suppressor of cytokine signaling-3; TLR2 and TLR4 messenger RNA (mRNA) and protein expression; mRNA expression of matrix metalloproteinase (MMP)-9 in MNCs; and plasma concentrations of endotoxin and MMP-9 increased significantly after glucose or water were consumed with the meal but not when orange juice was consumed with the meal. The generation of reactive oxygen species by polymorphonuclear cells was significantly lower when orange juice was added to the meal than when water or glucose was added to the meal.

Conclusions: The combination of glucose or water and the HFHC meal induced oxidative and inflammatory stress and an increase in TLR expression and plasma endotoxin concentrations. In contrast, orange juice intake with the HFHC meal prevented meal-induced oxidative and inflammatory stress, including the increase in endotoxin and TLR expression. These observations may help explain the mechanisms underlying postprandial oxidative stress and inflammation, pathogenesis of insulin resistance, and atherosclerosis. *Am J Clin Nutr* 2010;91:940–9.

INTRODUCTION

We previously showed that the intake of 75 g (300 kcal) glucose induced an acute increase in reactive oxygen species (ROS) generation and inflammation as reflected in an increase in nuclear transcription factor κB (NF-κB) binding, a decrease in the expression of inhibitory κB-α, and an increase in inhibitory κB kinases in peripheral blood mononuclear cells (MNCs) (1, 2). An increase in NF-κB binding is associated with an increase in tumor necrosis factor-α (TNF-α) expression, activator protein-1 binding, early growth response factor-1 expression and binding, plasma matrix metalloproteinase (MMP)-2 and -9, and tissue factor after glucose intake (3). In terms of oxidative and inflammatory stress, a similar response follows the intake of a high-fat, high-carbohydrate (HFHC) meal (4). In contrast, the intake of orange juice containing sucrose, glucose, and fructose (total sugar content: 75 g = 300 kcal) does not cause an increase in ROS generation or NF-κB binding (5). In addition, hesperetin and naringenin, 2 major flavonoids that are contained in orange juice, but not ascorbic acid, are able to suppress ROS generation by MNCs in vitro by >50% (5). Toll-like receptor (TLR) 2 is the specific receptor for lipopeptides and peptidoglycans from gram-positive bacteria, and TLR4 is the specific receptor for lipopolysaccharide (LPS) or endotoxin from gram-negative bacteria (6, 7). TLR4 was also shown to play an important role in the pathogenesis of atherosclerosis (8–11), diet-induced obesity, and the related insulin resistance (12, 13), whereas TLR2 was shown to be involved in ischemia-reperfusion–induced myocardial injury (14). In a recent study (15) we showed that there was a significant increase in plasma concentrations of endotoxin and an increase in TLR4 and TLR2 expression in MNCs after the intake of an HFHC meal. This increase of endotoxemia induced by a fatty meal was also confirmed in previous reports in human and rodents (16–18). This increase could contribute to and prolong the inflammatory response that follows the intake of such a meal. Whether this increase of endotoxemia is due to the lipid solubility of endotoxin and its absorption into the circulation with the fat contained in the meal or is secondary to other factors such as the inflammation of the intestinal epithelium is not clear. If it is secondary to other factors, the potential antiinflammatory effect

[1] From the Division of Endocrinology, Diabetes, and Metabolism, State University of New York at Buffalo, Buffalo, NY (HG and PD); Kaleida Health, Buffalo, NY (CLS, MU, KK, PV, SA, and PM); and Kyung Hee University, Seoul, Korea (PD).

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[3] Address correspondence to P Dandona, Diabetes-Endocrinology Center of Western NY, State University of New York at Buffalo, 3 Gates Circle, Buffalo, NY 14209. E-mail: pdandona@kaleidahealth.org.

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of orange juice intake could lower postprandial endotoxin increase.

In our recent study (15) we also showed that an HFHC meal induced an increase in the expression of the suppressor of cytokine signaling (SOCS)-3, a key protein responsible for interference with insulin signal transduction by causing the degradation of insulin receptor substrate-1. SOCS-3 is induced by proinflammatory cytokines TNF-α and interleukin (IL)-1β and IL-6. Proinflammatory meals may contribute to the pathogenesis of insulin resistance.

Because orange juice does not cause oxidative and inflammatory stress, and flavonoids in orange juice suppress ROS generation (5), we hypothesized that *1)* orange juice is able to lower the increase in ROS generation and the inflammatory response in MNCs and plasma after a HFHC meal, and *2)* the concentration of plasma endotoxin and TLR4 and SOCS-3 expression in MNCs, which increase after an HFHC meal, are reduced by the simultaneous intake of orange juice. Such a study is important because chronic oxidative stress and inflammation are the 2 basic mechanisms underlying atherosclerosis (19, 20). Obesity and diabetes are states of chronic oxidative and inflammatory stress and are major risk factors for atherosclerosis (21, 22). MNCs constitute the major cellular group (monocytes and T and B lymphocytes) that participate in intramural atherosclerotic inflammation and are known to be in a proinflammatory state in obese individuals who carry a high risk of atherogenesis and have a chronically elevated food intake (23). Furthermore, inflammatory factors contribute to interference with insulin signal transduction and insulin resistance.

SUBJECTS AND METHODS

Three groups of 10 healthy, normal-weight men and women (body mass index [in kg/m^2]: 20–25; age range: 20–40 y) were recruited for this study. All subjects presented for the investigation after an overnight fast to the Clinical Research Center, State University of New York at Buffalo. Subjects in the 3 groups ingested a 300-kcal drink of 75 g glucose (Glucola; Fisher Scientific, Pittsburgh, PA), orange juice ("Not From Concentrate" Florida Orange Juice; Florida Department of Citrus, Lakeland, FL), or water along with a 900-kcal HFHC meal (egg-muffin and sausage-muffin sandwiches and 2 hash-brown potatoes that contained 81 g carbohydrates, 51 g fat, and 32 g protein). To compensate for the difference in volume between the drinks, subjects were asked to drink an extra 350 mL water with the glucose drink. All subjects were given 10–15 min to finish their drinks and meals. Blood samples were collected before and 1, 3, and 5 h after the intake of the food and drinks. The experimental protocol was approved by the Human Research Committee of the State University of New York at Buffalo, and each subject signed an informed consent. Recruitment for this study began in October 2006.

In a previous study (5), we used orange juice obtained from a local supermarket and used portions of the juice from 0.5- or 1-gal packages for multiple experiments. To minimize any potential for instability of orange-juice constituents (24–26), we used packages of recently produced "Not from Concentrate" Florida orange juice in the current study. Each package, once opened, was discarded after a single experiment.

