Protection of Humans by Plant Glucosinolates: Efficiency of Conversion of Glucosinolates to Isothiocyanates by the Gastrointestinal Microflora

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Abstract

Plant-based diets rich in crucifers are effective in preventing cancer and other chronic diseases. Crucifers contain very high concentrations of glucosinolates (GS; β-thioglucoside-N-hydroxysulfates). Although not themselves protective, GS are converted by coexisting myrosinases to bitter isothiocyanates (ITC) which defend plants against predators. Coincidentally, ITC also induce mammalian genes that regulate defenses against oxidative stress, inflammation, and DNA-damaging electrophiles. Consequently, the efficiency of conversion of GS to ITC may be critical in controlling the health-promoting benefits of crucifers. If myrosinase is heat-inactivated by cooking, the gastrointestinal microflora converts GS to ITC, a process abolished by enteric antibiotics and bowel cleansing. When single oral doses of GS were administered as broccoli sprout extracts (BSE) to two dissimilar populations (rural Han Chinese and racially mixed Baltimoreans) patterns of excretions of urinary dithiocarbamates (DTC) were very similar. Individual conversions in both populations varied enormously, from about 1% to more than 40% of dose. In contrast, administration of ITC (largely sulforaphane)-containing BSE resulted in uniformly high (70%–90%) conversions to urinary DTC. Despite the remarkably large range of conversion efficiencies between individuals, repeated determinations within individuals were much more consistent. The rates of urinary excretion (slow or fast) were unrelated to the ultimate magnitudes (low or high) of these conversions. Although no demographic factors affecting conversion efficiency have been identified, there are clearly diurnal variations: conversion of GS to DTC was greater during the day, but conversion of ITC to DTC was more efficient at night. Cancer Prev Res; 5(4); 603–11. ©2012 AACR.

Introduction

It is widely accepted that human consumption of plant-rich diets, and consequently lower intake of other foods, reduces the risk of developing cancer and other age-related chronic diseases (1, 2). This landmark scientific consensus not only supports the view that many major chronic diseases can be prevented, but also suggests an explicit dietary strategy for prevention. In a recent illustration, British vegetarians had relative risks of stomach, ovarian, bladder, lymphatic/hematopoetic, and “all” cancers that were 0.36, 0.69, 0.47, 0.55, and 0.88, respectively, compared with those of meat eaters (3). A significant refinement of this concept is the finding that one specific family of vegetables, the Cruciferae, is more highly protective than many other dietary plants. Thus, the risk of bladder cancer was approximately halved in men who ate high amounts of cruciferous vegetables compared with low quantities, but not in those who ate fruits and other vegetables or their combinations (4). Consumption of both raw and total cruciferous vegetables (highest vs. lowest quintile) was associated with more than 40% lower risk of lung cancer among smokers (5). Consumption of 5 or more servings compared with less than 2 servings per week of crucifers was associated with a 33% lower risk of non-Hodgkin’s lymphoma in women (6). There was a decreasing (31%–39%) association of prostate cancer with increased intake of crucifers in 2 separate studies (7, 8), as well as substantial reductions in colorectal cancer (9). Cruciferous vegetables include 2 noteworthy edible genera: Brassica (broccoli, cabbage, and cauliflower), and Raphanus (radish and daikon). Crucifers are unique in the Western diet because they are rich in glucosinolates (GS), which are believed to be largely responsible for their protective properties (10–14). GS are β-thioglucoside N-hydroxysulfates that are converted to isothiocyanates (ITC; also known as mustard oils) by myrosinases [E.C. 3.2.1.147],...
abundant enzymes that coexist, but are physically segregated in intact plant cells. Myrosinase does not occur in cells of mammals but is found in their gastrointestinal microflora. Upon damage to plant cells, myrosinases gain access to their relatively inert GS substrates and convert them to highly reactive and bitter tasting ITC that defend plants against injury by insects, bacteria, and fungi (see reviews refs. 15–17). Conversion of GS to ITC is one of the most ingenious methods that plants, especially crucifers, have evolved for protection against predators (15).

