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Glucoraphasatin and Glucoraphenin, a Redox Pair of Glucosinolates of Brassicaceae, Differently Affect Metabolizing Enzymes in Rats

JESSICA BARILLARI,*,† RENATO IORI,† MASSIMILIANO BROCCOLI,‡ LAURA POZZETTI,‡ DONATELLA CANISTRO,‡ ANDREA SAPONE,‡ BARBARA BONAMASSA,‡ GIAN LUIGI BIAGI,‡ AND MORENO PAOLINI‡

Agricultural Research Council-Research Institute for Industrial Crops, (CRA-ISCI), Via di Corticella 133, 40129 Bologna, Italy, and Department of Pharmacology, Molecular Toxicology Unit, Alma Mater Studiorum, University of Bologna, Via Irnerio 48, 40126 Bologna, Italy

Brassica vegetables are an important dietary source of glucosinolates (GLs), whose breakdown products exhibit anticancer activity. The protective properties of Brassicaceae are believed to be due to the inhibition of Phase-I or induction of Phase-II xenobiotic metabolizing enzymes (XMEs), thus enhancing carcinogen clearance. To study whether GLs affect XMEs and the role of their chemical structure, we focused on two alkylthio GLs differing in the oxidation degree of the side chain sulfur. Male Sprague−Dawley rats were supplemented (per oral somministration by gavage) with either glucoraphasatin (4-methylthio-3-butenyl GL; GRH) or glucoraphenin (4-methylsulfinyl-3-butenyl GL; GRE), at 24 or 120 mg/kg body weight in a single or repeated fashion (daily for four consecutive days), and hepatic microsomes were prepared for XME analyses. Both GLs were able to induce XMEs, showing different induction profiles. While the inductive effect was stronger after multiple administration of the higher GRH dosage, the single lower GRE dose was the most effective in boosting cytochrome P-450 (CYP)-associated monooxygenases and the postoxidative metabolism. CYP3A1/2 were the most affected isoforms by GRH treatment, whereas GRE induced mainly CYP1A2 supported oxidase. Glutathione S-transferase increased up to ∼3.2-fold after a single (lower) GRE dose and UDP-glucuronosyl transferase up to ∼2-fold after four consecutive (higher) GRH doses. In conclusion, the induction profile of these GLs we found is not in line with the chemopreventive hypothesis. Furthermore, the oxidation degree of the side chain sulfur of GLs seems to exert a crucial role on XME modulation.

KEYWORDS: Glucosinolates; glucoraphasatin; Brassicaceae; xenobiotic metabolizing enzymes; cancer chemoprevention

INTRODUCTION

Many epidemiological studies have shown an inverse relationship between *Brassica* vegetable consumption and cancer risk (*1*). The studies were generally not designed to test this specific hypothesis and suffer from some limitations (i.e., low accuracy of estimates of intake and kind of foodstuff). Nonetheless, a great number of experimental studies provide consistent evidence for a protective role of Brassicaceae and the phytochemicals contained therein (*1*). In a comprehensive overview, the International Agency for Research on Cancer (IARC, Lyon, France) concluded that compounds from Brassica vegetables could affect cancer risk by altering the activity of various xenobiotic metabolizing enzymes (XMEs) and by affecting important cellular mechanisms for cancer development, such as apoptosis (*1*).

The preventive effect of this family of vegetables is associated with some unique aspect of their biochemistry. The most distinctive characteristic of Brassicaceae is their content of high glucosinolate (GL) levels. When the plant tissue is damaged, either by pests or humans, the GLs are broken down by the endogenous enzyme myrosinase (β-thioglucoside glucohydrolase; EC 3.2.1.147) to release isothiocyanates (ITCs), a reactive class of compounds containing an N═C═S group (**Figure 1**).

A well-established body of evidence shows that the loss of myrosinase activity during the cooking process favors the passage of intact GLs to the colon, where they are degraded by the colonic microflora (*2*). Hence, humans are hardly ever exposed directly to ITCs by dietary consumption of Brassicaceae and, what is more, only one-third of the ingested GLs are converted to ITCs by intestinal β-thioglucosidases (*3*). In spite of this, all the scientific literature refers to in-vivo studies on

* To whom correspondence should be addressed. Tel: +39.051.6316863. Fax: +39.051.374857. E-mail: j.barillari@isci.it.
† Agricultural Research Council-Research Institute for Industrial Crops.
‡ Department of Pharmacology

