Timing of Antioxidant Vitamin Ingestion Alters Postprandial Proatherogenic Serum Markers

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Background—This study was designed to determine the optimal timing of vitamins E and C to prevent oxidative stress induced by a high-fat evening meal in type 2 diabetes.

Methods and Results—Eleven subjects were admitted on 4 occasions. Euglycemia was maintained for 24 hours by insulin infusion. Participants were fed a high-fat test supper equivalent to a McDonald’s Big Mac Meal. Blood was drawn for measurement of C-reactive protein (CRP), interleukin 6 (IL-6), plasminogen activator inhibitor-1 (PAI-1), malonyldialdehyde (MDA), and total radical antioxidant parameter (TRAP) before and during the 4 hours after the test meal. Studies were performed in random sequence with vitamin E 800 IU and vitamin C 1 g given either before breakfast or before supper in a double-blind manner on the day of the test meal. Control studies were performed with no vitamins and no test meal administered. There was a significant rise in CRP and PAI-1 after the test supper (P < 0.05 compared with “no meal”). Either presupper or prebreakfast vitamins E and C prevented the meal-induced rise in CRP (P = 0.03), although presupper vitamins were more effective (P = 0.03 compared with prebreakfast vitamins). Only prebreakfast vitamins prevented the meal-induced rise in PAI-1 (P = 0.006). There were no significant meal-related changes in the concentrations of IL-6, MDA, or TRAP.

Conclusions—The timing of administration of antioxidant vitamins has variable effects on markers of meal-induced inflammation and fibrinolysis. This observation may be one reason why cardiovascular disease prevention trials using these vitamins have reported conflicting results. (Circulation. 2003;108:24-31.)

Key Words: antioxidants ■ plasminogen activator inhibitor ■ protein, C reactive

Despite improvements in glycemic control and cardiovascular risk reduction, >70% of individuals with type 2 diabetes die of atherosclerotic diseases. Oxidative stress is the excessive production of oxygen free radical species, which damage surrounding molecules, leading to release of inflammatory mediators, oxidation of LDL, and a prothrombotic state. The oxidative stress cascade is a common pathogenic mechanism activated by many cardiovascular risks present in diabetes and may underlie the synergism between them. Vitamin E is a potent antioxidant that uses vitamin C to regenerate its antioxidant capabilities in the body.1 Vitamins E and C have been shown to inhibit oxidation of LDL;2 to lower the levels of plasminogen activator inhibitor-1 (PAI-1),3 C-reactive protein (CRP), and interleukin-6 (IL-6);4 and to restore arterial flow.5,6 However, prospective studies examining the efficacy of antioxidant vitamins in the prevention of atherosclerotic diseases have reported conflicting results.7–12

Epidemiological and mechanistic studies suggest that perturbations of the postprandial period are involved in the pathogenesis of macrovascular complications in diabetes. High-fat meals seem to be particularly damaging to the vasculature. The typical Western diet with 3 meals per day causes postprandial lipemia for 18 hours.13 Moreover, postprandial hypertriglyceridemia is an independent risk factor for vascular disease.14 Vitamins E and C have been shown to prevent the adverse changes in oxidative stress markers and endothelial function induced by a high-fat meal.5,15–17 One possible reason for the disappointing results seen in recent antioxidant prevention trials is suboptimal timing of administration of the antioxidant vitamins in relation to meals. For the majority of patients, the evening meal is the largest of the day,18 yet most people take antioxidant supplements in the morning. If the antioxidant properties are consumed during meals, then vitamins should be taken before the meal for optimal protection. Alternatively, if the beneficial properties of antioxidant vitamins require hours to become effective, then vitamins should be consumed as early in the day as possible. No studies are available demonstrating the optimal time to take daily vitamins E and C. This study was designed to determine the optimal timing of vitamins E and C to prevent oxidative stress induced by a high-fat evening meal in healthy volunteers with type 2 diabetes.

Methods

Subjects

The study enrolled 11 subjects with type 2 diabetes of ≥6 months’ duration. Subjects were excluded if they had known vascular disease,
uncontrolled hypertension (>140/90 mm Hg), or marked hyperlipidemia (serum LDL >4.1 mmol/L or serum triglycerides >7.8 mmol/L). Eligible patients had normal ECGs and normal hematology, electrolyte, and liver laboratory results at screening. Other important exclusion criteria were cigarette smoking or recent use of antioxidant supplements or aspirin. All patients gave written informed consent, and the study received approval from the University of New Mexico Institutional Review Board.