By coincidence, ITC have been recognized for many years as powerful protectors against carcinogenesis in animals, and these effects have been attributed to the induction of cytoprotective enzymes (18–20). A widely studied example and these effects have been attributed to the induction of as powerful protectors against carcinogenesis in animals, evolved for protection against predators (15). Conversion of GS to ITC is one of the most ingenious methods that plants, especially crucifers, have evolved for protection against predators (15).

The myrosinase reaction therefore also plays a central role in the protection of animal cells against cancer and other chronic diseases. Consumption of lyophilized boiling water extracts of fresh broccoli sprouts (BSE) is an efficient method for administering standardized doses of GS to human volunteers. If these preparations are treated with myrosinase from daikon sprouts, the resultant extracts deliver ITC, principally SF, derived from its GS precursor GR (19, 21).

Earlier clinical studies with this system have established that (i) administration of SF-rich BSE resulted in uniformly high urinary excretion of dithiocarbamate (DTC) metabolites that accounted for about 70% to 90% of the administered dose of SF in 24 hours (22). (ii) GS to DTC and ITC to DTC conversions in individual subjects are strictly proportional to GS and ITC doses, respectively (23, 24). (iii) When BSEs in which myrosinase had been heat-inactivated are administered to humans, conversion of GS to DTC is promoted by the microflora of the gastrointestinal tract. Evidence for this mechanism was obtained by suppression of bowel microflora with enteric antibiotics and mechanical bowel cleansing, which almost completely abolished conversion of GS to DTC, followed by very rapid recovery (within days; ref. 23).

In contrast to administration of SF-rich BSE, however, the conversion of GS to DTC was highly variable among individuals, ranging from a low of about 1% to a high of more than 40% of the administered dose (22, 25).

It is well established that ITCs, not GS, are principally responsible for regulating genes coding for proteins that protect cells against oxidative stress, inflammation, and electrophile toxicity (10–12, 20). Because the majority of cruciferous vegetables are generally cooked before eating, thus inactivating plant myrosinase, the efficiency with which the gastrointestinal microbial flora of individuals converts dietary GS to ITC may either limit or augment the beneficial effects of high GS consumption. A differential effect of raw versus cooked broccoli consumption is strongly supported by epidemiologic data (14). In the hope of decreasing risk of chronic disease, our ultimate goal is to devise interventions to enhance the efficiency of the conversion of GS to pharmacologically active ITC, especially in individuals who do not do so efficiently. Therefore, our current study examines the factors responsible for the bioavailability and metabolism of SF in humans consuming GR delivered in BSE. Because the principal GS in these preparations of BSE is GR, the precursor of SF, we refer from here on to the conversion process as occurring from GR to SF. SF is conjugated with glutathione in animal tissues by glutathione S-transferase, to form glutathionyl–SF (a DTC) which then undergoes further stepwise enzymatic degradation via the mercapturic acid pathway to N-acetylcysteinyl-SF which is excreted in the urine. SF and its DTC metabolites can be quantified collectively by a spectroscopic high-performance liquid chromatography (HPLC) method that depends on a cyclocondensation reaction with 1,2-benzenedithiol developed in our laboratory (26, 27). Determination of urinary excretion of unchanged SF and its DTC metabolites provides a quantitative measure not only of the overall bioavailability of SF but also of its DTC metabolites, some of which are probably also active as regulators of cytoprotective enzymes (28–30). Most of the conversion of single doses of GR to SF and DTC is complete within 24 hours (refs. 22, 24; and experiments reported below). We refer in this article to the “efficiency of conversion” of single doses of GR administered in BSE, to urinary metabolites which include free SF and its DTC conjugates, as measured by the cyclocondensation reaction, and express these as a percentage of administered dose. We recognize that the overall formation of urinary DTC from orally administered GR involves multiple processes, including intestinal absorption, enzymatic conversion of GR to SF, enzymatic conjugations of SF via the mercapturic acid pathway, and renal excretion. The rate-limiting step in “conversion efficiency” reflects largely the formation of SF from GR, as the efficiency of conversion of administered SF to DTC in human volunteers is very high (70%–90%). Conversion efficiency is therefore a reasonable index of bioavailability of the pharmacologically active chemical species.