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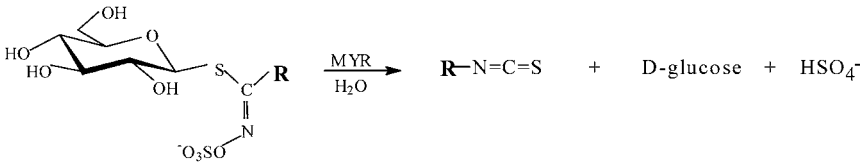

Trivial name	Chemical name	R side-chain
Glucoraphasatin (GRH)	4-methylthio-3-butenyl GL	$CH_3SCH=CHCH_2CH_2$-
Glucoraphenin (GRE)	4-methylsulfinyl-3-butenyl GL	$CH_3SOCH=CHCH_2CH_2$-

Figure 1. Myrosinase-catalyzed breakdown of GLs, releasing ITCs, and structures of the GL redox pair glucoraphasatin and glucoraphenin.

ITCs instead of GLs (*4*), overlooking the fact that the latter is the naturally occurring compound in the diet and that metabolism plays an important role in ITC bioavailability.

Some ITCs are recognized as potent inducers of Phase-II enzymes, but their ability to modulate XMEs varies by compounds. Although the effect has been attributed mainly to the presence of an −N═C═S group, it is believed that the side chain of their GL precursors may play a relevant role in modifying the lipophilicity and the electrophilicity (*5*). Among the sulforaphane analogues examined, the length of the carbon chain appeared to be of little importance, while the oxidation status of the sulfide had a pronounced effect on the inductive ability (*4*). However, in animal studies designed to investigate the possible mechanisms whereby natural compounds have a positive/negative effect on human health, it is essential that the appropriate compounds are selected for the study, unambiguously identifying which phytochemicals individuals are mainly exposed to through diet. Although it is common practice to focus on ITCs, this can produce misleading conclusions. In this regard, we previously found that glucoraphanin, the predominant GL in broccoli, did not confirm the potent induction of Phase-II enzymes that had been reported for its derivative sulforaphane in vivo (*6*).

With this in mind, having developed a method to obtain pure GLs at the gram scale (*7*), we investigated, in an animal model, the role of the oxidation degree of the side chain sulfur by comparing XME modulation following administration with glucoraphasatin (4-methylthio-3-butenyl GL; GRH) or glucoraphenin (4-methylsulfinyl-3-butenyl GL; GRE), a redox pair of GLs (**Figure 1**).

MATERIALS AND METHODS

Chemicals. GRH and GRE were purified from *Raphanus sativus* L. sprouts and seeds, respectively (*7*). Nicotinamide adenine dinucleotide phosphate in its oxidized and reduced forms ($NADP^+$ and NADPH), 1-chloro-2,4-dinitrobenzene, 7-ethoxyresorufin, *p*-nitrophenol, aminopyrine, glutathione, 16α-hydroxytestosterone, corticosterone, and androste-4-ene-3,17-dione, were purchased from Sigma Chemical Co. (St. Louis, MO). Glucose 6-phosphate, glucose 6-phosphate dehydrogenase, and cytochrome *c* were from Boehringer-Mannheim (Germany). Pentoxyresorufin and methoxyresorufin were purchased from Molecular Probes (Eugene, OR). HPLC grade methanol, tetrahydrofuran, and dichloromethane were acquired from Labscan Ltd (Co. Dublin, Ireland), 7α-, 6β-, and 16β-hydroxytestosterone were from Sterlaloid (Wilton, NH), and 6α-, 2α-, and 2β-hydroxytestosterone were a generous gift from Dr. P. Gervasi (CNR Pisa, Italy). All other chemicals were of the highest purity commercially available.

Treatment of Animals and Preparation of Subcellular Fractions. Male Sprague−Dawley rats (Harlan-Italy), weighing 280 ± 10 g, were housed under controlled conditions (12 h light−dark cycle, 22 °C, 60% humidity). They were fed with a rodent chow and had tap water ad libitum. GRH and GRE were dissolved in saline and were administered orally by gavage at 24 or 120 mg/kg body weight (b.w.), in single or repeated doses (daily for four consecutive days). The lower 24 mg/kg b.w. dose is twice as much as the GRH amount (*8*) to which humans can be exposed by a massive consumption of Japanese white radish (*9*), estimated to be about 55 g per day per person in Japan (*10*). The higher 120 mg/kg b.w. dose as well as the time of treatment were in accordance with previous studies on glucoraphanin, administered as pure compound (*6*) or as a component of broccoli sprout extract (*11*). Control animals received the vehicle (saline) only. Seven rats were used in each group. Rats were fasted for 16 h before being sacrificed humanely in accordance with approved Italian Ministerial procedures appropriate to the species. Livers were immediately removed and processed separately, and the S9 fraction (9000*g*) was then prepared as described elsewhere (*12*). The postmitochondrial supernatant fraction was then centrifuged for 60 min at 105 000*g*. The pellet was suspended in 0.1 M $K_2P_2O_7$ and 1 mM EDTA (pH 7.4) and was centrifuged again for 60 min at 105 000*g* to give the final microsomal fraction. Washed microsomes were then suspended with a hand-driven Potter Elvehjem homogenizer in a 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 20% (v/v) glycerol. Fractions were immediately frozen in liquid nitrogen and were stored at −80 °C until use.