Study Protocol

The study protocol had a randomized, placebo-controlled, crossover design (Figure 1). Subjects were admitted to the General Clinical Research Center (GCRC) on 4 separate evenings. Participants’ usual diabetes medications were withheld for the duration of the inpatient studies. Euglycemia was maintained for 24 hours before the test meal with premeal subcutaneous insulin lispro and a continuous regular insulin intravenous infusion. Subjects were fed a standardized dinner at 6 PM and a snack at 10 PM, after which they fasted until morning. On the second hospital day, subjects consumed a standardized breakfast at 8 AM and a standardized lunch at 1 PM. At 6 PM, participants were fed a high-fat test supper equivalent to a McDonald’s Big Mac Meal (70 g fat). The test meal was prepared in the GCRC metabolic kitchen and consisted of a beef patty, bun, American cheese, Thousand Island dressing, pickles, lettuce, French fried potatoes (prepared with reheated cooking oil), and an ice-cream shake. The meal contained 70.5 g fat and 104 g carbohydrate and had an energy content of 1314 kcal. Throughout the test meal period, the insulin infusion was continued at the individual basal rate noted to maintain overnight euglycemia for each subject. Arterialized venous blood was drawn for analysis before and during the 4 hours after the test meal (6 PM to 10 PM). Studies were performed in random sequence with natural vitamin E (RRR-α-tocopherol) 800 IU and vitamin C (ascorbic acid) 1 g, given either before breakfast or before the test supper on the day of the studies. Control studies were performed with no vitamins and no test meal. During the “no-meal” study arm, subjects fasted from 6 to 10 PM (equivalent to the postprandial period during the other test meal arms). The no-meal arm was performed to control for diurnal variation in the serum markers studied.

Study Measurements

Plasma levels of IL-6 and CRP were determined as markers of inflammation in the postprandial period. PAI-1 activity was measured by the glucose oxidase method (Analogix Instruments USA). Plasma high-sensitivity (hs) CRP was determined by chemiluminescence with an Immulite instrument (DPC Cirrus Inc). This hsCRP assay has a range of 0.01 to 25 mg/dL and an interassay and intra-assay CV of <5%. IL-6 levels were measured by a highly sensitive quantitative sandwich enzyme immunoassay technique (R&D Systems, Inc) with an interassay and intra-assay CV of <5%. Plasma PAI-1 activity was measured with a Chromotrizine immunoassay (Biopool International) with a detection range of 2 to 50 IU/mL and a precision of <4%. Plasma free (unbound) MDA was assayed by gas chromatography–mass spectrometry, which has a sensitivity of 0.01 μM/mL. The TRAP assay used a modification of the method of Ghiselli et al19 and reagents from the Total Antioxidant Status kit by Randox. Plasma vitamin E and C concentrations were determined by high-performance liquid chromatography and autoanalyzer, respectively. Both plasma triglyceride and cholesterol levels were measured colorimetrically with kits from Alfa Wasserman.

Statistical Methods

Excursions for the study measurements were expressed as the change from the presupper baseline levels. Area under the curve (AUC) was calculated by use of the linear trapezoidal rule. The primary efficacy variables assessed were (1) overall postprandial levels, as determined by the excursion AUC, and (2) peak postprandial excursion levels for hsCRP, IL-6, PAI-1, MDA, and TRAP. Secondary variables included plasma levels of glucose and vitamins E and C. Lipid standardized vitamin E levels were calculated by dividing the plasma vitamin E level by the sum of the plasma triglyceride and total cholesterol concentrations (vitamin E/triglycerides + total cholesterol). Data were analyzed by use of SAS (SAS Institute Inc). Parameters were compared among the various groups by ANOVA for repeated measures with post hoc Student’s t test for paired data. All data are reported as mean±SEM.

Results

Subject Characteristics

Eight men and 3 women (5 Hispanic, 4 white, 2 Native-American) were studied. Mean (±SD) age, body mass index, and duration of diabetes were 55±9 years, 30±7 kg/m², and 11±8 years, respectively. The subjects had a mean (±SD) HbA₁c of 8.2±1.9% and mean fasting glucose of 9.1±4.8 mmol/L. Seven of the patients were managed with oral hypoglycemic agents (n=5 for sulfonlureas, n=2 for metformin), and 4 were treated with insulin for diabetes control. The mean (±SD) fasting lipid profile of the subjects included LDL cholesterol of 3±1 mmol/L, HDL cholesterol of 1.6±0.8 mmol/L, and triglycerides of 3.3±2.1 mmol/L.