MNC isolation

Blood samples were collected in tubes containing sodium-EDTA as an anticoagulant. A total of 3–5 mL anticoagulated blood sample was carefully layered over 3.5 mL Lympholyte medium (Cedarlane Laboratories, Hornby, Canada) and centrifuged to separate the cells. A top band consisted of MNCs, and a bottom band consisted of polymorphonuclear cells (PMNs). The cells were carefully collected. This method provided yields of >95% pure PMN and MNC suspensions.

ROS-generation measurement by chemiluminescence

A total of 500 μL PMNs or MNCs (2×10^5 cells) were delivered into a Chronolog Lumi-Aggregometer cuvette (Chronolog, Havertown, PA). Luminol was added and followed by 1.0 μL of 10 mmol/L formylmethionyl leucinyl phenylalanine. In this assay system, the release of superoxide radical, as measured by chemiluminescence, was shown to be linearly correlated with that measured by the ferricytochrome C method (27). The interassay CV of this assay was 8%. We further established that the biological variation in ROS generation in normal subjects was ≈6% for readings obtained 1–2 wk apart.

Western blotting

MNC total cell lysates were prepared and electrophoresis, and immunoblotting was carried as previously described (4). Polyclonal or monoclonal antibodies against the NADPH oxidase subunit p47^{phox} (BD Biosciences, San Jose, CA), TLR2 (Imgenex, San Diego, CA), TLR4, SOCS-3 (Abcam Inc, Cambridge, MA), p38 mitogen-activated protein (MAP) kinase, phosphorylated tyrosine 182 of p38, and actin (Santa Cruz Biotechnology, Santa Cruz, CA) were used, and the membranes were developed with SuperSignal Chemiluminescence reagent (Pierce Chemical, Rockford, IL). Densitometry was performed with Molecular Analyst software (Biorad, Hercules, CA), and all values were corrected for loading with actin.

Total RNA isolation and real-time reverse transcriptase polymerase chain reaction

Total RNA isolation and reverse transcriptase polymerase chain reaction were performed as previously described (23, 28). The expression of MMP-9, TLR2, and TLR4 messenger RNA (mRNA) was measured. The specificity and size of the polymerase chain reaction products were tested by confirming the melting temperature at the end of the amplifications and by running it on a 2% agarose gel. All values were normalized to the expression of 3 housekeeping genes (β-actin, ubiquitin C, and cyclophilin A).

Measurement of plasma concentrations of endotoxin and serum MMP-9

Plasma and food endotoxin concentrations were measured by a commercially available kit [Cambrex Limulus Amebocyte Lysate (LAL) kit; Lonza Inc, Walkersville, MD]. The assay is a quantitative, endpoint assay for the detection of gram-negative bacterial endotoxin. LPS from the sample reacts enzymatically with a proenzme in the LAL reagent that leads to its activation

and the production of a colored peptide from the chromogenic substrate reagent over a short incubation period that can be read at 405–410 nm. Briefly, plasma samples were diluted 5-fold and heat treated at 75°C for 10 min to minimize interference by from plasma lipoproteins. Treated samples, standards, and blanks (no sample or no LAL reagent) were incubated with LAL reagent for 10 min at 37°C followed by incubation with the substrate for 5 min. The reaction was stopped, and absorbance from the blanks were subtracted from the samples, and standard values and concentrations were calculated. This assay has a sensitivity range of 0.1–1.0 EU/mL. Normal values measured in our laboratory from lean subjects ranged from 0.15 to 0.35 EU/mL. Plasma samples used for endotoxin measurements were stored in endotoxin-free glass tubes to prevent the loss of endotoxin to plastic tubes walls. All materials used for the assay were rendered endotoxin-free. MMP-9 concentrations were measured from serum by an immunoassay kit from R&D Systems (Minneapolis, MN).

Measurement of LPS content in the meal, glucose, and orange juice

Ingredients of the HFHC meal were mixed and homogenized with glucose or orange juice. Dilutions comparable with the amounts ingested from the meal, glucose, and orange juice were prepared in plasma or endotoxin-free water. Plasma was used as a diluent to maintain a similar testing medium as in the post-challenge LPS measurements. LPS concentrations were measured as described by using the LAL assay. Background and food color absorbance were measured from blank samples (no sample or no LAL reagent) and subtracted from the sample absorbance, and the endotoxin content was calculated (n = 3 each).

Measurement of plasma-glucose and insulin concentrations

Glucose concentrations were measured in plasma by the YSI 2300 STAT Plus glucose analyzer (YSI, Yellow Springs, OH), and insulin was measured by an enzyme-linked immunosorbent assay kit (Diagnostics Systems Laboratories Inc, Webster, TX).

Statistical analyses

Statistical analyses were conducted with SigmaStat software version 3.1 (SPSS Inc, Chicago, IL). Data are represented as means ± SEs. Baseline concentrations were compared by using Student's t test. The percentage of change from baseline was calculated, and statistical analysis for change from baseline was carried out by using one-factor repeated-measures analysis of variance (RMANOVA) followed by the Holm-Sidak post hoc test. Two-factor RMANOVA analysis, followed by Turkey's post hoc test, was used for all multiple comparisons between different groups.

RESULTS

Effect of the different meal and drink combinations on plasma-glucose and insulin concentrations

When a 900-kcal HFHC meal was ingested with water, glucose, or orange juice, there was a significant rise in glucose concentrations with water and glucose ($P < 0.05$; **Figure 1**A) but not with orange juice. Insulin concentrations increased significantly by 6-, 11, and 9-fold ($P < 0.001$; Figure 1B) after the meal with water, glucose, or orange juice, respectively. The increase in insulin concentrations was significantly higher when glucose or orange juice was taken with the meal compared with when water was taken with the meal ($P < 0.01$, 2-factor RMANOVA).