Aminopyrine *N*-Demethylase (APND). This was determined by quantification of CH_2O release, following a previously defined method (*13*). The total incubation volume was 3 mL, consisting of 0.5 mL of 50 mM aminopyrine and 25 mM $MgCl_2$, 1.48 mL of 0.60 mM $NADP^+$, 0.33 mM glucose 6-phosphate in 50 mM Tris-HCl buffer (pH 7.4), 0.02 mL glucose 6-phosphate dehydrogenase (0.93 U/mL), and 0.125 mL of sample (0.5 mg of protein). After 5 min of incubation at 37 °C, the yellow color, developed by the reaction of formaldehyde released with the Nash reagent, was read at 412 nm ($\epsilon = 8\ mM^{-1}\ cm^{-1}$) (*14*).

Pentoxyresorufin *O*-Dealkylase (PROD), Ethoxyresorufin *O*-Deethylase (EROD), and Methoxyresorufin *O*-Demethylase (MROD). The reaction mixture consisted of 0.025 mM $MgCl_2$, 200 mM pentoxyresorufin, 0.32 mg of liver microsomal proteins, and 130 mM NADPH in 2.0 mL of 0.05 M Tris-HCl buffer (pH 7.4). Resorufin formation at 37 °C was calculated by comparing the rate of increase in relative fluorescence to the fluorescence of known amounts of resorufin (excitation 562 mm, emission 586 nm) (*15*). EROD and MROD activities were measured in the same way as described for the pentoxyresorufin assay, except that the concentration of substrates was 1.7 mM ethoxyresorufin and 5 mM methoxyresorufin (*16*).

***p*-Nitrophenol Hydroxylase (pNFH).** This was determined in a final volume of 2 mL containing 2 mM *p*-nitrophenol in 50 mM Tris−HCl buffer (pH 7.4), 5 mM $MgCl_2$, and a NADPH-generating system consisting of 0.4 mM $NADP^+$, 30 mM isocitrate, 0.2 U of isocitrate dehydrogenase, and 1.5 mg of proteins. After 10 min at 37 °C, the

Table 1. Phase-I Xenobiotic Metabolizing Enzymes in the Liver of Rats Treated with Glucoraphasatin or Glucoraphenin[d]

		glucoraphasatin				glucoraphenin			
		single dose		repeated doses		single dose		repeated doses	
parameters	control	24 mg/ kg b.w.	120 mg/ kg b.w.	24 mg/kg b.w.	120 mg/ kg b.w.	24 mg/ kg b.w.	120 mg/ kg b.w.	24 mg/ kg b.w.	120 mg/ kg b.w.
aminopyrine *N*-demethylase (CYP3A1/2) nmol mg^{-1} min^{-1}	5.50 ± 0.63	8.99 ± 1.15[a]	9.04 ± 0.85[a]	12.65 ± 0.81[a]	13.13 ± 1.75[a]	9.67 ± 0.53[a]	9.67 ± 0.60[a]	7.11 ± 0.54[a]	7.22 ± 0.52[a]
methoxyresorufin*O*-demethylase (CYP1A2) pmol mg^{-1} min^{-1}	10.33 ± 0.09	12.03 ± 0.65[a]	13.08 ± 0.34[a]	13.67 ± 1.91[a]	16.88 ± 0.59[a]	20.67 ± 1.62[a]	11.71 ± 0.86[c]	17.90 ± 1.56[a]	10.79 ± 0.66
ethoxyresorufin *O*-deethylase (CYP1A1) pmol mg^{-1} min^{-1}	33.34 ± 1.95	38.42 ± 2.29[a]	38.80 ± 3.05[a]	40.15 ± 1.07[a]	50.31 ± 2.62[a]	36.97 ± 3.24	24.35 ± 2.90	35.28 ± 1.94	42.85 ± 3.35
pentoxyresorufin *O*-dealkylase (CYP2B1/2) pmol mg^{-1} min^{-1}	7.21 ± 0.75	7.45 ± 1.62	7.90 ± 1.39	8.04 ± 1.85[c]	8.42 ± 1.85[c]	10.92 ± 1.83[b]	7.97 ± 0.59[b]	7.75 ± 1.62	7.53 ± 0.75[c]
***p*-nitrophenolhydroxylase** (CYP2E1) nmol mg^{-1} min^{-1}	0.64 ± 0.03	0.65 ± 0.03	0.45 ± 0.17[a]	0.72 ± 0.04[a]	0.54 ± 0.14[a]	0.94 ± 0.09[a]	0.44 ± 0.02[a]	0.83 ± 0.10[a]	0.34 ± 0.04[a]

