Orange Juice or Fructose Intake Does Not Induce Oxidative and Inflammatory Response

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OBJECTIVE — We have previously shown that 300 kcal from glucose intake induces a significant increase in reactive oxygen species (ROS) generation and nuclear factor-κB (NF-κB) binding in the circulating mononuclear cells in healthy normal subjects. We hypothesized that the intake of 300 calories as orange juice or fructose, the other major carbohydrate in orange juice, would induce a significantly smaller response than that of glucose.

RESEARCH DESIGN AND METHODS — Four groups (eight subjects each) of normal-weight subjects were given a 300-cal drink of glucose (75 g), fructose (75 g), or orange juice or water sweetened with saccharin (control group) to drink, and then blood samples were collected.

RESULTS — There was a significant increase in ROS generation by mononuclear cells (by 130 ± 18%, $P < 0.001$), polymorph nuclear cells (by 95 ± 22%, $P < 0.01$), and in NF-κB binding in mononuclear cells by 82 ± 16% ($P < 0.01$) over the baseline after 2 h of glucose intake. These changes were absent following fructose, orange juice, or water intake. There was significantly lower ROS generation and NF-κB binding following orange juice, fructose, and water compared with glucose ($P < 0.001$ for all). Furthermore, incubation of mononuclear cells in vitro with 50 mmol/l of the flavonoids hesperetin or naringenin reduced ROS generation by 52 ± 7% and 77 ± 8% ($P < 0.01$), respectively, while fructose or ascorbic acid did not cause any change.

CONCLUSIONS — Caloric intake in the form of orange juice or fructose does not induce either oxidative or inflammatory stress, possibly due to its flavonoids content and might, therefore, represent a potentially safe energy source.

Diabetes Care 30:1406–1411, 2007

Our previous work has shown that the intake of glucose (75 g = 300 kcal), cream (33 g = 300 kcal), and a fast-food meal (900 kcal) induce an increase in reactive oxygen species (ROS) generation by peripheral blood mononuclear cells in parallel with an increase in intranuclear nuclear factor-κB (NF-κB) DNA binding, a decrease in inhibitor κBα, and an increase in inhibitor κB kinase (IKK)α and IKKβ expression and IKK activity (1–3). Intravenous infusion of glucose also results in an increase in proinflammatory cytokines, if endogenous insulin secretion is inhibited concomitantly with somatostatin (4). Glucose intake also results in an increase in proinflammatory transcription factors, AP-1 and Egr-1, and the corresponding genes activated by them: matrix metalloproteinase (MMP)-2, MMP-9, tissue factor, and plasminogen activator inhibitor-1 in normal subjects (5) and in vitro (6,7).

This raises the question whether caloric intake in any form induces oxidative stress and inflammation and whether the type of response is determined by the source of these calories. This is important since obesity, the result of excessive macronutrient intake, is characterized by an excess of oxidative stress and inflammation (8–10). Furthermore, the restriction of caloric intake in obese subjects results in a reduction of oxidative stress (8,9) and inflammatory mediators. A 48-h fast in normal subjects results in a 35% reduction in ROS generation in 24 h and >50% reduction in 48 h (11). Clearly, therefore, caloric intake is a major source of oxidative and inflammatory stress. Since atherosclerosis, the major cause of cardiovascular death, is associated with oxidative stress and inflammation in the arterial wall (12), the search for foods that are least likely to cause oxidative stress and inflammation must be pursued.

Citrus juices, especially orange juice, have been recommended by several health and nutrition groups as a healthy source of calories, and their intake is associated with improved lipid profile and a reduced risk of cardiovascular disease (13,14). Furthermore, orange juice is a rich source of flavonoids and vitamin C (15,16), which may suppress ROS generation and inflammatory processes. It is also possible that flavonoids contained in orange juice may reduce or prevent oxidative stress and inflammation induced by macronutrients like glucose, fructose, and sucrose contained in it. Therefore, we hypothesized that orange juice intake induces less oxidative stress and inflammation than an equicaloric amount of glucose.

RESEARCH DESIGN AND METHODS

— Four groups, eight each, of healthy normal-weight subjects (BMI 20–25 kg/m^2), aged 20–40 years, were recruited for this study. Three

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Received for publication 11 July 2006 and accepted in revised form 10 March 2007.

Published ahead of print at http://care.diabetesjournals.org on 23 March 2007. DOI: 10.2337/dc06-1458.