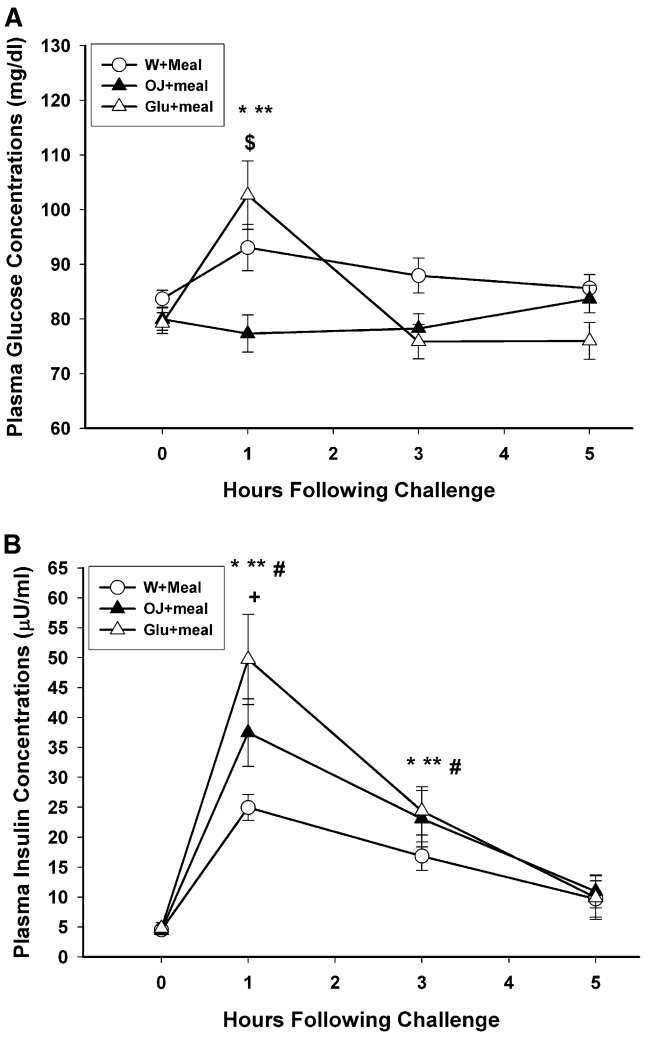

FIGURE 1. Mean (±SE) change in plasma glucose (A) and insulin (B) concentrations after intake of a 900-kcal high-fat, high-carbohydrate meal and a 300-kcal drink of orange juice (OJ+meal), glucose (Glu+meal), or water (W+meal) in normal subjects. $^{*\cdot**\cdot\#}P < 0.05$ [repeated-measures ANOVA (RMANOVA)] compared with baseline values after W+meal, Glu +meal, or OJ+meal, respectively; $^{+,\$}P < 0.05$ (2-factor RMANOVA) for comparison between W+meal or Glu+meal and OJ+meal treatments, respectively. P for interaction (treatment × time) = 0.011 for glucose with differences at 1 h between groups; P for interaction (treatment × time) = 0.027 for insulin with differences at 1 h between groups. n = 10 each.

Effect of meal and drink combinations on ROS generation by MNCs and PMNs

There was no significant difference in basal ROS generation or basal concentrations of any other tested inflammatory markers between the groups (**Table 1**). There was a significant mean (±SE) increase in ROS generation by MNCs from baseline (by

TABLE 1
Oxidative stress and concentrations of inflammatory mediators at baseline[1]

	HFHC + water	HFHC + glucose	HFHC + orange juice
ROS-PMN (mV)	110 ± 25	118 ± 29	107 ± 26
ROS-MNC (mV)	142 ± 36	169 ± 48	157 ± 32
$p47^{phox}$ protein[2,3]	1.72 ± 0.38	1.54 ± 0.42	1.71 ± 0.55
P38 protein[2,3]	1.22 ± 0.33	1.52 ± 0.38	1.42 ± 0.41
Phosphorylated p38 protein/p38 ratio[2]	0.31 ± 0.12	0.25 ± 0.14	0.38 ± 0.16
TLR2 protein[3]	0.46 ± 0.18	0.52 ± 0.24	0.43 ± 0.19
TLR4 protein[3]	0.65 ± 0.22	0.61 ± 0.25	0.68 ± 0.28
SOCS-3 protein[3]	0.28 ± 0.13	0.25 ± 0.14	0.25 ± 0.15
TLR2 mRNA[4]	0.92 ± 0.25	1.21 ± 0.31	1.04 ± 0.29
TLR4 mRNA[4]	1.28 ± 0.34	1.13 ± 0.21	1.21 ± 0.23
SOCS-3 mRNA[4]	0.52 ± 0.14	0.47 ± 0.11	0.53 ± 0.09
MMP-9 mRNA[4]	0.71 ± 0.16	0.66 ± 0.18	0.63 ± 0.22
Plasma MMP-9 (ng/mL)	357 ± 35	342 ± 32	369 ± 31
Plasma endotoxin (EU/mL)	0.23 ± 0.02	0.32 ± 0.08	0.28 ± 0.06

[1] All values are means ± SEs. n = 10 each. HFHC, high-fat, high-carbohydrate meal; ROS-PMN, reactive oxygen species generation by polymorphonuclear cells; ROS-MNC, ROS generation by mononuclear cells; TLR, Toll-like receptor; mRNA, messenger RNA; SOCS, suppressor of cytokine signaling; MMP, matrix metalloproteinase. Student's t test was used for testing differences between group baseline values. There were no significant differences between groups (t test).

[2] Protein concentrations were measured by Western blotting.

[3] Densitometry was corrected for loading and reported as a ratio to actin.

[4] mRNA expression was measured by reverse transcriptase polymerase chain reaction, corrected to 3 housekeeping genes (β-actin, ubiquitin C, and cyclophilin A), and reported as relative units.

62 ± 41%, 63 ± 25%, and 47 ± 21%, $P < 0.05$; **Figure 2**A) when the meal was taken with water, glucose, or orange juice, respectively. However, ROS generation by PMNs that was induced after intake of the orange juice and meal was significantly lower compared with the increase after intake of either glucose and the meal or water and the meal ($P < 0.05$, 2-factor RMANOVA).

Effect of the different meal and drink combinations on $p47^{phox}$ and p38 MAP kinase and phosphorylated p38 MAP kinase protein in MNCs

Glucose or water intake with the meal caused a significant increase in the protein expression of $p47^{phox}$ (by 94 ± 9% and 103 ± 13% over the baseline values, respectively; $P < 0.05$; **Figure 3**) in the MNCs, whereas intake of orange juice with the meal did not induce any significant changes in $p47^{phox}$ concentrations. In addition, glucose or water intake with the meal induced a significant increase in p38 MAP kinase protein concentrations (by 74 ± 15% and 47 ± 11% over the baseline values, respectively, $P < 0.05$; Figure 3) and p38 MAP kinase phosphorylation (by 76 ± 13% and 52 ± 8% over the baseline values, respectively; $P < 0.05$; Figure 3) in the MNCs, whereas intake of the meal and orange juice did not induce any significant changes in the expression of p38 MAP kinase protein or phosphorylated MAP kinase.