[a] Significantly different compared with corresponding control ($P < 0.01$). [b] Significantly different compared with corresponding control ($P < 0.025$). [c] Significantly different compared with corresponding control ($P < 0.05$), Wilcoxon's rank method. [d] Values are the mean ± SD of seven independent experiments performed on seven different animals. Rats received GRH or GRE in single or repeated doses (daily for four consecutive days). See Materials and Methods for details and experimental procedures.

reaction was stopped by the addition of 0.5 mL of 0.6 N perchloric acid. Precipitated proteins were removed by centrifugation, and 1 mL of supernatant was then mixed with 1 mL 10 N NaOH. Absorbance at 546 nm was immediately measured and 4-nitrocatechol was determined (ϵ =10.28 mM^{-1} cm^{-1}) (*17*).

Testosterone Hydroxylase (TH). *Incubation and Isolation.* Incubation mixtures contained liver microsomes (equivalent to 1−2 mg of protein), 0.6 mM $NADP^+$, 8 mM glucose 6-phosphate, 1.4 U glucose 6-phosphate dehydrogenase, and 1 $MgCl_2$, in a final volume of 2 mL 0.1 M phosphate Na^+/K^+ buffer (pH 7.4). The mixture was preincubated for 5 min at 37 °C. The reaction was performed at 37 °C by shaking and was started by the addition of 1 mM testosterone (final concentration) in methanol. After 10 min, the reaction was stopped with 5 mL ice-cold dichloromethane and 12 nmol corticosterone (internal standard) in methanol. After 1 min of vortexing, phases were separated by centrifugation at 2000*g* for 10 min, and the aqueous phase was extracted once more with 2 mL dichloromethane. The organic phase was extracted with 0.02 N NaOH to remove lipid constituents, was dried over anhydrous sodium sulfate, and was transferred to a small tube. Dichloromethane was evaporated at 37 °C under nitrogen, and the dried samples were stored at −20 °C until use. The samples were dissolved in 100 μL methanol and were analyzed by HPLC (*18*).

HPLC Separation And Quantification. Chromatographic separation was performed using a system consisting of a high-pressure pump (Waters Model 600E, Multisolvent Delivery System), a single injection valve (Rheodyne Model 7121, Cotati, CA) with a 20 μL sample loop, and an ultraviolet (UV) detector (254 nm, Waters Model 486, Tunable Adsorbance Detector) connected to an integrator (Millenium 2010, Chromatography Manger). For reverse-phase separation of metabolites, a NOVA-PAK C18 analytical column (60 Å, 4 mm, 3.9 × 150 mm, Waters) was used as the stationary phase. The mobile phase consisted of solvent A [7.5% (v/v) tetrahydrofuran in water] and solvent B [7.5% (v/v) tetrahydrofuran and 60% (v/v) methanol in water] at a 1 mL/min flow rate. Metabolite separation was performed by a gradient from 30 to 100% (v/v) of solvent B over 30 min. The eluent was monitored at 254 nm, and the area under the absorption band was integrated. The concentration of metabolites was determined by the ratio between respective metabolite peak areas and corticosterone (the internal standard) and in comparison with the calibration curves obtained with synthetic testosterone derivatives (*19*, *20*)

Glutathione *S*-Transferase (GST). The incubation mixture for measuring GST activity contained 1 mM glutathione, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) in methanol, and 0.025 mL of sample in a final volume of 2.5 mL 0.1 M phosphate Na^+/K^+ buffer (pH 6.5). The product of the reaction of the thiol group of glutathione with the electrophilic group of CDNB was read at 340 nm (ϵ =9.6 mM^{-1} cm^{-1}) (*21*).

UDP-Glucuronosyl Transferase (UGT). UGT activity was determined kinetically using 1-naphthol as substrate (final concentration, 50 mM) by the continuous fluorometric (excitation 293 mm, emission 335 nm) (*22*) monitoring of 1-naphtholglucuronide production in the presence of 1 mM uridine−5′-diphosphoglucuronic acid.

Protein Concentration. Protein concentration was determined according to the method described by Lowry et al. (*23*) and revised by Bailey (*24*), using bovine serum albumin as a standard and diluting samples 200 times to provide a suitable protein concentration.