Additional information for this article can be found in an online appendix at http://dx.doi.org/10.2337/dc06-1458.

Abbreviations: CRP, C-reactive protein; IKK, inhibitor κB kinase; NF-κB, nuclear factor-κB; ROS, reactive oxygen species.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

groups were given a single 300-kcal challenge of glucose, fructose, or orange juice, whereas subjects from the fourth group were given only water sweetened with saccharin. All subjects were given 10 min to finish their drinks. A fasting blood sample was collected before and at 1, 2, and 3 h following the drink intake. An institutional review board–approved consent form was obtained from all subjects.

Mononuclear cell isolation

Blood samples were collected in Na-EDTA as an anticoagulant. Anticoagulated blood samples (3.5 ml) were carefully layered over 3.5 ml of Lympholyte medium (Cedarlane Laboratories, Hornby, ON, Canada) and centrifuged to separate the cells. A top band consisting of mononuclear cells and a bottom consisting of polymorph nuclear cells were collected. This method yields >95% pure polymorph nuclear cell and mononuclear cell suspensions.

ROS generation measurement by chemiluminescence

Polymorph nuclear cells (500 μl) or mononuclear cells (2×10^5 cells) were delivered into a Chronolog Lumiaggregometer cuvette. Luminol was then added, 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 has been shown to be linearly correlated with that measured by the ferricytochrome C method. The interassay coefficient of variation of this assay is 8%. We have further established that the biological variation in ROS generation in normal subjects is ~6% for readings obtained 1–2 weeks apart.

NF-κB DNA binding activity

Nuclear NF-κB DNA binding activity was measured by electrophoretic mobility shift assay. Nuclear extracts were prepared from mononuclear cells and by high-salt extraction. The specificity of the bands was confirmed by supershifting these bands with specific antibodies against Rel-A (p65) and p50 (Santa Cruz Biotechnology, Santa Cruz, CA) and by competition with cold oligonucleotides.

Measurement of plasma glucose, insulin, and C-reactive protein concentrations

Glucose levels were measured in plasma by YSI 2300 STAT Plus glucose analyzer (Yellow Springs Instruments, Yellow Springs, OH), and insulin was measured by an enzyme-linked immunosorbent assay kit (Diagnostics Systems Laboratories, Webster, TX). Serum C-reactive protein (CRP) was measured using an enzyme-linked immunosorbent assay kit from Alpha Diagnostic International (San Antonio, TX).

ROS generation by mononuclear cell in vitro

Freshly isolated mononuclear cells from fasting normal subjects were incubated for 1 h in PBS containing 5 mmol/l glucose with either fructose (5 mmol/l), vitamin C (0.250 mmol/l ascorbic acid), or the flavonoids hespertin (50 μmol/l) and naringenin (50 μmol/l) dissolved in DMSO. Cells were washed once, and ROS assay was carried as previously described. Controls included cells incubated with glucose with or without DMSO. This experiment was repeated four times, and data represent the means ± SE. Inhibition of ROS generation by mononuclear cells was evaluated by incubating freshly isolated mononuclear cells with the NADPH oxidase inhibitor DPI (Sigma, San Antonio, TX) at a concentration of 10–1,250 nmol/l for 30 min. Cells were washed once and ROS generation measured.

Statistical analysis

Statistical analysis was conducted using SigmaStat software (SPSS, Chicago, IL). All the data are represented as means ± SE. Statistical analysis from baselines was carried out using Holm-Sidak one-way repeated-measures ANOVA. Dunnett's two-factor ANOVA method was used for all multiple comparisons between different groups. Student's *t* tests for impaired data were used to compare Δglucose-to-Δinsulin ratio 1 h after glucose or orange juice intake. ROS generation from the in vitro experiment was compared between the treatments using paired *t* test analysis.

