Bioavailability of Carotenoids and Tocopherols from Broccoli: *In Vivo* and *In Vitro* Assessment

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Fruits and vegetables are the major sources of biologically active compounds, and carotenoids and tocopherols constitute important groups in human diets. Bioavailability is a critical feature in the assessment of the role of micronutrients in human health, and the approaches to this issue include *in vitro* and *in vivo* methods. Our aim was to evaluate the bioavailability of carotenoids and tocopherols present in broccoli and to compare *in vitro* and *in vivo* approaches. Fourteen apparently healthy volunteers consumed 200 g broccoli once a day for seven days. Blood samples were drawn at baseline and after intervention to determine changes in lutein, β-carotene, and α- and γ-tocopherol as relevant phytochemicals provided with this vegetable. Broccoli also was subjected to simulated gastrointestinal digestion to assess changes related to preabsorptive processes. Analytes in serum and at each phase of the digestion were assayed by high-performance liquid chromatography. During the intervention, the amounts supplied daily ranged from 2.4 to 3.1 mg lutein, 1.4 to 1.8 mg β-carotene, 4.5 to 6.8 mg α-tocopherol, and 0.8 to 1.8 mg γ-tocopherol.

Significant changes in serum in both men and women were observed only for lutein, whereas for γ-tocopherol a significant change was detected in women. No changes were observed for α-tocopherol, β-carotene, retinol, the α-tocopherol-to-cholesterol ratio, or serum lipids. Using the *in vitro* model, more than 75% of lutein, β-carotene, γ-tocopherol, and α-tocopherol remained at the duodenal phase, whereas incorporation into the supernatants accounted for <20% of the initial content in food. Regular consumption of broccoli at dietary levels increased serum concentrations of lutein and γ-tocopherol without affecting α-tocopherol or β-carotene status in serum. The behavior of these phytochemicals under *in vitro* gastrointestinal conditions does not fully explain the changes observed *in vivo*. Exp Biol Med 231:1733–1738, 2006

Key words: bioavailability; carotenoids; tocopherols; human study

Introduction

Fruits and vegetables are the major sources of biologically active compounds (i.e., phytochemicals), and an increased consumption is recommended. Among these compounds, carotenoids and tocopherols constitute important groups in human diets that, in addition to their vitamin activity, display several other biological activities, including antioxidant capacity, blue light filtering, modulation of immune function, and regulation of cell differentiation and proliferation (1–4).

Interest in the bioavailability of vitamins and other food components has greatly increased because of the existence of undernourished populations and groups at risk of developing micronutrient deficiencies (i.e., the elderly) and the epidemiologic evidence suggesting protective effects against several diseases (i.e., cancer and cardiovascular diseases). Another factor is the increasingly important role of the food industry in developing new products with added nutritional value and their potential impact on public health and the nutritional status of the population (5).

Bioavailability is a critical feature in the assessment of the role of vitamins in human health. Approaches to the study of the bioavailability of food components can be broadly classified as *in vitro* and *in vivo* methods, each having its pros and cons. *In vitro* models based on human physiology have been developed as simple, inexpensive, and reproducible tools to study digestive stability, micellization, intestinal transport, and metabolism and to predict the bioavailability of different food components (i.e., ascorbic acid, carotenoids, chlorophylls, and polyphenols; Refs. 6–11). However, several food- and host-related factors are capable of influencing vitamin bioavailability at different points (12), and, thus, *in vitro* methodology for

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bioavailability assessment and its potential predictive value regarding human absorption of phytochemicals should be validated in different *in vivo* situations.

The application of *in vitro* digestion models may be of relevance in understanding the bioavailability of phytochemicals in humans as well as in the food industry, including food technology, functional foods, and product design. However, comparison of *in vitro* digestion models is complicated, since it usually is difficult to ascertain which model provides the most accurate bioaccessibility values in terms of the human situation unless the models can be compared with *in vivo* studies (13). Most of the previous studies have dealt with single nutrients (i.e., lutein) from different foods or supplements or have used pharmacokinetic approaches (single-dose) to assess the relative bioavailability. Regarding tocopherols, similar studies performed in humans using green vegetables at achievable dietary levels as a source of α- and γ-tocopherol are scarce. Moreover, to our knowledge, information regarding the bioavailability of simultaneously present and co-ingested phytochemicals, both *in vitro* and *in vivo*, is lacking. Thus, to lend support to both the reliability of this approach for the study of food-related determinants of the bioavailability of carotenoids and tocopherols from fruits and vegetables, as well as to its potential applicability to other food matrices and components, our objective was to evaluate in humans the bioavailability of the major carotenoids and tocopherols present in broccoli and compare two complementary approaches: *in vitro* and *in vivo* protocols.