Statistical Analysis. Statistical evaluation was performed using the Wilcoxon's rank test to assess significant differences in the considered parameters between the groups of treated animals compared with controls (*25*).

RESULTS

Phase-I Metabolism. All rats remained in good health throughout the experiments, and no significant differences were seen in body weight gain among the different groups. No differences were recorded in either absolute or relative liver weight between the rats undergoing the different treatments and controls (data not shown). The effects of the administration of the two GLs on Phase-I linked monooxygenases in rat liver were assessed using both a single probe toward specific CYP isoforms and testosterone as a multibioprobe of many CYPs, being metabolized in a stereo- and regioselective manner by different isoforms. In general, the results show that while in rats supplemented with GRH the inductive effect was more marked after multiple administration of the higher dosage, a single administration of the lower GRE dose was more effective in inducing CYP-associated reactions.

Table 1 reports the various CYP-supported microsomal mixed function oxidases in liver subcellular preparations derived from rats after single or repeated administration (daily for four consecutive days) of either GRH or GRE (24 or 120 mg/kg b.w.). CYP3A1/2 were the isoforms most affected by GRH treatment. The preferential CYP3A1/2-linked monooxygenase (APND) was markedly and significantly ($P < 0.01$) induced up to 1.8-fold by single GRE administration (24 mg/kg b.w.) and up to 2.4-fold by repeated GRH treatment (higher dose). The CYP1A2-supported monooxygenase (MROD) showed the greatest inductive effect for single GRE treatment, as it was significantly ($P < 0.01$) increased up to 1.6-fold after repeated GRH administration (higher dose) and up to 2-fold at the lower GRE dose. CYP1A1-linked (EROD) monooxygenase was significantly ($P < 0.01$) induced by GRH treatment (up to 1.5-fold with 120 mg/kg b.w. in repeated fashion) only. The CYP2B1/2-supported (PROD) oxidase showed the same pattern of response as the other CYPs, but the increase was slight (up to 1.2 and 1.5-fold, respectively, after GRE and GRH administration). On the contrary, the induction profile of CYP2E1-linked (pNFH) monooxygenase was different compared to the other Phase-I activities. At the higher dose, both GLs were able

Table 2. Phase-I Xenobiotic Metabolizing Enzymes in the Liver of Rats Treated with Glucoraphasatin or Glucoraphenin[d]

		glucoraphasatin				glucoraphenin			
		single dose		repeated doses		single dose		repeated doses	
-linked monooxygenases	control	24 mg/kg b.w.	120 mg/kg b.w.	24 mg/kg b.w.	120 mg/kg b.w.	24 mg/ kg b.w.	120 mg/ kg b.w.	24 mg/ kg b.w.	120 mg/ kg b.w.
6α-testosterone hydroxylase (CYP1A1) pmol mg^{-1} min^{-1}	0.02 ± 0.01	n.d.	n.d.	n.d.	n.d.	0.06 ± 0.01[a]	0.03 ± 0.01	0.04 ± 0.01[a]	0.04 ± 0.01[a]
7α-testosterone hydroxylase (CYP2C11,CYP1A1/2) pmol mg^{-1} min^{-1}	29.97 ± 4.05	42.42 ± 5.05[a]	42.75 ± 4.52[a]	37.06 ± 4.99[c]	37.76 ± 4.21[c]	45.66 ± 4.15[a]	27.77 ± 3.21	39.68 ± 4.01[a]	47.95 ± 4.12
6β-testosterone hydroxylase (CYP1A1/2,CYP3A1/2) pmol mg^{-1} min^{-1}	1.38 ± 0.22	1.81 ± 0.21[b]	1.82 ± 0.31[b]	1.63 ± 0.29[c]	1.63 ± 0.21[c]	2.56 ± 0.25[a]	1.47 ± 0.25	1.60 ± 0.19[c]	1.54 ± 0.17
16α-testosterone hydroxylase (CYP2B1,CYP2C11) pmol mg^{-1} min^{-1}	2.44 ± 2.18	2.62 ± 3.11	2.86 ± 2.99[a]	2.83 ± 3.13[c]	3.02 ± 3.82[a]	5.37 ± 0.65[a]	3.89 ± 0.39[a]	2.61 ± 0.31	2.93 ± 0.35[c]
16β-testosterone hydroxylase (CYP2B1) pmol mg^{-1} min^{-1}	19.03 ± 2.05	31.75 ± 3.25	27.73 ± 3.17[a]	35.54 ± 4.01[a]	38.06 ± 4.12[a]	36.19 ± 4.11[a]	21.09 ± 1.95[c]	22.69 ± 2.76[c]	22.59 ± 2.93
2α-testosterone hydroxylase (CYP2C11) pmol mg^{-1} min^{-1}	1.96 ± 2.12	2.05 ± 2.63	2.33 ± 2.56[a]	2.29 ± 2.95[a]	2.43 ± 2.62[a]	3.78 ± 0.41[a]	2.71 ± 0.05[a]	1.93 ± 0.35	2.20 ± 0.28
2β-testosterone hydroxylase (CYP1A1,CYP3A1/2) pmol mg^{-1} min^{-1}	88.78 ± 9.18	133.42 ± 15.05	112.63 ± 14.12	119.16 ± 23.67[a]	121.76 ± 12.98[a]	183.20 ± 13.05[a]	101.35 ± 11.05[a]	107.90 ± 12.11[a]	113.35 ± 14.08
4-androsten-3,17-dione (CYP3A1) nmol mg^{-1} min^{-1}	0.72 ± 0.05	0.73 ± 0.08	0.76 ± 0.11	0.72 ± 0.09	0.78 ± 0.08	1.25 ± 0.02[a]	0.92 ± 0.06[a]	0.79 ± 0.18	0.73 ± 0.15