RESULTS

Effect of the different treatments on plasma glucose and insulin concentrations

Plasma glucose concentrations increased from a mean of 94 ± 6 mg/dl to 129 ± 21, 109 ± 20, and 94 ± 10 mg/dl at 1, 2, and 3 h, respectively, after glucose intake and from 90 ± 4 mg/dl to 116 ± 9, 102 ± 5, and 92 ± 5 mg/dl at 1, 2, and 3 h, respectively, following orange juice ($P < 0.001$ repeated-measures ANOVA for both) (online appendix Fig. 1 [available at http://dx.doi.org/10.2337/dc06-1458]). There was no significant difference in glucose values between glucose and orange juice intake. There was no significant change in glucose concentration following fructose or water intake. Plasma insulin concentrations increased from 9.5 ± 2.44 μU/ml to 47.0 ± 8.3, 20.5 ± 8.3, and 13.6 ± 5.6 μU/ml at 1, 2, and 3 h, respectively, following glucose and from 6.1 ± 1.8 μU/ml to 50.3 ± 13.1, 19.6 ± 12.4, and 4.3 ± 1.5 μU/ml at 1, 2, and 3 h, respectively, following orange juice ($P < 0.001$ repeated-measures ANOVA for both) (online appendix Fig. 2). Fructose intake increased insulin concentrations from 8.8 ± 1.3 μU/ml to 17.0 ± 5.2, 13.1 ± 3.9, and 8.9 ± 1.8 μU/ml at 1, 2, and 3 h, respectively ($P < 0.01$ repeated-measures ANOVA). There was no difference in the change in insulin values following glucose and orange juice intake when compared by two-factor repeated-measures ANOVA, but, when compared with fructose, changes in insulin concentrations following both glucose and orange juice were significantly greater ($P < 0.05$). There was a trend toward a greater insulin concentration following orange juice for a given glucose concentration compared with glucose intake. This was best expressed as a ratio between the increase in insulin and glucose concentrations at 1 h. This ratio was significantly greater following orange juice (Δinsulin-to-Δglucose ratio at 1 h = 1.77 ± 0.32 vs. 1.12 ± 0.25; $P = 0.033$ unpaired *t* test).

Effect of the different treatments on ROS generation by mononuclear and polymorph nuclear cells

There was a significant increase in ROS generation by mononuclear and polymorph nuclear cells by 130 ± 18% ($P < 0.001$ repeated-measures ANOVA) (Fig. 1*A*) and 95 ± 22% ($P < 0.01$ repeated-measures ANOVA) (Fig. 1*B*), respectively, over the baseline within 2 h of glucose intake. There was no significant change in ROS generation by mononuclear or polymorph nuclear cells following fructose, orange juice, or water intake. When ROS generation was compared between the groups, there was a significant difference between glucose intake and all of orange juice, fructose, and water intake ($P < 0.01$ for all, two-factor repeated-measures ANOVA). There was no significant difference in ROS generation between orange juice, fructose, and water intake.