Materials and Methods

Subjects. Fourteen apparently healthy volunteers (seven men and seven women; ages 20–35 years) were enrolled in the dietary intervention study. All participants were required to have biochemical and hematological profiles and serum levels of vitamins A and E and carotenoids within accepted reference ranges (Table 1; Ref. 14), whereas exclusion criteria included the use of vitamin and/or herbal supplements, dieting, chronic medication, or intercurrent disease or infection that could alter the bioavailability or the status of the compounds of interest.

The dietary intervention study consisted of the intake of 200 g broccoli once a day, at lunch or dinner, for 7 days. Broccoli was purchased in two batches and was prepared as edible portions (200 g) to be supplied to the volunteers. The broccoli processing conditions were uniform for all subjects and involved the use of microwave ovens (800 W; 5 mins of cooking plus 5 mins left standing). Subjects were asked to consume the standard portions together with a fixed amount of olive oil (10 ml). No other changes were made in the diet or the lifestyle of the participants except the requirements that they avoid the consumption of other green vegetables (i.e., spinach and swiss chard) or fortified foods (i.e., juices) and that they keep a record of their diets to check the compliance with these dietary recommendations. Overnight fasting blood samples were obtained before and after the intervention study for analysis of vitamins A and E and carotenoids. The study protocol was approved by the Comité Etico de Investigación Clínica of the Hospital Universitario Puerta de Hierro, and all subjects were informed and gave their signed consent.

Standards and Reagents. Unless otherwise stated, all reagents and materials used in the *in vitro* protocol and the analysis of blood samples for vitamins A and E and carotenoids were purchased from Sigma (Madrid, Spain), VWR Internacional Eurolab (Mollet del Vallés, Spain) and Carlo Erba (Madrid, Spain). Zeaxanthin, 9-*cis*-β-carotene and 13-*cis*-β-carotene were generously supplied by DMS (formerly Hoffmann-La Roche, Basel, Switzerland).

***In Vitro* Digestion and Phytochemical Analysis.** The *in vitro* digestion model was based on that of Oomen et al. (15) and optimized to assess the bioavailability of phytochemicals from foods (Granado-Lorencio et al.; Ref. 16) Commercially available broccoli (*Brassica oleracea*), purchased to be supplied to the volunteers as a source of free carotenoids and tocopherols, was studied (number of batches = 4, two per batch) to assess the stability and isomerization of carotenoids and tocopherols under *in vitro* conditions. Briefly, broccoli was prepared as for a meal (microwave, 5 mins) and was homogenized with a kitchen blender for 1 min to simulate mastication. Samples (in triplicate) of approximately 10 g were transferred to a flask, and a saliva solution (9 ml, pH 6.5) containing organic and inorganic components and α-amylase (145 mg) was added, after which the samples were incubated in a shaking water bath (37°C, 95 opm) for 5 mins. Gastric juice (13.5 ml) with organic and inorganic solutions, mucin (1 g), bovine serum albumin (1 g), and pepsin (1 g) from porcine stomach was added. The pH was adjusted to 1.1, and the solution was incubated for 1 hr. Duodenal juice (25 ml, organic plus inorganic solutions, containing 3 g porcine pancreatin) and bile solution (9 ml, containing 0.6 g bovine bile) were introduced after neutralization of the pH (7.8), and the human pancreatic lipase (1 unit), colipase (12.5 μg), cholesterol esterase (5 units), phospholipase A_2 (50 μl), and taurocholate salts (19.9 mg) were added. The final volume was approximately 65 ml, and the mixture was incubated for 2 hrs. Transfer from the duodenal digesta to the aqueous-micellar phase was estimated by calculating the proportion of phytochemicals in the supernatants after low-speed centrifugation (5000 *g* for 20 mins). The entire procedure was performed under dimmed light. At each step, aliquots (~1 ml) were collected in duplicate, extracted before and after chemical (KOH) hydrolysis, and analyzed by high-performance liquid chromatography (14, 17).