Materials and Methods
Preparation of broccoli sprout extract
Preparation of extracts was essentially as described previously (22, 31–33). Briefly, broccoli sprouts were grown from selected seeds with adequate GR levels to yield 3-day-old fresh green sprouts with levels of at least 6 μmol of GR per gram. Seeds were surface-disinfected and grown in a commercial green sprouting facility that adheres to U.S. Food and Drug Administration mandated sanitary regulations for sprout production. After 3 days of growth in which water and light were the only inputs, an aqueous extract was prepared in a steam-jacketed kettle at a food processing facility (Oregon Freeze Dry). Sprouts were plunged into boiling deionized water and maintained at more than 95°C for 30 minutes and the sprout residues removed by filtration. The aqueous extract containing about 5 μmol of GR per mL, and essentially no SF, was frozen rapidly, and lyophilized in industrial freeze driers. Total GR titer of the resulting powder was at least 200 μmol/g as assayed.
Urine collection, measurement of SF metabolites, and compliance

Urine volumes were measured and aliquots were taken for measurement of creatinine (Hagerstown Medical Laboratory, Hagerstown, MD) and for cyclocondensation, which was done as described by Ye and colleagues (27). We discarded 8 clearly incomplete urine collections, as judged by creatinine measurements, of a total of 139 collections.

Analysis of SF metabolites

SF metabolites were analyzed by isotope dilution mass spectrometry as described by Egner and colleagues (36) and were measured in the first overnight urines collected from participants randomized to receive a daily dose of 400 μmol GR in BSE for 14 days. Details of the study conducted in Qidong, China, have been reported (25).

Statistics

Descriptive statistics, as well as ANOVA, trend analyses (nptrend, ranksum), pairwise correlations (pwcorr), Students t-test comparisons, and random intercept linear mixed effect models to evaluate between-person covariate effects were all made using Stata 10.0 (StataCorp.).

Results and Discussion

Efficiency of conversion of GS to ITC

Reliable quantitative conversion efficiencies were obtained on 45 healthy volunteers living in Baltimore (27 women and 18 men, aged 21 to 87 years). Thirteen subjects were evaluated only once, whereas 32 subjects were tested from 2 to 7 times, to provide a total of 131 observations. At 8 am each subject received a single oral dose of broccoli sprout extract (BSE) containing 200 μmol of GS of which 85% was GR, the precursor of SF. Total 24-hour urine was collected in 2 aliquots: for the first 8 hours and the subsequent 16 hours (completeness of urine collections was confirmed by creatinine determinations). The sum of the urinary excretion of nonmetabolized SF and its DTC metabolites was determined by the cyclocondensation reaction (27). These determinations permitted comparisons with the conversions of GR to SF metabolites in 2 earlier studies; (i) a safety and tolerance study in which cohorts of 3 subjects each received similar BSE preparations containing either 25 or 100 μmol of GR, every 8 hours for 7 days, and in which complete urine collections were obtained during each 8-hour interval for 7 days (21 samples; ref. 22). (ii) Our study in China in which 99 healthy volunteers received doses of BSE extract containing 400 μmol of GR nightly for 14 days, and overnight urines (approximately 12 hours) were collected after each dose (25). Comparisons of conversion efficiencies between studies that used a wide range of oral doses of GS are valid, as at least over a dose range of 25 to 200 μmol GS, there is a strict linear relation between DTC excretion and dose, and the time courses of excretion of DTC over this range of doses were also similar (22).

Magnitude of total conversions

In the 131 observations obtained on 45 subjects, the range of total 24-hour DTC excretions following a single 200 μmol glucosinolate dose varied enormously, from 2.2 to 81.3 μmol of DTC (i.e., 1.1%–40.7% of dose) with a mean of 23.5 μmol (11.8% of dose). The distribution of magnitudes of conversions was skewed dramatically toward lower efficiencies. Thus, more than 80% (106 of 131) of the conversion determinations were below the mid point value (20.9% of dose), and only 9 of the 45 subjects exceeded this level (Fig. 1A). High conversion is therefore uncommon.