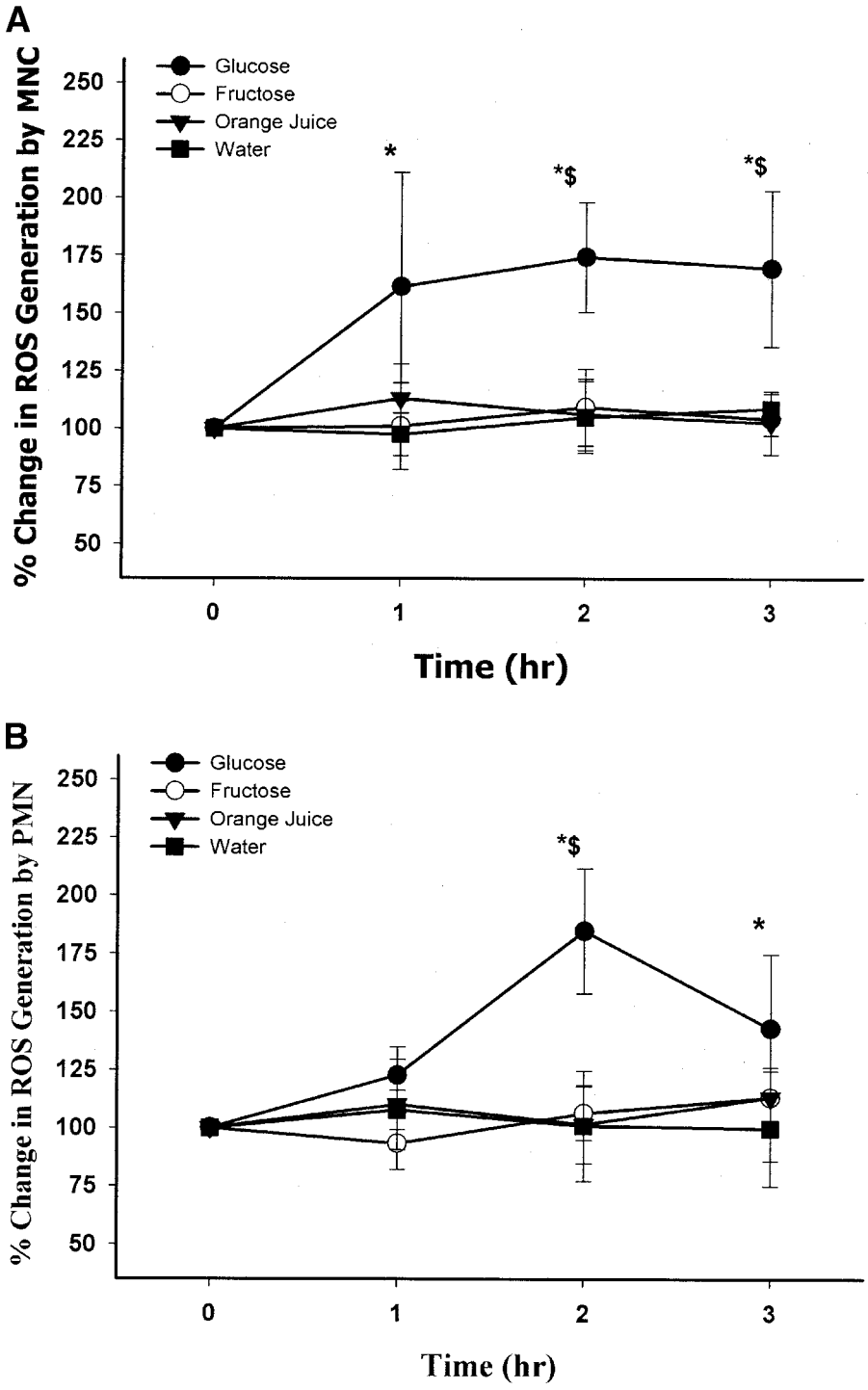

Figure 1—*ROS generation by mononuclear cells (MNC) (*A*) and polymorph nuclear cells (PMN) (*B*) following glucose, fructose, orange juice, and water ingestion.* *P < *0.01 repeated-measures ANOVA for glucose;* $P < *0.01 two-factor repeated-measures ANOVA between glucose and all other groups.* n = *8 for all.*

Effect of the different treatments on NF-κB DNA binding in the mononuclear cells

NF-κB DNA binding was increased significantly by 82 ± 16% over the baseline ($P < 0.01$ repeated-measures ANOVA) (Fig. 2) within 2 h of glucose intake. There was no significant increase in NF-κB binding following fructose, orange juice, or water intake. When NF-κB DNA binding changes were compared between the groups, there was a significant difference in NF-κB binding following glucose intake when compared with baseline and to orange juice, fructose, and water intake ($P < 0.01$ for all, two-factor repeated-measures ANOVA). There was no significant difference in NF-κB binding between orange juice, fructose, and water intake.

Effect of 300-kcal intake on plasma CRP concentration

Although none of the challenges induced a significant change in plasma concentrations of CRP over the 3-h observation period, there was a trend toward a fall ($P < 0.1$ repeated-measures ANOVA) and a significant fall in CRP concentrations within 1 h of orange juice intake (1,305 ± 375 vs. 1,219 ± 321 ng/ml, $P = 0.044$ paired *t* test) (Fig. 3). The change in CRP concentrations following orange juice was also significantly different compared with water intake ($P < 0.01$ two-factor repeated-measures ANOVA).

Effect of orange juice components on ROS generation by mononuclear cells in vitro

ROS generation by mononuclear cells freshly isolated from normal subjects was measured individually following a 1-h incubation with various components or orange juice. There was no significant change in ROS generation by mononuclear cells following incubation with 5 mmol/l fructose or 0.25 mmol/l ascorbic acid when compared with control cells incubated with glucose alone. ROS generation was significantly lower when mononuclear cells were incubated with hesperetin (by 52 ± 7%, $P = 0.004$) or naringenin (by 77 ± 8%, $P = 0.002$), the two major falvonoids in orange juice, when compared with control cells incubated with glucose and DMSO alone (Fig. 4).