For vitamins A and E and carotenoid analysis in serum, samples were processed as described elsewhere (14). Briefly, 0.5 ml serum was mixed with 0.5 ml ethanol containing internal standard (retinyl acetate), vortexed, and extracted twice with 2 ml methylene chloride/hexane (1:5). Organic phases were pooled, evaporated to dryness, and

Table 1. Characteristics of the Subjects and Serum Concentrations at Entrance and at the End of the Intervention Study[a]

	At entrance[b]	At the end of intervention[b]	*P* value[c]
No. of subjects (male/female)	14 (7/7)	—	—
Age (years)	24 (21, 27)	—	—
BMI (kg/m^2)	23.1 (22, 25)	—	—
Total cholesterol (m*M*)	4.16 (3.72, 4.63)	4.16 (3.75, 4.58)	0.96
HDL cholesterol (m*M*)	1.52 (1.34, 1.73)	1.55 (1.42, 1.71)	0.76
LDL cholesterol (m*M*)	2.28 (1.89, 2.69)	2.22 (1.76, 2.66)	0.80
Triglycerides (m*M*)	0.80 (0.54, 1.05)	0.84 (0.65, 1.04)	0.74
Retinol (μ*M*)	1.79 (1.43, 2.16)	1.81 (1.49, 2.13)	0.95
Lutein (μ*M*)	0.20 (0.17, 0.24)	0.30 (0.25, 0.36)	0.001
Zeaxanthin (μ*M*)	0.050 (0.037, 0.062)	0.037 (0.025, 0.049)	0.001
β-cryptoxanthin (μ*M*)	0.30 (0.19, 0.40)	0.25 (0.16, 0.34)	0.037
Lycopene (μ*M*)	0.66 (0.54, 0.78)	0.54 (0.47, 0.61)	0.005
α-carotene (μ*M*)	0.080 (0.060, 0.101)	0.075 (0.057, 0.093)	0.22
β-carotene (μ*M*)	0.40 (0.17, 0.63)	0.40 (0.19, 0.60)	0.73
α-tocopherol (μ*M*)	24.7 (22.0, 27.4)	25.0 (22.3, 27.7)	0.73
α-tocopherol/cholesterol (μmol/mmol)	6.8 (6.1, 7.4)	6.81 (6.3, 7.4)	0.88
γ-tocopherol (μ*M*)	0.54 (0.41, 0.66)	0.62 (0.44, 0.81)	0.43

[a] BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
[b] Values given as mean (95% CI).
[c] ANOVA and nonparametric test (Wilcoxon signed rank test).

reconstituted to be injected (THF/EtOH) onto the high-performance liquid chromatography. The chromatographic system consisted of a Spheri-5-ODS column (Applied Biosystems, San Jose, CA) with gradient elution of acetonitrile/methanol (85/15) for 5 mins to acetonitrile/methylene chloride/methanol (70/20/10) for 20 mins. Ammonium acetate (0.025 *M*) was added to the methanol. Detection was carried out by a photodiode array (Model 996; Waters Associates, Milford, MA) set at 294 nm for tocopherols and 450 nm for carotenoids. Using this method, α-tocopherol, γ-(+β)-tocopherol (under these chromatographic conditions the two vitamers coelute) and δ-tocopherol, *trans*-lutein, zeaxanthin, 13/15-*cis*-lutein, α-carotene, all-*trans*-β-carotene, 9-*cis*-β-carotene, and 13/15-*cis*-β-carotene, among other carotenoids, can be determined simultaneously. Identification of compounds was carried out by comparing retention times with those of authentic standards and on-line UV–visible spectra.

For vitamins A and E and carotenoid analysis, samples from each individual (obtained before and after the intervention) were analyzed on the same day to reduce analytical variability. The short- and long-term precision and accuracy of the analytical method was within accepted values, as verified periodically through our participation in the Fat-Soluble Quality Assurance Program conducted by the National Institute of Standards and Technology (NIST; Gaithersburg, MD). Biochemical markers were monitored throughout the study by analyses performed in the General Biochemistry Laboratory of the hospital according to quality-controlled standardized methods.

Statistics. In order to achieve consistency in the results from the different *in vitro* experiments, the parameters evaluated (i.e., stability) were expressed as percentages of the initial concentrations, and descriptive statistics were used (mean, median, 95% confidence interval [95% CI], etc.). *In vitro* results were interpreted on the basis of data from crude and saponified extracts.