By comparison, in our 2006 study (22) in which subjects were dosed every 8 hours for 7 days, the mean excretion efficiencies for all 21 determinations in 3 individuals receiving doses of 25 μmol BSE GR were 17.5% (range 5.87%–41.2%), and in those receiving doses of 100 μmol GR were 18.5% (range 4.14%–38.3%). The finding of large between-subject differences led to the recognition that there seemed...
The kinetics of conversion patterns

In a small number of subjects from whom additional urine samples were collected at both shorter (<8 hours) and longer (>24 hours) time intervals, we observed (i) all of the DTC derived from a single dose of GR dose was excreted within 24 hours. (ii) Median peak excretion in all of the tests occurred at about 8 hours, but the excretion patterns during the 24-hour period were dramatically different among individuals. Whereas in some subjects more than two-thirds of the total excretion of DTC occurred in the first 8 hours after dosing, in others two-thirds of total DTC excretion occurred during the 8–24-hour collection period. There is therefore a continuum of conversion velocities ranging from fast to slow. Furthermore, the ratios of the rates of DTC excretion per hour during these collection periods varied greatly among subjects. We selected a random sample of 13 of the 131 conversion determinations, ranging from low to high conversion efficiency to examine the relation between rate and total quantity of conversion (Fig. 2). The ratio of DTC excretion rates (during the first 8 hours to subsequent 16 hours) ranged enormously and apparently continuously from 0.55 to 28. Interestingly, there was absolutely no relation between the rates (slow to fast) of converter phenotypes, and the total magnitude (low to high) of conversion during the 24-hour time period (Fig. 2).

Variability in conversion efficiency between- and within-subjects over time

Figure 3 compares the large conversion efficiency differences between subjects as well as the greater consistency over time within subjects in 2 populations. In Baltimore (Fig. 3A) the conversion magnitudes on 2 cohorts who received 75 or 300 μmol of GR in 3 divided doses daily (3 subjects each) varied from about 5% to 30%, but the conversion within individuals over a 7-day period was much more consistent (22). A similar pattern was observed in China (25), where 99 subjects received 400 μmol of GS daily for 14 days. Figure 3B shows the excretion of DTC on 4 days for the 10 lowest and the 10 highest converters. Furthermore, there are very large quantitative differences between the highest and lowest converters, but values for individuals were much more consistent.
Human Conversion of Glucosinolates to Isothiocyanates

To establish and then validate protocols to assess the effects of potential interventions on the efficiency of conversion of GS to ITC, we compared the variances of the conversion efficiencies of repeated observations within subjects to those between-subjects, including only the first observation in those who had multiple tests. The intervals between retesting varied between a few days to 2.5 years. The first test for each subject is highlighted in Fig. 1A. The 131 observations in 32 subjects with multiple observations on the time course of DTC excretion rates in healthy hospitalized volunteers in which BSE containing 25 μmol of SF was given orally at 8-hour intervals (at 7 am, 3 pm, and 11 pm) for 7 days and compared them with rates observed when BSE delivering either 25- or 100-μmol doses was consistency among repeat observations on each individual, there were occasional very low conversions and the reasons for these anomalies are unclear. Nevertheless, a total analysis of all repetitive observations on individual subjects showed clearly that the interval between repetitive studies (from a few days to 2.5 years) did not explain this variability and provided the basis for power calculations for intervention studies (results not shown).