Effect of NADPH inhibition on ROS generation by mononuclear cells in vitro

There was a significant dose-dependent inhibition of ROS generation by mononuclear cells following 30 min of incubation in 5 mmol/l glucose with increasing concentrations (10–1,250 nmol/l) of the NADPH oxidase inhibitor DPI in DMSO (online appendix Table 1) compared with control cells incubated with glucose and DMSO alone. This experiment was repeated three times on mononuclear cells from three different normal subjects.

CONCLUSIONS— Our data confirm that the intake of 75 g glucose induces a significant increase in ROS generation and NF-κB binding by mono-

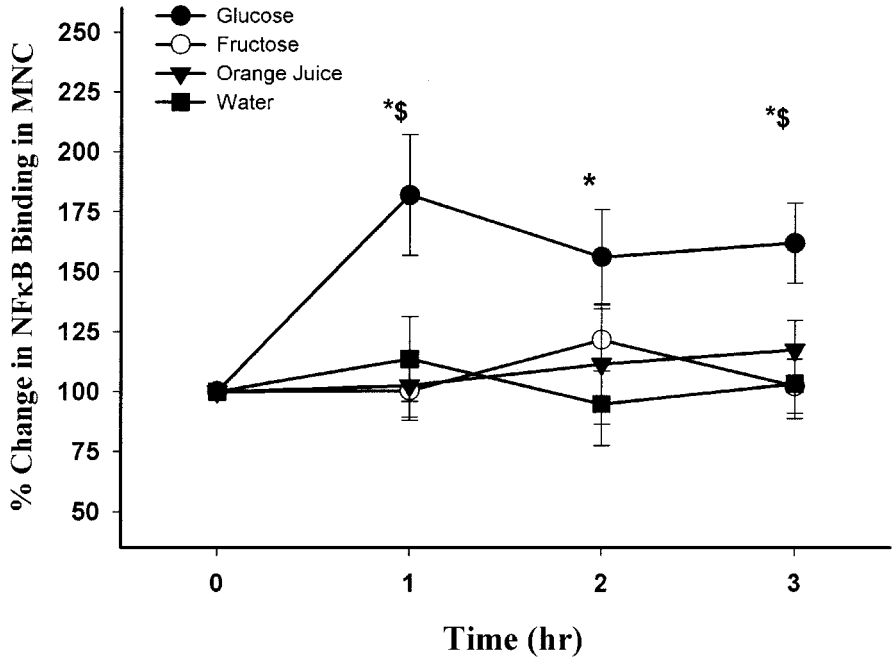

Figure 2—*Change in intranuclear NF-κB binding activity following glucose, fructose, orange juice, and water ingestion.* *P < *0.01 repeated-measures ANOVA for glucose;* $P < *0.01 two-factor repeated-measures ANOVA between glucose and all other groups.* n = *8 for all. MNC, mononuclear cells.*

nuclear cells at 1 and 2 h after intake. In contrast, equivalent amounts (300 cal) of either orange juice or fructose did not induce a change in either ROS generation or NF-κB binding. These data are important since they show for the first time that "safe" nutritional choices on the basis of minimizing postprandial oxidative and inflammatory stress can be made. It is relevant that alcohol intake (300 cal) also does not result in increased ROS generation or NF-κB binding as previously reported by us (17). However, alcohol cannot and should not be recommended as a major macronutrient source.

We have previously shown that the diphenylene iodonium (DPI), a specific inhibitor of NADPH oxidase (18), completely inhibits ROS generation by leukocytes in a dose-dependent manner (19). Our repeat experiments confirm this, as presented here. This indicates that in our assay system, ROS generation is mainly due to the activation of the NADPH oxidase, which is located mainly in the leukocyte membrane, in order to assist in bacterial killing following phogocytosis. Further testing with specific inhibitors of the mitochondrial electron transport chain is needed to totally exclude possible contribution of the mitochondria to ROS generation in our assay system. However, the fact that DPI dose-dependently inhibits the ROS generation in a cell-based assay system like ours implies that the majority of ROS detected in our system are the products of NADPH oxidase. Supporting this concept, we have previously shown that superoxide dismutase also markedly suppresses ROS generation in this system.