[a] Significantly different compared with corresponding control ($P < 0.01$). [b] Significantly different compared with corresponding control ($P < 0.025$). [c] Significantly different compared with corresponding control ($P < 0.05$), Wilcoxon's rank method. n.d., not detectable. [d] Values are the mean ± SD of seven independent experiments performed on seven different animals. Rats received GRH or GRE in single or repeated doses (daily for four consecutive days). See Materials and Methods for details and experimental procedures.

Table 3. Phase-II Xenobiotic Metabolizing Enzymes in the Liver of Rats Treated with Glucoraphasatin or Glucoraphenin[c]

		glucoraphasatin				glucoraphenin			
		single dose		repeated doses		single dose		repeated doses	
parameters	control	24 mg/ kg b.w.	120 mg/ kg b.w.	24 mg/ kg b.w.	120 mg/ kg b.w.	24 mg/ kg b.w.	120 mg/ kg b.w.	24 mg/ kg b.w.	120 mg/ kg b.w.
glutathione *S*-transferase nmol mg^{-1} min^{-1}	1.94 ± 0.16	2.16 ± 0.17[b]	2.80 ± 0.17[a]	2.86 ± 0.37[a]	3.38 ± 0.26[a]	6.20 ± 0.67[a]	2.92 ± 0.18[a]	4.79 ± 0.16[a]	3.30 ± 0.27[a]
UDP-glucuronosyl transferase nmol mg^{-1} min^{-1}	85.3 ± 1.8	90.9 ± 9.1[a]	141 ± 13[a]	135 ± 18[a]	166 ± 37[a]	157 ± 9[a]	95.8 ± 8[a]	143 ± 11[a]	114 ± 15[a]

[a] Significantly different compared with corresponding control ($P < 0.01$). [b] Significantly different compared with corresponding control ($P < 0.05$), Wilcoxon's rank method. [c] Values are the mean ± SD of seven independent experiments performed on seven different animals. Rats received GRH or GRE in single or repeated doses (daily for four consecutive days). See Materials and Methods for details and experimental procedures.

to significantly ($P < 0.01$) reduce the pNFH: GRH mainly in single treatment (−30% compared with the control) and GRE mainly in repeated dosage (−47% compared with the control). Conversely, at the lower dose, pNFH activity was slightly but significantly ($P < 0.01$) increased, mainly after the single GRE treatment (1.5-fold).

Table 2 reports the effects of GRH and GRE administration on rat hepatic monooxygenases, using testosterone as a multi-bioprobe. In agreement with the findings obtained by using single probes, GRE showed the greatest inducing effect after single treatment (lower dosage), while GRH did so after repeated administration (higher dose). At variance with this behavior, testosterone 7α-(CYP1A1/2-CYP2C11) and 6β-(CYP1A1/2-CYP3A1/2) hydroxylases increased mainly after single GRH administration and apparently were not related to the dose. With the exception of testosterone 16β-(CYP2B1) hydroxylase, all the oxidases were affected to a greater extent by GRE than GRH treatment. In particular, while the 16β-hydroxytestosterone-supported oxidase was mostly affected ($P < 0.01$) by GRH (reaching a 2-fold increase after 120 mg/kg b.w.), testosterone 2α-(CYP2C11) and 16α-(CYP2B1, CYP2C11) hydroxylases were noticeably and significantly ($P < 0.01$) increased mostly after GRE injection (up to 1.9- and 2.2-fold, respectively, at 24 mg/kg b.w.). Among the stereo- and regioselective hydroxylations of testosterone, the CYP1A1/2 (6α position) was the one most affected by GRE, reaching a 2.5-fold increase ($P < 0.01$) after single administration (24 mg/kg b.w.). The induction of this isoform was also sustained by the slight but significant ($P < 0.01$) enhancement of the hydroxylation in 7α position (up to 1.5-fold, at 24 mg/kg b.w.). A marked and significant ($P < 0.01$) induction of CYP3A1/2-CYP1A1/2-supported metabolism (measured as testosterone 6β- and 2β-hydroxylases, respectively, increased up to 1.9 and 2.1-fold, at 24 mg/kg b.w.) and androst-4-ene-3,17-dione-(CYP3A1)-linked monooxygenase (up to 1.7-fold, at lower dose) was recorded.