Sex-related differences in serum response upon broccoli ingestion were analyzed by ANOVA. Differences in serum concentrations at the end of the intervention period were assessed by ANOVA and nonparametric test (Mann-Whitney *U* test and Wilcoxon signed ranks test). Statistical significance was set at $P < 0.05$, and the analysis was performed with SPSS 8.0 statistical software for Windows (SPSS Inc., Chicago, IL).

Results

Human Study. Samples of the broccoli used in the study were analyzed in ready-to-eat form (i.e., after microwave processing and with olive oil). The amounts provided were estimated by analyzing the two batches of broccoli twice ($n = 4$, each one in triplicate), calculating the average content (mean, 95% CI) and using the confidence intervals to estimate the range of the dose provided. Average contents of the phytochemicals per 100 g broccoli were: 1373 μg *trans*-lutein (95% CI: 1200, 1546), 812 μg total β-carotene (95% CI: 729, 895), 2829 μg α-tocopherol (95% CI: 2262, 3396), and 672 μg γ-tocopherol (95% CI: 420, 924). Based on these data and the amount of broccoli consumed, average amounts of the phyochemicals supplied per day during the intervention study ranged between 2.4 and 3.1 mg lutein, 1.4 and 1.8 mg β-carotene, 4.5 and 6.8 mg α-tocopherol, and 0.8 and 1.8 mg γ-tocopherol.

Table 2. Recovery of Carotenoids and Tocopherols from *In Vitro* Digestion[a]

Digestion phase	β-carotene	Lutein	α-tocopherol	γ-tocopherol
Food, μg/100 g (mean, 95% CI)	812 (729, 895)	1373 (1200, 1546)	2829 (2262, 3396)	672 (420, 924)
Gastric[b]	77 (72–82)	88 (77–98)	90 (89–90)	60 (28–91)
Duodenal[b]	81 (71–99)	95 (82–103)	85 (68–97)	75 (42–97)
Supernatant[b]	18 (15–21)	10 (6–15)	20 (18–21)	16 (12–19)

[a] $n = 4$. Recovery expressed as percentage of the initial content in the food (as 100%).
[b] Values are given as mean (range).

Regarding the intervention, at entrance differences between sexes were observed only for γ-tocopherol (0.66 vs. 0.42 μ*M* for men and women, respectively; $P = 0.03$). Serum concentrations of carotenoids and tocopherols reached after consumption of broccoli for 7 days are shown in Table 1. On the group level, only the changes in lutein reached statistical significance. For γ-tocopherol, although a nonsignificant increment in serum concentrations was observed on the group level, this change was significant for women ($P = 0.014$), even when the final concentrations achieved did not differ between sexes. As shown in Table 1, the levels of other carotenoids not supplied with broccoli significantly decreased in serum (except for α-carotene), whereas no significant changes were observed for α-tocopherol, β-carotene, retinol, the α-tocopherol-to-cholesterol ratio, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, or triglycerides (Table 1).

In Vitro Assessment. Under the *in vitro* conditions employed, more than 75% of lutein, β-carotene, γ-tocopherol, and α-tocopherol contained in processed broccoli was recovered (stability) at the end of the duodenal phase, whereas incorporation into the supernatants (micellization) accounted for ≤20% of the initial content (Table 2). *cis*-Lutein and *cis*-β-carotene in the food did not increase throughout the digestion process (saliva, gastric, or duodenal phase), but in comparison with the duodenal content the percentages of *cis*-carotenoids in the supernantants were higher both for 13/15-*cis*-β-carotene (20%–27%) and *cis*-lutein (approximately 12% vs. 6% during the duodenal phase).

Discussion

The present study investigated the *in vivo* and *in vitro* bioavailability of carotenoids and tocopherols from broccoli. Broccoli was chosen because it is a frequently consumed dietary source of carotenoids and tocopherols, and also because of the interest in broccoli on the part of the food industry, especially with regard to broccoli's importance as a food for emerging technologies (i.e., minimally proccessed, modified atmospheres) and commercialization. The amount to be consumed was set at achievable dietary levels (i.e., 200 g/day) considered sufficient to provoke significant changes in the serum status of these phytochemicals.