Plasma Glucose and Triglyceride Excursions

Normal capillary blood glucose levels (~4 to 7 mmol/L) were maintained for 24 hours before the test meal. Mean (±SEM) presuppertime plasma glucose levels (5.3±0.02 mmol/L; range, 3.9 to 8.5 mmol/L) were similar for all study conditions. The postmeal glycemic excursions were similar for all arms in which a test supper was consumed (Figure 2A) and were significantly higher than the no-meal control study (P<0.001). Plasma triglyceride changes after the test meal are depicted in Figure 2B. Triglyceride levels rose after the high-fat test meal (P<0.05 compared with no-meal control). When vitamins were given before supper, there was a significantly higher integrated increment (AUC) in triglyceride levels compared with the no-vitamins study
day (28 versus 23 mmol/L per 4 hours, \( P < 0.03 \)). There was no significant difference between triglyceride levels on the presupper and prebreakfast vitamin administration study days.

**Plasma Levels of Vitamins E and C**

Prebreakfast vitamin administration resulted in significantly higher presupper levels of vitamins E and C compared with the other study conditions (\( P < 0.05 \)). Vitamin E levels remained constant throughout the posttest meal period with prebreakfast dosing (Figure 3A). Integrated postmeal lipid standardized vitamin E levels were higher with prebreakfast dosing than with any of the other study conditions (\( P < 0.01 \)) but were not different by 4 hours after the meal in the prebreakfast compared with the presupper vitamin study days (Figure 3B). There was a significant postmeal increment in lipid standardized vitamin E levels when the vitamins were given immediately before a meal (\( P = 0.007 \) compared with no vitamins). Vitamin C levels waned in the postprandial test period with prebreakfast compared with presupper vitamin administration (Figure 3C). The peak levels and integrated AUC for vitamin C were not different with prebreakfast or presupper vitamin administration but were significantly higher than the no-vitamin (\( P < 0.05 \)) and no-meal (\( P < 0.05 \)) control arms.

**Inflammatory Markers**

There was a significant increase from presupper CRP levels when no vitamins were administered on the day of the test meal (\( P < 0.05 \) for peak and AUC compared with each of the other study conditions) (Figure 4). Presupper or prebreakfast vitamins E and C prevented the meal-induced rise in CRP (\( P = 0.03 \) compared with no vitamins). Presupper vitamins were most protective, with CRP levels significantly lower than the fluctuations seen in the no-meal control arm (\( P < 0.01 \)) and lower than the prebreakfast vitamin administration arm. No significant correlations were found between the CRP excursions and the postmeal triglyceride or glucose excursions during the various study conditions. Circulating IL-6 concentrations trended upward during the studies, con-
Figure 3. Plasma levels of vitamin E (A), lipid standardized vitamin E (B), and vitamin C (C) during no-meal control arm (open triangle) and after a high-fat test supper with presupper vitamins E and C (closed square), prebreakfast vitamins E and C (closed triangle), and no vitamins (open square).
istent with the known circadian rhythm of this cytokine. However, IL-6 concentrations were not significantly different between any of the 4 study conditions.

**Fibrinolytic Activity**
Baseline premeal levels of PAI-1 activity were similar in all study conditions. An exaggeration of the normal diurnal decrease in plasma PAI-1 levels was seen in the no-meal control arm. In contrast, a significant rise in plasma PAI-1 was detected after the high-fat test suppers compared with the no-meal control arm ($P<0.05$). Only when vitamins E and C were given before breakfast was this meal-induced rise in PAI-1 prevented ($P=0.5$ compared with the no-meal, $P=0.006$ compared with presupper vitamins) (Figure 5). There were no significant correlations between the postmeal PAI-1 excursions and the triglyceride or glucose.

**Oxidative Stress Parameters**
Peak and integrated AUC for serum MDA levels were not significantly affected in the postprandial period when vitamins E and C were given immediately before the test supper compared with the other study conditions ($P<0.05$). However, the high-fat test meal did not cause significant alterations in MDA levels compared with background diurnal fluctuations (data not shown). Plasma TRAP values did not differ significantly between the study conditions.

**Discussion**
This study demonstrates that a high-fat evening meal induces an inflammatory and hypofibrinolytic state in subjects with type 2 diabetes. A rise in the plasma levels of CRP and PAI-1 activity, not explained by background diurnal fluctuations, was observed when a high-fat meal was consumed in the absence of antioxidant vitamins. Presupper administration of vitamins E and C was superior to prebreakfast vitamin administration in normalizing plasma levels of CRP, although vitamins taken at either time were effective inhibitors of the rise in CRP induced by a high-fat supper. In contrast, only

![Figure 4](image_url)

**Figure 4.** Change from presupper baseline levels of CRP during no-meal control arm (open triangle) and after a high-fat test supper with presupper vitamins E and C (closed square), prebreakfast vitamins E and C (closed triangle), and no vitamins (open square).