Effect of the different meal and drink combinations on MMP-9 mRNA in MNC and MMP-9 concentrations in plasma

Glucose or water intake with the meal caused a significant increase in the mRNA expression of MMP-9 by 99 ± 18% and 119 ± 33% over baseline values at 1 and 3 h, respectively ($P < 0.02$, **Figure 4**A), in the MNCs, whereas orange juice with the meal induced no change in MMP-9 mRNA. The plasma MMP-9 concentration increased after glucose and meal intake by 32 ± 12% over the baseline value at 3 h (from 342 ± 32 to 423 ± 43 ng/mL, $P < 0.05$; Figure 4B) and after water and meal intake by 41 ± 13% over the baseline value at 3 h (from 357 ± 35 to 415 ± 37 ng/mL, $P < 0.05$; Figure 4B), whereas it did not change significantly after intake of orange juice and the meal (from 369 ± 31 to 378 ± 26 ng/mL).

Effect of the different meal and drink combinations on the expression of TLR2 and TLR4 and SOCS-3 proteins in MNCs

The intake of the meal with glucose or water resulted in an increase in TLR2 mRNA in the MNCs by 87 ± 27% and 44 ± 11% over the baseline values, respectively ($P < 0.05$; **Figure 5**A) and in an increase in TLR4 mRNA in the MNCs by 56 ± 11% and 34 ± 10% over the baseline values, respectively ($P < 0.05$; Figure 5B). There was also a concomitant increase in TLR2 and TLR4 proteins by 54 ± 26% and 44 ± 18% over the baseline values, respectively, after meal and glucose intake and by 48 ± 20% and 45 ± 19% over the baseline values, respectively, after water and meal intake ($P < 0.05$; **Figure 6**). The intake of the meal with orange juice did not result in any significant change in TLR2 or TLR4 mRNA or protein. Similarly SOCS-3 protein increased significantly after meal intake with water and glucose by 39 ± 14% and 43 ± 12% ($P < 0.05$; **Figure 7**) over the baseline values, respectively, whereas there was no significant change when the meal was combined with orange juice.

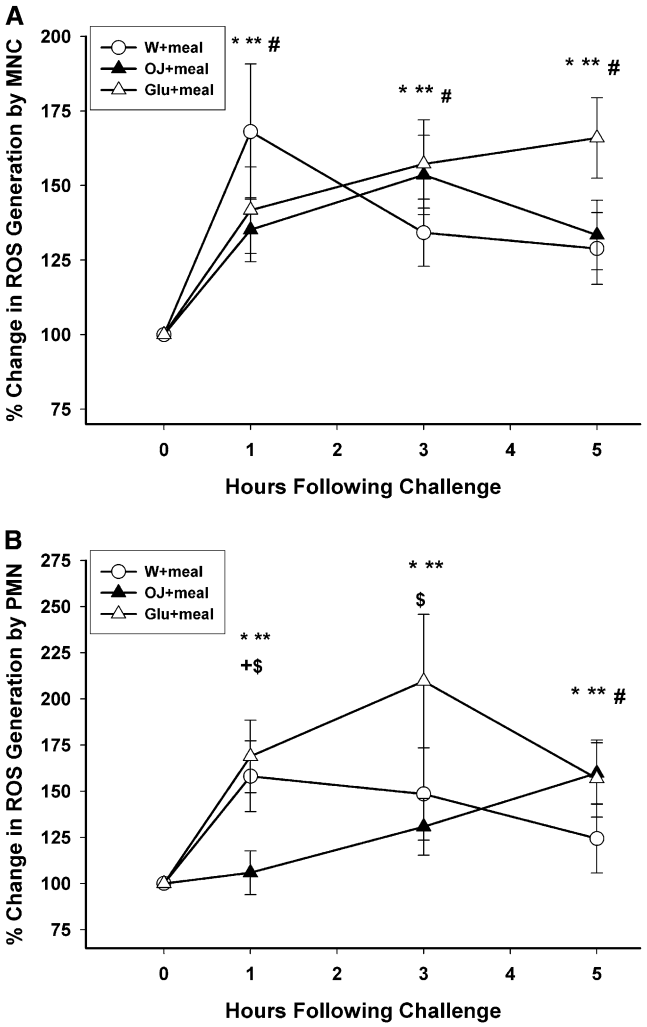

FIGURE 2. Mean (±SE) change in reactive oxygen species (ROS) generation by mononuclear cells (MNC) (A) and polymorphonuclear cells (PMN) (B) after intake of a 900-kcal high-fat, high-carbohydrate meal and a 300-kcal drink of orange juice (OJ+meal), glucose (Glu+meal), or water (W+meal) in normal subjects. $^{*,**,\#}P < 0.05$ [repeated measures ANOVA (RMANOVA)] compared with baseline values after W+meal, Glu+meal, or OJ+meal, respectively; $^{+,\$}P < 0.05$ (2-factor RMANOVA) for comparison between W+meal or Glu+meal and OJ+meal treatments, respectively. *P* for interaction (treatment × time) = 0.151 for ROS generation by MNCs; *P* for interaction (treatment × time) = 0.039 for ROS generation by PMNs with differences between groups at 1 and 3 h. $n = 10$ each.

Endotoxin content of the HFHC meal, glucose drink, and orange juice and plasma endotoxin concentrations after different meal and drink combinations

There was no detectable endotoxin in the glucose drink. The concentration of endotoxin in orange juice was 85 ± 21 EU/mL, and thus, the total amount of endotoxin in a single drink of orange juice was 55,250 EU. The endotoxin content of water was 21 ± 10 EU/mL. The total endotoxin content of the HFHC meal was 12,600 EU (data not shown). After the intake of the HFHC meal and glucose drink, the plasma concentration of endotoxin increased from 0.32 ± 0.08 to 0.50 ± 0.14 EU/mL (70 ± 21% over the baseline value at 3 h, $P < 0.05$; **Figure 8**), whereas it increased from 0.23 ± 0.02 to 0.36 ± 0.03 EU/mL (60 ± 16% over the baseline value at 5 h, $P < 0.05$; Figure 8) after water and meal intake. On the other hand, there was no increase after the intake of the HFHC meal and orange juice despite a much larger endotoxin load (12,600 + 55,250 = 67,850 EU). Considering that the amount of endotoxin is extremely small (<10 μg) in comparison with a total estimated amount of 1 g in the gut (17), it is unlikely that the LPS content of food contributes significantly to the increase in plasma endotoxin concentrations.