In the present human study, mean serum levels of lutein and γ-tocopherol increased (by approximately 50% and 16%, respectively), although on the group level only the change in lutein reached statistical significance. Compared with β-carotene, the greater serum lutein response is consistent with serum responses reported in other human studies using green vegetables (18–21) and with the preferential incorporation into chylomicrons and a higher bioavailability of lutein and zeaxanthin reported in humans (18, 19, 22). Nevertheless, the lack of a significant effect on serum β-carotene levels was somewhat unexpected, since a 28% increase in serum levels of β-carotene has been reported after 4 days of broccoli consumption (19). In contrast to that cited study, the lack of a significant increase may be related to the smaller amount of broccoli consumed and, thus, the dose of β-carotene supplied (1.4–1.8 vs. 1.7–24.6 mg/day in Ref. 19). Nevertheless, the lack of variation in serum β-carotene may be related to the effect of dietary intervention and relatively high serum levels of the volunteers at the start of the study (mean: 0.40; 95% CI: 0.17, 0.63). In this respect, although the subjects were advised to avoid only other green vegetables and other relevant contributors (i.e., β-carotene–enriched juices) during the study, it could be that the amount of broccoli consumed (β-carotene supplied) had been only enough to compensate for their habitual intake of β-carotene from other food sources. This explanation is supported by the decrease in the serum levels of all other carotenoids, a finding that suggests both a good compliance with the protocol and a true change in the habitual food patterns of the volunteers.

As shown, stability during the *in vitro* digestion was similar for both carotenoids, although β-carotene was, on average, slightly better incorporated into the supernatants. This small difference could compensate somewhat for the distinct content in the broccoli so that a similar response in serum could be expected. However, in the human study, lutein significantly increased, whereas β-carotene did not change, a finding not expected given the similar *in vitro* behavior of both carotenoids. Thus, based on the present *in vitro* results, plausible explanations for the different serum

responses indicate the involvement of host-related factors, such as the differential biologic actions of lutein and β-carotene (i.e., provitamin A capacity) in the body. Although the volunteers showed adequate serum retinol levels that did not change during the study, it is known that about two-thirds of the β-carotene ingested may be recovered as retinyl esters in the postprandial state (23). Thus, a differential first-pass metabolic effect at the intestinal level also may have contributed to the differences in the serum responses of lutein and β-carotene.

Regarding tocopherols, broccoli (plus 10 ml olive oil) contained an important amount of α- and γ-tocopherol, and its consumption accounted for more than 30% of the recommended dietary intake of α-tocopherol (23). Under these conditions, only serum γ-tocopherol increased, whereas no change was observed for α-tocopherol, even after correction for total cholesterol concentration (i.e., α-tocopherol-to-cholesterol ratio). Again, although this lack of increase may relate to the dose supplied (i.e., insufficient to provoke a significant increase in serum), γ-tocopherol did show an increase (moderate at the group level but significant in women) and, thus, the dose does not seem to be a plausible single contributing factor, even assuming some dietary changes during the study.

As with carotenoids, the *in vitro* study showed a similar recovery of α- and γ-tocopherol at the end of duodenal phase and in their incorporation into supernatants, a finding that is consistent with the nonselective absorption of different tocopherol isomers in the human gastrointestinal tract (24, 25). Moreover, the estimate of approximately 20% as the proportion of α-tocopherol to be present in the supernatants is consistent with some figures reported for absorption of α-tocopherol from meals (23). Thus, based on the *in vitro* behavior, the serum response of α-tocopherol was expected to be higher than that of γ-tocopherol in the human study. However, since serum levels were measured under overnight fasting conditions, plasma concentrations depended upon secretion from the liver (23). In this respect, it is well known that liver discriminates between tocopherols, with a preferential incorporation of α-tocopherol isomer into very low-density lipoproteins through the action of an α-tocopheryl-transfer protein (23–25), illustrating again a first-pass (liver) effect. Thus, despite the lack of biodiscrimination observed during preabsorptive processes (*in vitro* model), the differential control of the intracellular pool of the two isomers may explain, at least in part, the serum responses observed in the present human assay.

In summary, short-term consumption of broccoli at dietary levels by apparently healthy young volunteers significantly increased the serum concentrations of lutein and γ-tocopherol, and the *in vitro* protocols have proved to be a rapid and cost-efficient alternative to the initial screening of bioavailability of micronutrients and phytochemicals (26). However, the present results indicate that food-related factors affecting bioavailability of carotenoids and tocopherols may be approached using *in vitro* methods, whereas host-related factors and different physiologic processes, including health and nutritional status, first-pass metabolism, intracellular regulation, and homeostatic control, may limit the comparability and the predictive value of *in vitro* models. In this respect, the two approaches should be considered complementary, but not necessarily interchangeable.

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