![Figure 5](image_url)

**Figure 5.** Change from presupper baseline levels of PAI-1 activity during no-meal control arm (open triangle) and after a high-fat test supper with presupper vitamins E and C (closed square), prebreakfast vitamins E and C (closed triangle), and no vitamins (open square).
prebreakfast vitamins E and C were effective in blocking the meal-stimulated peak in PAI-1 activity.

Studies have shown that both postmeal hyperglycemia and postprandial hypertriglyceridemia constitute independent risk factors for cardiovascular disease.\textsuperscript{14-20} Patients with type 2 diabetes have exaggerated postmeal hyperglycemia. In addition, type 2 diabetes subjects with moderate fasting hypertriglyceridemia (as in the present study) demonstrate greater postprandial increments in triglyceride levels.\textsuperscript{21} Thus, type 2 diabetes subjects are at particular risk for a constellation of atherogenic postprandial disturbances. The mechanisms by which excessive postprandial glucose and lipid levels promote atherogenesis are numerous. Evidence exists that hyperglycemia increases free radical production by protein (including lipoprotein) glycation, glucose autoxidation, and activation of the polyol pathway.\textsuperscript{17} Postprandial lipemia also causes oxidative stress and vascular dysfunction.\textsuperscript{15} Furthermore, ingestion of a meal high in fat used for deep frying in a commercial fast food restaurant may worsen postprandial oxidative stress.\textsuperscript{16} The cooking oil used in the present study was reheated on $\geq10$ occasions before preparation of the test meals, as is the common practice in fast-food restaurants. High-fat meals such as the supper used in the present study are obviously contrary to nutritional guidelines proposed by the American Heart Association and American Diabetes Association but contribute significantly to a typical Western diet.\textsuperscript{18} Patients with diabetes consume a higher proportion of energy from fat, and the consumption of fast foods such as French fries and hamburgers is increasing.\textsuperscript{18}

Vitamin E is a lipid-soluble, chain-breaking antioxidant. Vitamin C acts in conjunction with vitamin E by regenerating the reduced form of $\alpha$-tocopherol.\textsuperscript{1} Natural RRR-$\alpha$-tocopherol, as used in this study, is twice as potent as the synthetic form of vitamin E.\textsuperscript{22} The scientific rationale for the use of antioxidant vitamins in the prevention of cardiovascular disease comes from experimental and observational studies. Specifically in patients with type 2 diabetes, vitamin E supplementation can retard LDL oxidation, lower MDA and CRP levels, and improve endothelial flow.\textsuperscript{2,4,5,23} Early prospective trials evaluating vitamin E for cardiovascular disease prevention reported encouraging results.\textsuperscript{2,7-9} Subsequent large interventional studies do not support a benefit from antioxidant supplementation.\textsuperscript{10-12} Explanations for these discrepant findings include varying dietary vitamin content and doses of vitamins, incorrect combination or formulation of vitamins, noncompliance (most studies do not measure vitamin levels), inadequate duration of follow-up to detect a benefit, and the presence of preexisting atherosclerosis in the study groups. A recent study reported that the bioavailability of oral vitamin E is maximal when it is given with food.\textsuperscript{24} In the present study, vitamins were given either with breakfast or with supper to ensure maximal absorption and antioxidant capacity. We propose that the time of administration of antioxidant vitamins in relation to a high-fat meal is an important variable to consider in the design of clinical trials with vitamins E and C. Current assays do not distinguish between the active reduced and ineffective oxidized forms of vitamin E. Despite sufficient plasma levels, the antioxidant capability of vitamin E may be consumed by earlier meals.

CRP is an exquisitely sensitive acute-phase reactant produced by the liver in response to cytokines, predominantly IL-6. IL-6 is a circulating multifunction protein produced by multiple tissues, including mononuclear cells, endothelial cells, and adipocytes. Circulating IL-6 concentrations display a high-amplitude circadian rhythm with peak values at night.\textsuperscript{25} In the absence of inflammation, CRP concentrations remain stable over long periods of time and are not subject to time-of-day variability, which may explain why CRP is a more accurate predictor of cardiovascular risk than IL-6.\textsuperscript{25-27} Commercially available hsCRP assays are necessary for risk assessment of cardiovascular disease. Baseline hsCRP concentration is a powerful biomarker for the prediction of atherosclerotic diseases.\textsuperscript{28} Vitamin E supplementation partially corrects elevated CRP and IL-6 levels in patients with type 2 diabetes.\textsuperscript{4} The present study demonstrated a significant reduction of the meal-induced rise in CRP with presupper or prebreakfast vitamins E and C compared with the no-vitamins arm. In addition, evening vitamin dosing resulted in a significant drop in CRP concentrations compared with the slight diurnal decrease seen in the no-meal control study. Although the half-life of CRP is 19 hours, the clearance approximates a monoexponential function with a steeper drop in levels over the initial part of the curve.\textsuperscript{29} If the hepatic production of CRP is halted by presupper vitamins and rapid initial clearance proceeds, this could explain the mechanism of the 14% reduction in CRP levels seen in the postprandial period in this study arm. We observed that vitamin E levels were stable but vitamin C levels waned after prebreakfast compared with presupper vitamin ingestion. Thus, our data suggest that ambient vitamin C may be necessary to regenerate the reduced form of vitamin E to facilitate maximal antioxidant and anti-inflammatory protection after a high-fat evening meal.