Phase-II Metabolism. Table 3 reports the effects of the administration of the two GLs on GST and UGT, selected as Phase-II enzyme markers. As recorded for Phase-I linked monooxygenases, GRE treatment provoked the greatest inducing effect after the single lower dosage, while GRH did so after repeated administration of the higher dose. Concerning GST activity, this increased mainly after GRE administration, reaching a 3.2-fold increase ($P < 0.01$) in rats treated with a single 24 mg/kg b.w. GRE. As regards UGT activity, both the GLs showed the same behavior as GST, but the magnitude of the response was more homogeneous; the activity was significantly ($P < 0.01$) increased up to 1.8-fold after a single 24 mg/kg b.w. GRE and up to 1.9-fold after repeated administration of 120 mg/kg b.w. GRH.

DISCUSSION

As a consequence of the emerging interest in the protective and health-promoting influence of GLs and their metabolic products, the gram scale availability, thanks to our method (*7*), of both GLs of the GRH/GRE redox pair stimulated us to evaluate the importance of the sulfur oxidation status in the side chain for their biological activity, testing the naturally occurring compounds in Brassica vegetables in an experimental animal model.

To the best of the authors' knowledge, this is the first study reporting the effect of GRH and GRE on hepatic XMEs in rodents. A significant inductive effect was observed for both Phase-I and Phase-II metabolizing enzymes with both the GLs. The marked difference we found in the outcomes of GRH and GRE treatments seems to exclude an interconversion between the two GLs or their metabolites, a phenomenon observed with erucin and sulforaphane (*26*). On the contrary, the different XME induction profile suggests that the side chain sulfur oxidation degree can have a crucial role in XME modulation.

In accordance with our previous data on glucoraphanin, differing from GRE by the saturated side chain only, XMEs are affected by GLs (*6*), but there is no evidence that GLs themselves are direct inducers of Phase-II enzymes, and the literature reports that activity is achieved only after their conversion to ITCs (*2*). Because humans are exposed to ITCs mainly through consumption of GLs, we used an in-vivo model mimicking the route of absorption and metabolism of GLs following Brassicaceae consumption. As stated above, it is well-known that GLs are metabolized, at least in part, to ITC by intestinal microflora in humans (*3*) and rodents (*27*). Although this metabolic pathway has been observed for several compounds of this class in rats (*2*), the fate of untransformed GLs is not yet known; we cannot exclude that it might have contributed (i.e., by other metabolic pathways) to the biological activity. Moreover, the experimental routine use of ITCs, instead of GLs, misrepresents the quantitative exposure of humans, since bioavailability is markedly different (*3*).

The induction of Phase-II enzymes in connection with intrinsic mechanisms used by living organisms to deactivate potentially carcinogenic molecules is one of the most emphasized strategies in cancer chemoprevention (*28*). This approach, originally termed "electrophile counterattack" by Prestera et al. (*29*), now focuses on the nuclear erythroid 2-related factor 2 (Nrf2)-antioxidant response element (ARE) signaling pathway that regulates a battery of antioxidant and "detoxification" enzymes at the transcriptional level. In our model, the two GLs acted as bifunctional inducers, markedly affecting both postoxidative and various CYP-dependent metabolisms. In evaluating the biological outcomes linked to CYP modulations, the different expressions of the monooxygenases should be carefully considered. For example, an induction of about 2-fold in the O-demethylation of methoxyresurufin from 10 to 20 pmol mg^{-1} min^{-1} represents a net increase of only 10 pmol mg^{-1} of protein per unit time. The corresponding 2-fold induction of activities such as the O-demethylation of aminopyrine from 5 to 10 nmol mg^{-1} min^{-1} represents a distinct (powerful) increase of 5000 pmol mg^{-1} $min^{-1.}$ In this regard, even if in-vivo experiments cannot reproduce actual human exposure, some considerations about the employed doses can be helpful for a better understanding of these results. As stated above, the lower 24 mg/kg b.w. dose was extrapolated from the daily intake of GRH in the Japanese diet (*8*−*10*). This dose of GRH was appreciably able to induce GST and UGT, and markedly CYP3A1/2, mainly in repeated fashion. When the dose was increased to 120 mg/kg b.w., the effect was also enhanced; however, the extension of treatment from 1 to 4 days was the major determinant in the enhancement of the inductive effects for both CYPs and Phase-II enzymes in GRH treated rats. Consistent with this, it has been reported that long-term administration can maximize the inductive effect of GL-derived ITCs; even if tissue-specific responses were documented, bladder GST increased with the dose of allyl-ITC over 21 days of duration of the experiment (*30*).