DISCUSSION

Our data show, for the first time to our knowledge, that the intake of orange juice with an HFHC meal prevented the marked increases in ROS generation and other inflammatory indexes, which occurred after the intake of the HFHC meal in combination with water or a glucose drink. We also observed that there was an increase in TLR2 and TLR4 expression after the HFHC meal in association with an increase in the endotoxin concentration. Finally, there was a significant increase in SOCS-3 expression after intake of the meal with water and the meal with glucose. These increases are prevented by the concomitant intake of orange juice with the HFHC meal. Thus, the intake of orange juice was able to neutralize the oxidative and inflammatory stress caused by the HFHC meal and the associated increases in plasma endotoxin concentrations and the expression of TLR4, the receptor for endotoxin, and TLR2. Furthermore, the orange juice intake prevented a significant increase in SOCS-3 after the meal. These data make the issue of macronutrient-induced postprandial inflammation a more complex one because, in our search for safe noninflammatory macronutrients, we have to consider safe combinations and those foods that buffer or neutralize the proinflammatory effects of other foods. These issues of postprandial inflammation are relevant to atherogenesis because postprandial hyperglycemia and hypertriglyceridemia were shown to be predictive of cardiovascular events (29, 30).

Several other inflammatory indexes were significantly lower when comparing the orange juice plus meal group with the glucose plus meal or water plus meal groups. There was a significant increase in MMP-9 mRNA after intake of the meal and glucose or water, whereas there was no such increase after the intake of orange juice and the meal. Because we previously showed that plasma MMP-9 concentrations increase after a glucose challenge (3) and after a meal (31), our current data indicate that this increase occurs at the transcriptional level. Water or glucose with the meal also induced an increase in the plasma MMP-9 concentration. This increase was not observed after the intake of orange juice with the meal. MAP kinase (p38) expression and phosphorylation increased after intake of both water and glucose with the meal but not after intake of orange juice and the meal. p38 MAP kinase is proinflammatory and may be involved in atherogenesis (32, 33).

We also showed that, after the intake of glucose or water with the meal, there was a significant increase in plasma endotoxin concentrations, whereas the intake of the meal with orange juice prevented this increase. Although the magnitude of the increase in endotoxin concentrations is not likely to cause any clinical symptoms, it may contribute to the oxidative and inflammatory stress after a meal, especially because there was a concomitant increase in TLR4 expression. An increase in TLR4 expression in MNCs with a concomitant increase in endotoxin concentration would enhance inflammation induced by fatty foods because

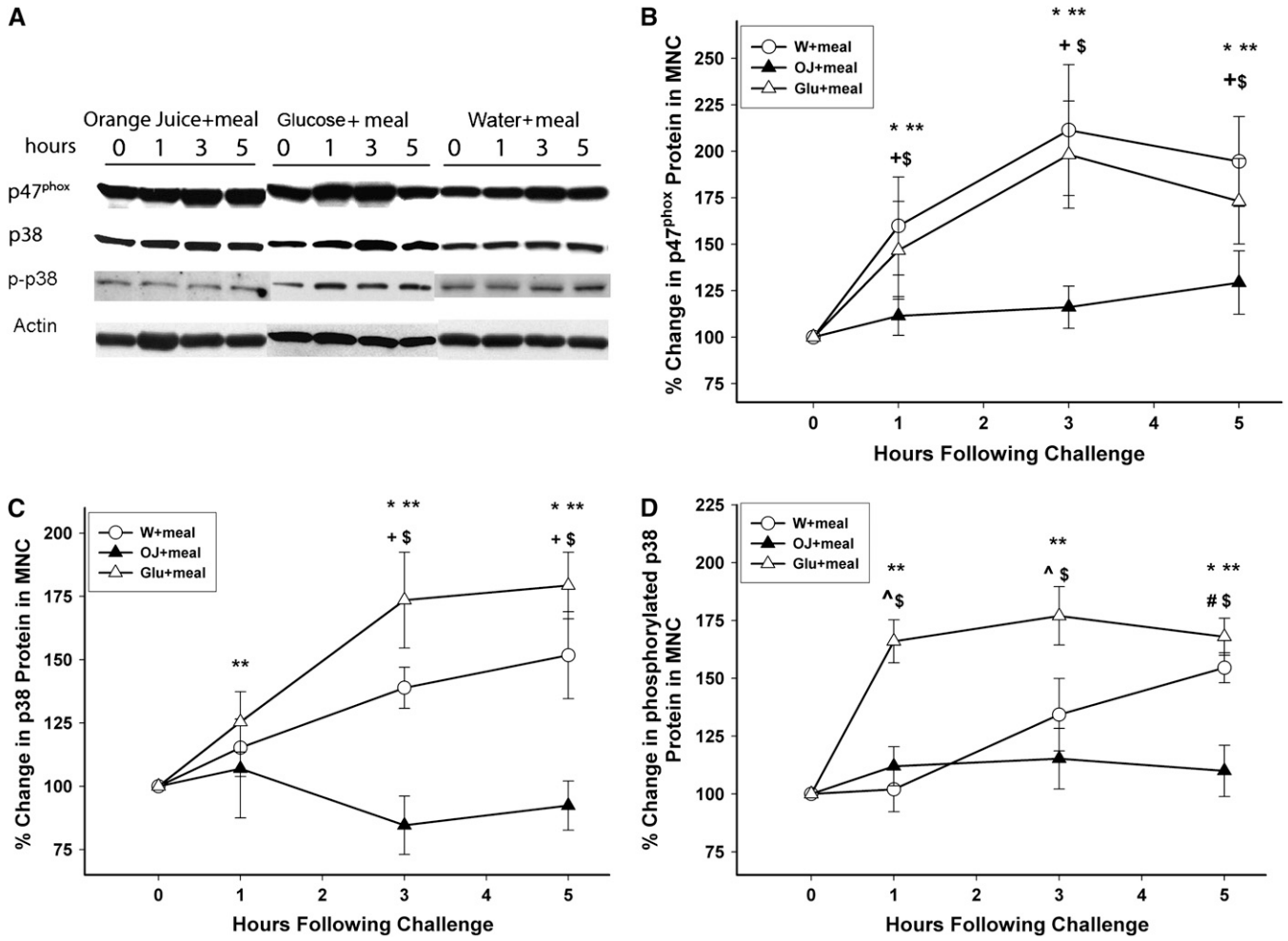

FIGURE 3. Western blotting representative gel and densitometry for the change in NADPH oxidase subunit ($p47^{phox}$) (A and B), p38 mitogen-activated protein kinase (MAPK) (A and C), and phosphorylated p38 MAPK protein (A and D) after intake of a 900-kcal high-fat, high-carbohydrate meal and a 300-kcal drink of orange juice (OJ+meal), glucose (Glu+meal), or water (W+meal) in normal subjects. Values in B–D are means ± SEs. $^{*,**,\#}P < 0.05$ [repeated measures ANOVA (RMANOVA)] compared with baseline values after W+meal, Glu+meal, or OJ+meal, respectively; $^{\wedge}P < 0.05$ (2-factor RMANOVA) for comparisons between W+meal and Glu+meal; $^{+,\$}P < 0.05$ (2-factor RMANOVA) for comparisons between W+meal or Glu+meal and OJ+meal treatments, respectively. *P* interaction (treatment × time) = 0.21 for $p47^{phox}$; *P* for interaction (treatment × time) = 0.031 for p38 with differences between the groups at 3 and 5 h; *P* for interaction (treatment × time) = 0.28 for phosphorylated p38. $n = 10$ each. MNC, mononuclear cells.