It is intriguing that the absence of an increase in ROS generation and NF-κB binding following orange juice intake was observed inspite of an increase in plasma glucose concentration, which was not significantly different from that observed following glucose. This raised the question whether the presence of flavonoids and vitamin C exerted an ROS and NF-κB binding suppressive effect or that fructose, the other major sugar in orange juice, may be suppressive of ROS generation and NF-κB binding. Therefore, we tested these possibilities in a separate series of experiment conducted in vitro. We were able to demonstrate that fructose and ascorbic acid did not suppress ROS generation, whereas hesperetin and naringenin inhibited ROS generation by 52 and 77%, respectively, at micromolar concentrations. Clearly, therefore, the two major flavonoids in orange juice might mediate the suppression of glucose-induced ROS generation. This area requires further investigation including work in vivo.

Since fructose is a major sugar in fruits and other vegetable products, it would be worth investigating the potential anti-inflammatory effect of food products rich in fructose. It is also of interest

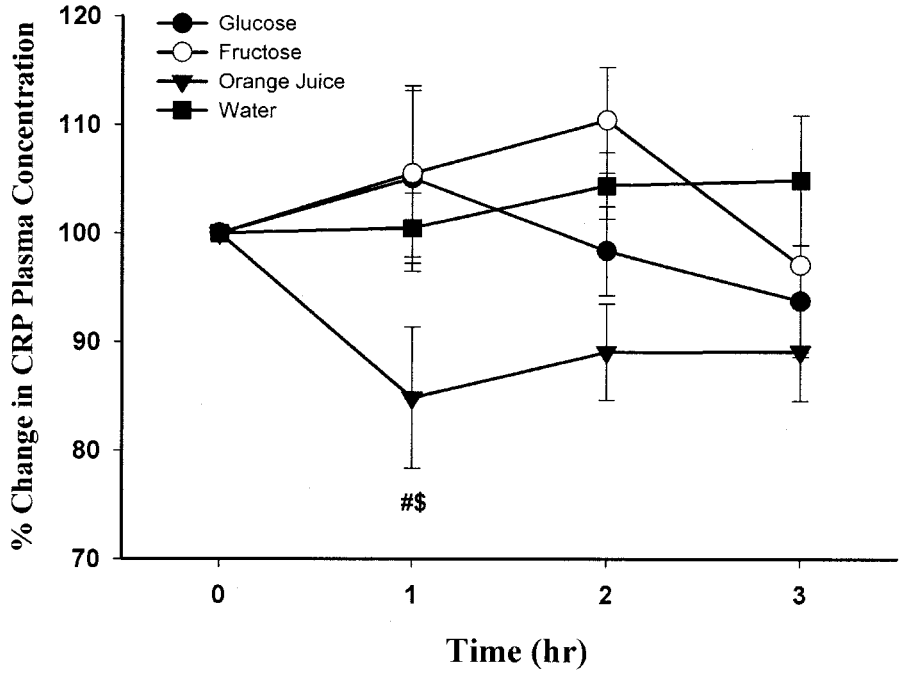

Figure 3—*Change in plasma CRP concentrations following glucose, fructose, orange juice, and water ingestion.* #P < *0.05 paired* t *test for orange juice;* $P < *0.01 two-factor repeated-measures ANOVA between orange juice and water.* n = *8 for all.*