Diurnal variation in conversion/conjugation efficiencies

We have now analyzed in greater detail our previous observations on the time course of DTC excretion rates in healthy hospitalized volunteers in which BSE containing 25 μmol of GR and measurement of 24-hour DTC excretion. The results are expressed as percent of administered dose. The intervals between tests varied from a few days to 2.5 years.
of GR was administered on the same schedule (22). Urine was collected during the corresponding 8-hour intervals. Three subjects were in each cohort. There is a marked and regular periodicity in the DTC excretion patterns during each day (Fig. 5A–C). Whereas for the SF treatment, the mean urinary DTC excretion for all subjects was lowest during the morning (7 am to 3 pm) and rose during the subsequent two 8-hour intervals, the excretion rate of GR metabolites showed the opposite pattern, falling progressively from day to night. These marked diurnal variations were nearly constant on each day of the 7 days studied and are summarized graphically in Fig. 5D and E which compare the mean DTC excretions during the 3 daily time periods for the last 6 days for the 2 cohorts that received SF-rich and GR-rich BSE, respectively. The SF conversions are thus based on 18 measurements for each time period, and the GR conversions for each time period are based on 36 measurements. For ease of comparison, we have normalized the 7 am to 3 pm (morning) DTC excretion values for all treatment groups to 100%. Compared with the morning values, the corresponding mean DTC excretion values for SF administration rose to 170% and 175% for the afternoon (3 pm to 11 pm) and nighttime (11 pm to 7 am), respectively (Fig. 5D). In contrast, following GR administration, the afternoon and the nighttime excretions were 81.8% and 66.6%, respectively. Differences in conversion by time of dosing were highly significant by one-way ANOVA (F = 62.1, P < 0.00009; Fig. 5E).

Because the formation of the DTC from GR presumably proceeds through SF as an obligatory intermediate, and the conversion of SF to DTC increases during the 24-hour day, although the overall conversion of GR to DTC declines during this time period, it seems likely that the rate-limiting step in the latter decline occurs during the conversion of GR to SF. It also seems possible that the actual decrease in conversion efficiency of GR to SF during the night may be even larger.

Multiple processes are involved in the conversion of GR to DTC and their excretion in the urine. Most important is the participation of the gastrointestinal microbial flora which differs between individuals and within individuals over time. However, given the absence of any significant differences in the bacterial community structure of high and low ITC excreters reported recently (37), and the fact that conversion efficiency rapidly recovers from antibiotic challenge (23), the formidable task of detailed mapping of these microbial communities may not provide adequate insight into how the conversion of GR to SF can be enhanced in low converters. Other processes include the absorption and hydrolysis of GR to SF in the gastrointestinal tract, the conjugation of SF with glutathione, and its subsequent sequential degradation and N-acetylation of cysteine as part of the mercapturic acid pathway (38). Diurnal variation of renal excretion rates of these metabolites with substantial participation of tubular secretion (27) also may play a role in the diurnal kinetics of the observed conversion/excretion patterns. Thus diurnal changes in gastrointestinal mobility and in renal clearance may be involved. Diurnal changes in the activities of glutathione transferases are less likely to be responsible because polymorphism in at least some of these enzymes did not seem to influence SF metabolite excretion patterns (25). In light of the recently described dramatic effects of circadian rhythm on UV-induced skin carcinogenesis (39), the observations reported herein may have special relevance.

**Relation of conversion efficiencies to demographic characteristics**

Sample size was too small to make any but the most basic observations on the correlations between conversion efficiencies and demographic characteristics for the 131 observations on the 45 subjects studied, which were as follows: Gender (27 female, 18 male); Race: African-Americans 19, Caucasians 25, Asians 1; Age 21 to 87 years (mean 46.0); body mass index (BMI): 20.7 to 46.0 (mean 29.2); stool frequency per week: 2.3 to 14 (mean 8.5). A random intercept, linear mixed effect model used to evaluate
within-cluster and between-cluster covariate effects verified that there were no significant correlations of DTC excretion magnitudes with BMI, stool frequency, age, number of repeat visits, or race. Gender was only of marginal significance ($t = 2.06$, $P = 0.046$) with mean conversion by males (47 observations on 18 subjects) of 31.7 μmol (15.8% of dose), and mean conversions by females (84 observations on 27 subjects) of 18.9 μmol (9.5% of dose). In contrast, there was no relation of gender to efficiency of conversion of GS to total DTC in the 99 subjects studied in Qidong, China (25), nor was there an effect in our smaller China study (31). A more intensive examination of demographic factors influencing conversion/excretion efficiencies is therefore justified.