TLR4 is the receptor for endotoxin (12, 13). Therefore, it is of interest that orange juice suppresses TLR4 (and TLR2) expression while also reducing plasma endotoxin increase after the HFHC meal. This is also relevant to the pathogenesis of atherosclerosis and insulin resistance because TLR4 and TLR2 were incriminated in the pathogenesis of atherosclerosis and insulin resistance. The expression of TLR4 is observed in atherosclerotic plaques, whereas TLR4 deletion protects mice from atherosclerosis and insulin resistance induced by a high-fat diet (10, 12, 13).

The data presented in this article emphasize that the intake of glucose and a HFHC meal are profoundly and rapidly proinflammatory, that this process occurs at the cellular and molecular level, that specific proinflammatory genes are activated after the intake of glucose and a HFHC meal, and that these changes are observed in MNCs that participate in vascular inflammation. Vascular inflammation is an essential component of atherosclerosis (19, 34). It is possible that such postprandial changes may become permanent if a subject partakes of similar meals repetitively as a regular habit. The choice of safe foods that are not proinflammatory may provide protection from the unending cycle of postprandial and cumulative inflammation. This choice may lower the risk of atherosclerosis and resistance to insulin. In this context, orange juice is not only noninflammatory on its own but it also reduces or even eliminates the inflammation caused by an HFHC meal.

This potent effect of orange juice is probably attributable to its flavonoids, naringenin, and hesperidin because they exert a significant ROS suppressive effect (5) in vitro at concentrations of 50 μmol/L. These concentrations are consistent with a flavonoid content of 5–10 mg/100 mL in the consumed orange juice in our studies, assuming a complete absorption from the gut and its distribution in 5.0 L. However, the concentrations usually achieved in plasma after orange juice consumption are far lower than the concentrations used in vitro. The potent effects of these flavonoids were observed in experimental animal models (35–38) in relation to endotoxin-induced inflammation in vivo and in cells in vitro. However, further studies elucidating the

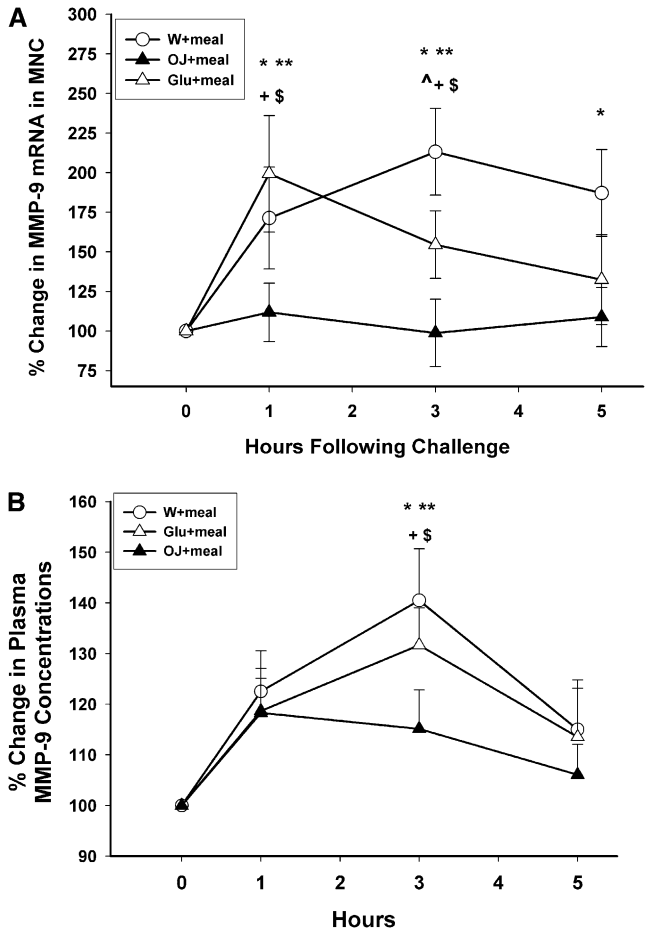

FIGURE 4. Mean (±SE) change in matrix metalloproteinase (MMP)-9 messenger RNA (mRNA) expression in mononuclear cells (MNC) (A) and plasma concentration (B) after intake of a 900-kcal high-fat, high-carbohydrate meal and a 300-kcal drink of orange juice (OJ+meal), glucose (Glu+meal), or water (W+meal) in normal subjects. $^{*,**}P < 0.05$ [repeated measures ANOVA (RMANOVA)] compared with baseline values after W+meal, Glu+meal, or OJ+meal, respectively; $^{\wedge}P < 0.05$ (2-factor RMANOVA) for comparisons between W+meal and Glu+meal; $^{+,\$}P < 0.05$ (2-factor RMANOVA) for comparison between W+meal or Glu+meal and OJ+meal treatments, respectively. P for interaction (treatment × time) = 0.032 for MMP-9 mRNA with differences between the groups at 1 and 3 h; P for interaction (treatment × time) = 0.028 for plasma MMP-9 with differences between the groups at 3 h. n = 10 each.

antiinflammatory effects of these flavonoids in the human in vivo are required.

We were surprised to find no increase in glucose concentrations at 1 h after the orange juice intake. Indeed, even when orange juice was taken with the fast-food meal, there was no change in glucose concentrations. Although we do not have any data on blood glucose concentrations between 0 and 60 min, and it is possible that there may have been a peak of glucose during this period, it is also quite clear that there was no evidence of a significant change in blood glucose concentrations after orange juice intake at 60 min. In contrast, blood glucose concentrations were still elevated in the groups drinking either glucose or water with the meal. We tested the possibility that orange juice might interfere with our glucose assay. Our data show that adding orange juice to water, a glucose standard, or plasma did not interfere with glucose measurement (data not shown). We previously reported a higher insulin-to-glucose ratio after orange juice intake compared with that after glucose intake (5). However, in that report, there was a greater increase in glucose concentrations after orange juice intake. This difference is probably the result of the use of recently pasteurized, well-refrigerated orange juice in the current study in contrast to orange juice from a large can of reconstituted juice from a supermarket that was used repeatedly. Clearly, more experiments need to be done to address glucose-insulin relations after orange juice intake and the possible mechanisms underlying this preliminary observation. It is possible that incretin mechanisms are involved in the genesis of this phenomenon.