PAI-1 is the primary physiological inhibitor of clot lysis. Circulating PAI-1 levels are elevated in patients with coronary artery disease and in type 2 diabetes.\textsuperscript{30} PAI-1 levels in the highest quintile are predictive of future coronary events.\textsuperscript{31} The increased PAI-1 detected in coronary atherectomy specimens from type 2 diabetic subjects may contribute to the higher mortality and restenosis rate after coronary angioplasty.\textsuperscript{32} Hyperinsulinemic subjects exhibit an exaggeration of the classic afternoon decrease in PAI-1 activity, as we observed in the present study.\textsuperscript{33} PAI-1 levels are not affected by inflammation,\textsuperscript{33} which is consistent with our discordant results for antioxidant vitamin protection for CRP and PAI-1. PAI-1 release is stimulated by combined hyperinsulinemia, hyperglycemia, and hypertriglyceridemia,\textsuperscript{34} a constellation of metabolic derangements that is found in the postprandial period in type 2 diabetes. This study is the first published demonstration of meal-induced increased concentrations of PAI-1 activity. Our finding that prebreakfast vitamins E and C can blunt the meal-induced rise in PAI-1 is consistent with the known beneficial effects of antioxidants on PAI production from endothelial cells\textsuperscript{35} and with a recent study that showed a significant reduction in PAI-1 levels with high-dose $\alpha$-tocopherol supplementation in type 2 diabetes patients.\textsuperscript{3} Presupper vitamins may have been absorbed too slowly to prevent the rapid hyperglycemia- and hypertriglyceridemia-
induced production of PAI-1. Because PAI-1 is produced predominantly by endothelial cells (compared with CRP production by the liver), it may take longer for vitamin E to become available at the site of PAI-1 production.

MDA is a stable terminal metabolite of oxidized lipids. The level of MDA commonly expressed as thiobarbituric acid-reactive substances overestimates MDA concentrations up to 10-fold.17 The present study used gas chromatography–mass spectrometry, which is not subject to this inaccuracy. Studies have shown increased MDA and decreased TRAP at baseline and after a liquid meal in type 2 diabetes.17 Improved glycemic control and vitamin E supplementation can only partially correct MDA levels in type 2 diabetes.23 The high-fat meal in the present study did not induce significant changes in the levels of MDA. However, coadministration of vitamins E and C in the evening caused a significant decline in MDA concentration in the postprandial period. The preceding 24 hours of normoglycemia and the continuous basal insulin infusion used in this protocol may have masked meal-stimulated elevations in MDA. A limitation of the present study is the absence of a direct measure of free radical production by mononuclear cells. TRAP is a measure of the antioxidant status of extracellular fluids. In this study, there were no significant changes in the plasma antioxidant status of the subjects, as measured by TRAP.

We conclude that the timing of the ingestion of antioxidant vitamins is important for optimal efficacy in preventing meal-induced inflammation and hypofibrinolysis in type 2 diabetes. When taken in the morning, vitamins E and C protect against the elevations in PAI-1 and CRP induced by an evening high-fat meal. Ingestion of antioxidant vitamins immediately before a high-fat meal does not protect against postprandial hypofibrinolysis. However, presupper vitamins are superior to prebreakfast vitamins in lowering evening levels of CRP. We hypothesize that sufficient levels of vitamin C are required to regenerate the antioxidant capacity of vitamin E in the evening. The optimal combination of antioxidant vitamins requires further study but on the basis of our findings may include morning administration of RRR-α-tocopherol and slow-release ascorbic acid. The present study may explain the variable results obtained in antioxidant prevention trials. These studies used vitamin E with and without vitamin C at various doses and at variable times relative to meals. Our results suggest that the timing, dosage, and combination of antioxidant vitamins for the most favorable profiles of surrogate markers of atherogenesis need to be determined before large-scale clinical trials are initiated.

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References

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