Patterns of SF metabolites

The cyclocondensation reaction provides generic quantification of ITC and their DTC metabolites (Fig. 6A) but does not distinguish their individual components. Availability of mass spectrometry for determining individual DTC metabolites provided a means for determining whether the quantitative differences in the excretion patterns observed among subjects were related to changes in patterns of metabolism (36). The 2 major metabolites of SF (N-acetylcysteinyl–SF and cysteinyl–SF) and free SF, all tracked monotonically with increasing total DTC excretion in our study in China (25). This is shown clearly in Fig. 6B which leads to the conclusion that conversion phenotypes neither result from nor create differences in the relative proportion of each of the major SF metabolites (Fig. 6A). When evaluated by use of pairwise correlations, urinary excretion of free SF, and its major mercapturic acid conjugates cysteinyl–SF, and N-acetylcysteinyl–SF, and the minor metabolite cysteinylyglycyl–SF were each highly correlated with total DTC excretion ($P = 0.00009$). The glutathionyl–SF conjugate (the SF metabolite found in lowest abundance in the urine) was not correlated with total DTC excretion ($P = 0.147$). There was no difference in the relative abundance of any of these compounds, or the less abundant cysteinylyglycyl–SF and glutathione–SF (data not shown), irrespective of conversion phenotype. Furthermore, none of these metabolites were correlated with the absence of either glutathione S-transferase (GST) genotypes GSTT1, GSTM1, or the absence of both enzymes. Also, there was no interaction between GST genotypes and total DTC excretion and no disproportionate representation of GST genotype in either high- or low-efficiency converters.

Conclusions

A major contributor to the protective effects of cruciferous vegetable in reducing the risk of cancer and other chronic diseases is believed to be their high content of GS. Whereas GS themselves are not significant chemoprotectors, they are converted by coexisting plant myrosinases

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Figure 6. Urinary excretion of SF and its metabolites (A), in the first overnight urine collections (approximately 12 hours) from 98 subjects in Qidong, China, who received 400 μmol of GR daily for 14 days (25, 36). (B) Urinary excretion of free SF (○), and its major mercapturic acid conjugates: cysteinyl–SF (▲), and N-acetylcysteinyl–SF (▲), as a function of total DTC excretion. Measurements of metabolites were for a single day and are expressed as nmol of the specified metabolite and normalized per mg creatinine.
and the gastrointestinal microflora of humans to ITCs, which are widely recognized as potent protective agents. The bioavailability of ITCs from GS is relatively constant in individual subjects upon repeated determinations over time but varies dramatically between individuals (ranging from 1%–40% of administered dose). Surprisingly, similar patterns of bioavailability have been observed in 2 highly dissimilar (with respect to genetics, diet, and environment) populations: rural Han Chinese and a mixture of Caucasian and African-American residents of Baltimore. High conversion efficiencies above 20% of dose are rare. The reasons underlying these differences between individual subjects remain unexplained, yet the potential health benefits of interventions to increase the bioavailability of ITCs from GS require serious consideration. The only solid clue as to factors contributing to these differences is that conversion is considerably higher during the day than at night. This may need to be considered when addressing the timing of GS administration to humans in clinical trials and in diet-based prevention strategies. The complex interactions of these diurnal cycles of microbial metabolism in the gut lumen, with the daily cycling of mammalian enzymes that are directly associated with carcinogenesis (e.g., those enzymes involved in the repair of DNA damage; 39), suggests that circadian rhythmicity may have profound implications for cancer prevention. Although our observations suggest that the gut microbiome exerts primary control over GR processing, there are a large number of interacting external factors that can affect conversion and use of these phytochemicals.

We have therefore identified a potential limitation to the protective benefits of crucifers in reducing chronic disease risk: low efficiency of conversion of GS to ITC by the gastrointestinal microflora of most individuals globally.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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