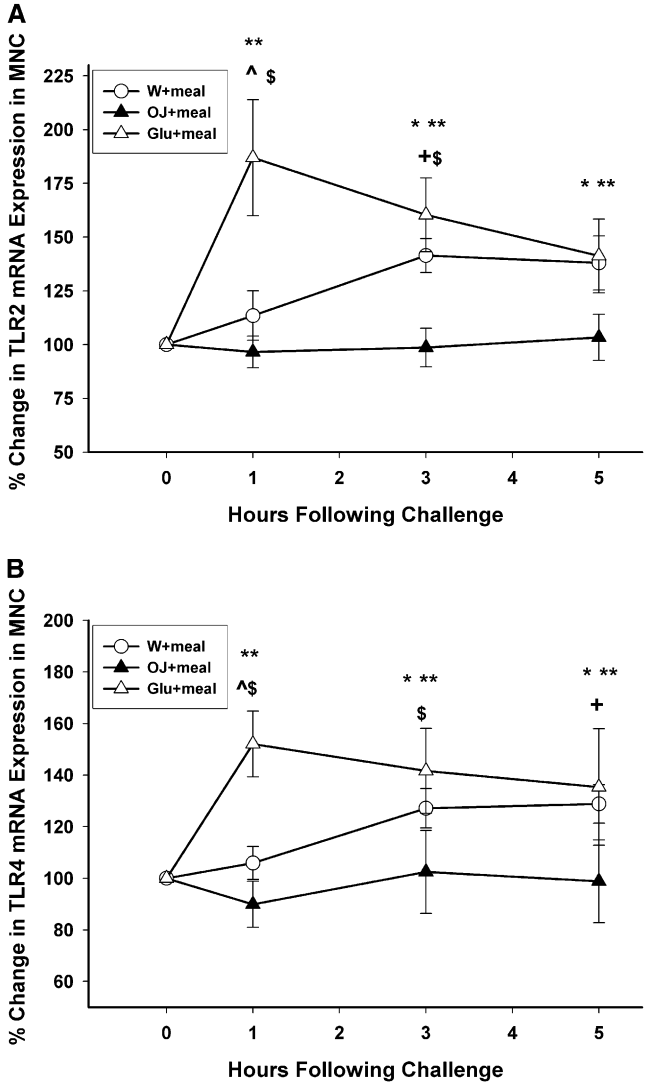

FIGURE 5. Mean (±SE) change in Toll-like receptor (TLR) 2 expression (A) and TLR4 messenger RNA (mRNA) expression (B) in mononuclear cells (MNC) after intake of a 900-kcal high-fat, high-carbohydrate meal and a 300-kcal drink of orange juice (OJ+meal), glucose (Glu+meal), or water (W+meal) in normal subjects. $^{*,**}P < 0.05$ [repeated measures ANOVA (RMANOVA)] compared with baseline values after W+meal, Glu +meal, or OJ+meal, respectively; $^{\wedge}P < 0.05$ (2-factor RMANOVA) for comparisons between W+meal and Glu+meal; $^{+,\$}P < 0.05$ (2-factor RMANOVA) for comparison between W+meal or Glu+meal and OJ+meal treatments, respectively. P for interaction (treatment × time) = 0.034 for TLR2 mRNA with differences between the groups at 1 and 3 h; P for interaction (treatment × time) = 0.027 for TLR4 mRNA with differences between the groups at 1, 3, and 5 h. n = 10 each.

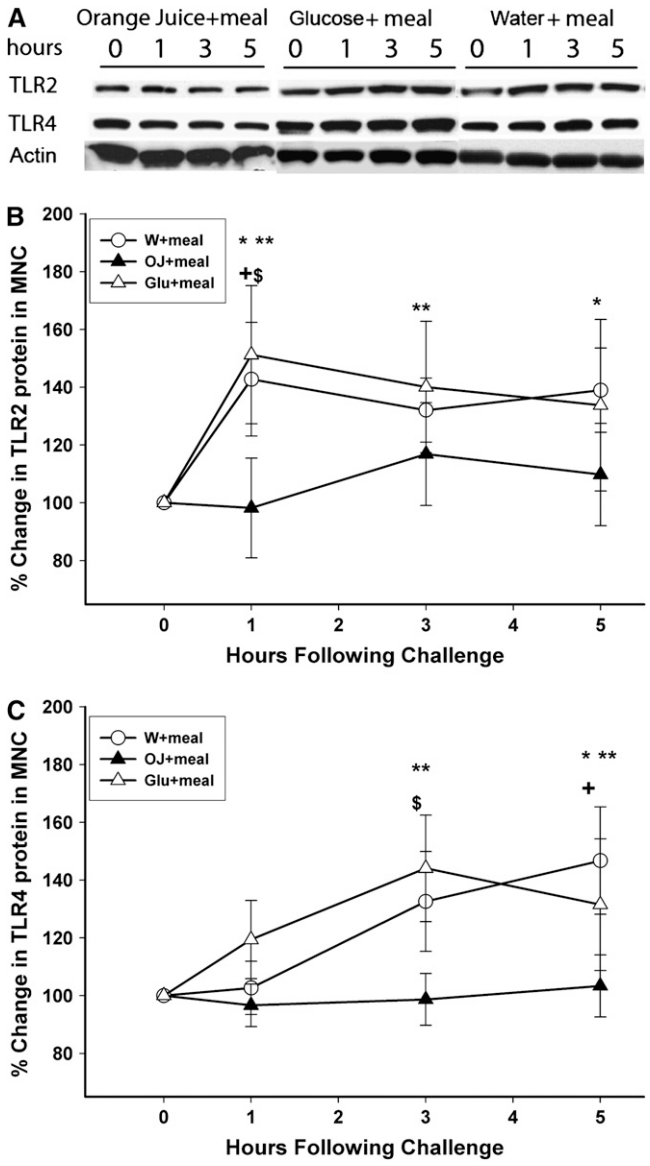

FIGURE 6. Western blotting representative gel and densitometry for the change in Toll-like receptor (TLR) 2 protein (A and B) and TLR4 protein (A and C) in mononuclear cells (MNC) after intake of a 900-kcal high-fat, high-carbohydrate meal and a 300-kcal drink of orange juice (OJ+meal), glucose (Glu+meal), or water (W+meal) in normal subjects. Values in B and C are means ± SEs. $^{*,**,***}P < 0.05$ [repeated measures ANOVA (RMANOVA)] compared with baseline values after W+meal, Glu+meal, or OJ+meal, respectively; $^{+,\$}P < 0.05$ (2-factor RMANOVA) for comparison between W+meal or Glu+meal and OJ+meal treatments, respectively. P for interaction (treatment × time) = 0.041 for TLR2 protein with differences between the groups at 1 h; P for interaction (treatment × time) = 0.044 for TLR4 protein with differences between the groups at 3 and 5 h. n = 10 each.