Conversely, a single administration of GRE was more effective in inducing both Phase-I and Phase-II enzymes. The lower 24 mg/kg b.w. dose was able to strongly induce GST and UGT, while CYP1A2 was the most affected CYP isoform. The increase of both dose and time of treatment may have mitigated the effects of GRE. This aspect need to be carefully considered, since cancer chemopreventive agents are expected to be chronically administered.

One possible explanation for the pattern of induction following GRE treatments may be related to a simultaneous toxic effect on microsomal components. This is because the measured monooxygenases are the result of the general balance between two concomitant phenomena such as the induction (linked to increased gene transcription, mRNA, or protein stabilization) and toxicity (linked to an increased production of toxic metabolites or an overgeneration of reactive oxygen species, ROS, derived from the CYP catalytic cycle): the latter can thus overcome induction itself at higher dose (*31*).

The reasons for the different behavior of GRE with respect to GRH still need to be clarified. The chemical structures of the two GLs (**Figure 1**) show that the oxidation status of sulfur is the only difference between the two compounds. Data are accumulating to show that much of the GL-derived ITC activity stems from cell response to chemical stress. GL-derived ITCs affect the glutathione-related antioxidant system in different ways (*32*). While they transcriptionally stimulate antioxidative enzymes as well as nonenzymatic proteins, leading to enhanced protection against oxidative stressors, they also directly alkylate and deplete cellular thiols and elevate ROS, leading to cellular damage (*32*). The dichotomous effects on oxidative stress are not merely a reflection of the dose, even if increasing dosage exacerbates the stress itself (*32*). We previously showed that GRH, but not GRE, possesses an antioxidant direct capacity against hydrogen peroxide and radical compound in chemical assays (*7*). Although not directly related to its radical scavenging activity, the reducing capacity of GRH is of concern in explaining the different response of XMEs to test GLs. Compounds triggering an ARE response can modify the cellular redox status (*33*), and it could be possible that the balance between the antioxidative and oxidative impulses is the key to explain these findings.

Taking into account the different weight of the different enzymatic units, as stated above, the strong induction of the postoxidative enzymes GST and UGT is concomitant with the modest enhancement of CYP1A2-linked monooxygenase in GRE treated rats as well as of the marked induction of CYP3A1/2 in GRH treated rats. The induction of Phase-I enzymes causes general concern since, in addition to the inevitable perturbation of endogenous metabolism and cell related functions where these catalysts are physiologically involved, it could increase the bioactivation of ubiquitous protoxins, substrates of the boosted isoforms. In this connection, the phenomenon of chemical bioactivation depends on the nature of the substrate and not on the kind of XME involved (*34*). Hence, it is conceivable that if on one hand XME modulation (e.g., any modulation of Phase-II or of Phase-I enzymes) can

lead to an increase of detoxification of certain chemicals, it can at the same time increase the bioactivation of other procarcinogens to which humans are simultaneously exposed. This point of view is of great interest, considering that the liver is the main biotransforming human tissue and is exposed daily to a large quantity of protoxins (including protoxics, promutagens, and procarcinogens of natural and synthetic origins) present in food (i.e., additives, contaminants, and cooking process derivatives) and the air (i.e., environmental pollutants) or is linked to some unhealthy life styles, such as smoking and excessive alcohol intake.

Despite the above, there is growing interest in dietary Phase-II enzyme inducers as a means of reducing cancer incidence and cardiovascular diseases (*35*). To further emphasize this point, we would like to recall that several GL-derived ITCs known to trigger the ARE as an acute effect transiently increase ROS and deplete glutathione, altering the intracellular redox. A shift in the redox balance can signal post-translational regulation of multiple cellular pathways associated with cell death or survival, as well as upregulation of detoxification enzymes (*33*). All the systems may be envisaged to work in concert to synergize the protective effect of GLs and their metabolic products.

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