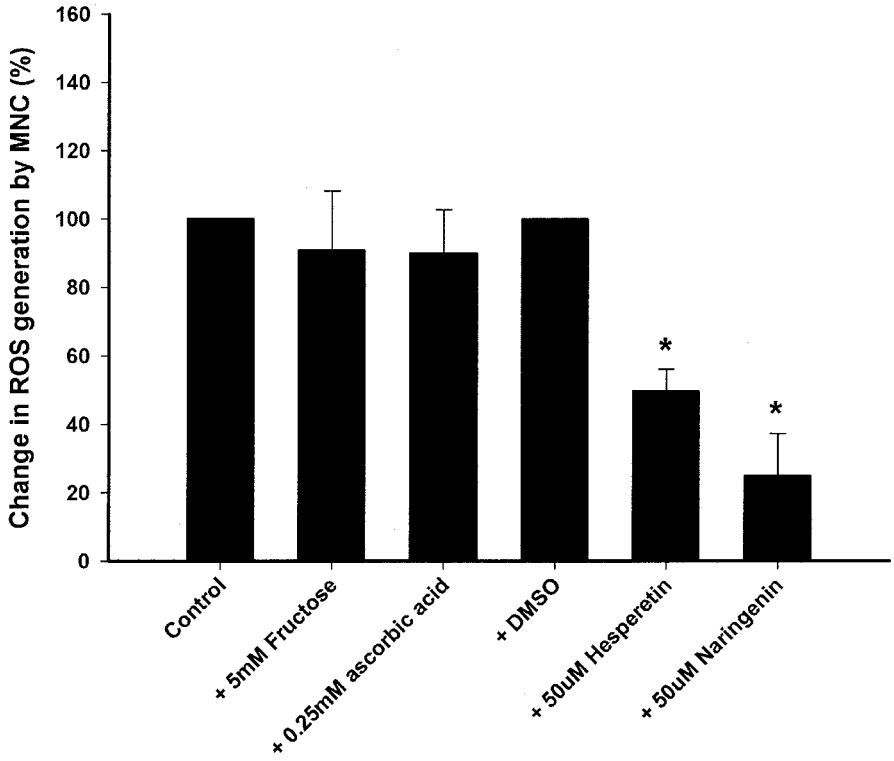

Figure 4—*ROS generation by mononuclear cells (MNC) in vitro following 1 h of incubation with orange juice components (fructose [5 mmol/l], vitamin C [0.25 mmol/l ascorbic acid], hespertin [50 μmo/l], and naringenin [50 μmol/l]). Control cells were incubated for 1 h with 5 mmol/l glucose alone or with DMSO.* *P $<$ *0.01 paired* t *test.* n = *4; data represents means* ± *SE.*

that 75 g fructose did not alter plasma glucose concentrations significantly, while it did induce a small but significant increase in plasma insulin concentrations at 1 h. It is possible that fructose is taken up and metabolized by the β-cell, resulting in a small release of insulin in fashion similar to that observed with glucose. The absence of oxidative and inflammatory stress following fructose is intriguing since fructose diets in animals induce insulin resistance. However, the quantities of fructose contained in high-fructose diets are far greater than those contained in a 300-cal drink of orange juice.

It is appropriate to state that all calories in orange juice are from sugars: glucose (30%), fructose (30%), and sucrose (40%) (20). Sucrose is hydrolyzed in the gastrointestinal tract by disaccharidases into an equal number of glucose and fructose molecules. Thus, for all practical purposes, orange juice has glucose and fructose as the two major sugars in equal amounts. It is, therefore, intriguing that although the average increase in glucose and insulin concentrations following orange juice are not significantly different from those following glucose, the average increase in insulin concentrations calculated as Δinsulin-to-Δglucose ratio at 1 h for each subject was significantly higher following orange juice intake. This suggests that orange juice may be more insulinogenic compared with glucose and that changes in glucose concentrations might not always totally predict plasma insulin response. Similar observations have been reported following the intake of orange juice and other fruit juices in terms of plasma glucose and insulin responses in type 2 diabetic patients (21). A higher insulin response may contribute, independently from the flavonoids effect, to the absence of oxidative stress and inflammation following orange juice since insulin reduces ROS generation, NF-κB activation, and CRP concentrations in humans in vivo (22,23).

The data on the effect of orange juice on plasma CRP concentrations are interesting. Although orange juice did not induce a significant fall in CRP over the observation period of 3 h, there was a trend (P = 0.1 repeated-measures ANOVA) toward a reduction and a significant fall at 1 h ($P < 0.05$ paired t test) from baseline. There was also a highly significant ($P < 0.01$ two-factor repeated-measures ANOVA) difference between the effect of water and orange juice on CRP concentrations following their intake. The clinical significance of a fall of CRP by 10–15% for a period of 2–3 h following orange juice is not clear. Studies on the long-term effect of orange juice intake in high-risk groups are necessary to evaluate the clinical implications of a fall in CRP.

Our data are relevant to patients with diabetes since oxidative (24) and inflammatory (25) stress are markedly increased in this condition and may contribute to accelerated atherosclerosis. Clearly, the choice of foods that either do not increase or actually decrease oxidative and inflammatory stress in diabetic subjects is important. Our findings also raise the issue of whether hypoglycemia in diabetic patients should be treated with glucose or orange juice. Further studies are required.

In conclusion, *1*) orange juice or fructose taken in equicaloric amounts to 75 g glucose does not cause either oxidative stress or inflammation in contrast to glucose; *2*) the two flavonoids hesperetin and naringenin have a ROS-suppressive effect in vitro, which needs to be confirmed in vivo; *3*) ascorbic acid does not exert this effect; and *4*) there are ways of avoiding postprandial oxidative stress and inflammation by making appropriate choices. The search for safe noninflammatory foods and diets must continue.

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