The induction of oxidative stress and inflammation after the excessive intake of HFHC meals is potentially atherogenic and is known to impair endothelial function. Postprandial hypertriglyceridemia and hyperglycemia impairs the flow-mediated vasodilation of the brachial artery and is associated with increased ROS generation and inflammation (39, 40). Increased ROS generation can reduce the bioavailability of nitric oxide as superoxide combines with nitric oxide to form peroxynitrite, which might have a proconstrictor effect on blood vessels along with a platelet proaggregatory effect because nitric oxide exerts

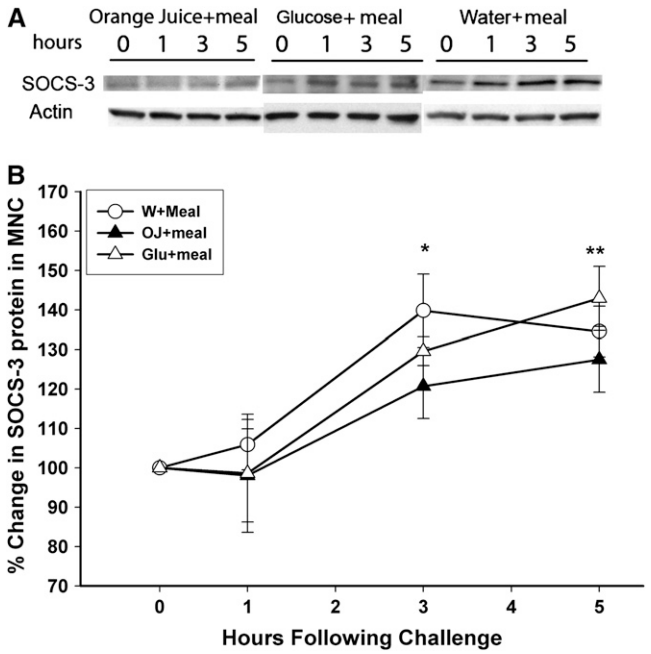

FIGURE 7. Change in suppressor of cytokine signaling (SOCS)-3 (A and B) protein in mononuclear cells (MNC) after intake of a 900-kcal high-fat, high-carbohydrate meal and a 300-kcal drink of orange juice (OJ+meal), glucose (Glu+meal), or water (W+meal) in normal subjects. Values in B are means ± SEs. $^{*,**,***}P < 0.05$ [repeated measures ANOVA (RMANOVA)] compared with baseline values after W+meal, Glu+meal, or OJ+meal, respectively. P for interaction (treatment × time) = 0.364. n = 10 each.

a vasodilatory effect on blood vessels and an antiaggregatory effect on platelets (41). Hyperglycemia was also shown to attenuate the vasodilatory effect of insulin in the coronary circulation in patients with type 1diabetes (42). The monocyte and the T and B lymphocytes contained in the MNC fraction are known to

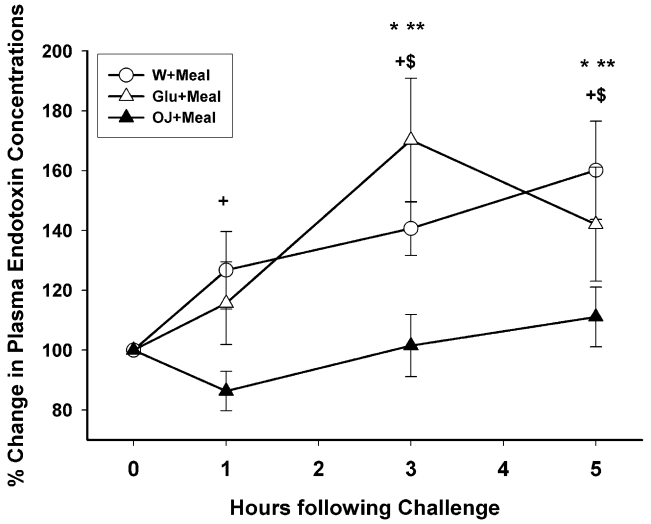

FIGURE 8. Mean (±SE) change in plasma endotoxin concentrations after intake of a 900-kcal high-fat, high-carbohydrate meal and a 300-kcal drink of orange juice (OJ+meal), glucose (Glu+meal), or water (W+meal) in normal subjects. $^{*,**,***}P < 0.05$ [repeated measures ANOVA (RMANOVA)] compared with baseline values after W+meal, Glu+meal, or OJ+meal, respectively; $^{+,\$}P < 0.05$ (2-factor RMANOVA) for comparisons between W+meal or Glu+meal and OJ+meal treatments, respectively. P for interaction (treatment × time) = 0.134. n = 10 each.

participate in atherogenesis (19). The suppressive effect of orange juice on ROS generation and inflammation in MNCs and plasma is important in this context because it could potentially lead to the prevention of endothelial dysfunction and atherosclerosis.

In conclusion, the intake of orange juice in combination with an HFHC meal prevents an increase in ROS generation and the inflammatory response in MNCs in contrast to the increase in both of these indexes after the HFHC meal with glucose or water. Consistent with these observations, MMP-9 mRNA expression and p38MAP kinase expression and phosphorylation increased after intake of glucose or water plus the meal but not after intake of orange juice plus the meal. Glucose or water intake with the meal also resulted in an increase in plasma endotoxin concentrations and increased expression of TLR4 and TLR2. In addition, there was an increase in SOCS-3 induced after the intake of the meal and either water or glucose. Such increases were absent after intake of orange juice and the meal. Thus, there may be food products that may be noninflammatory and protective against the proinflammatory effects of other foods. These observations are relevant to the role of postprandial inflammation in the pathogenesis of atherosclerosis, cardiovascular disease, and insulin resistance.

The authors' responsibilities were as follows—PD: study conception and design, statistical analysis, and manuscript preparation; HG: study conception and design, recruitment of subjects, protocol implementation, sample and statistical analysis, data acquisition, and manuscript preparation, editing, and revision; PM: study design, recruitment of subjects, protocol implementation, and manuscript preparation; VP: study design, recruitment of subjects, and protocol implementation; MU: recruitment of subjects, protocol implementation, sample acquisition, processing, analysis, and data acquisition; KK: sample acquisition, processing, analysis, and data acquisition; and CLS and SA: sample acquisition, processing, analysis, data acquisition, and manuscript editing and revision. PD is supported by the Florida Department of Citrus. HG, CLS, MP, KK, PV, SA, and PM had no conflicts of interest.

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