JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Induction of Phase II Detoxification Enzymes in Rats by Plant-Derived Isothiocyanates: Comparison of Allyl Isothiocyanate with Sulforaphane and Related Compounds

REX MUNDAY* AND CHRISTINE M. MUNDAY

AgResearch, Ruakura Agricultural Centre, Private Bag 3123, Hamilton, New Zealand

Plants of the family Brassicaceae contain high levels of glucosinolates. The latter compounds are degraded to isothiocyanates, some of which have been shown to be potent inducers of phase II detoxification enzymes in vitro. In the present study, the ability of six plant-derived isothiocyanates (allyl isothiocyanate, iberverin, erucin, sulforaphane, iberin, and cheirolin) to increase tissue levels of the phase II detoxification enzymes quinone reductase (QR) and glutathione *S*-transferase (GST) in a variety of rat tissues has been compared. At the low dose level employed (40 μmol/kg/day), cheirolin was without effect in any tissue. All of the other isothiocyanates, however, increased GST and QR activities in the duodenum, forestomach, and/or the urinary bladder of the animals, with the greatest effects being seen in the urinary bladder. With the exception of cheirolin, little difference was observed in the inductive activity of the various isothiocyanates. Phase II enzymes are known to protect against chemical carcinogenesis, and the selectivity of isothiocyanates in inducing such enzymes in the bladder is of interest in view of recent epidemiological studies showing a decreased incidence of cancer of this organ in individuals with a high dietary intake of *Brassica* vegetables.

KEYWORDS: Isothiocyanates; quinone reductase; glutathione *S*-transferase; urinary bladder; rat

INTRODUCTION

There is much evidence that elevated tissue levels of phase II detoxification enzymes are associated with decreased susceptibility to chemical carcinogenesis (*1*−*3*). These enzymes, which include quinone reductase (QR, DT-diaphorase, NAD(P)H:quinone oxidoreductase, EC 1.6.99.2), the glutathione *S*-transferases (GST, EC 2.5.1.18), epoxide hydrolase (EC 3.3.2.3), and UDP-glucuronosyltransferase (EC 2.4.1.17), detoxify many harmful chemicals, converting them to hydrophilic metabolites that are readily eliminated from the body. They also potentiate the antioxidant defenses of tissue, providing increased resistance to "active oxygen" species (*4*). Phase II enzymes are highly inducible in animals and in humans (*5*, *6*).

Isothiocyanates are recognized as potent inducers of phase II enzymes. These substances are formed from glucosinolates, which are widely distributed among plants of the family Brassicaceae, which includes the familiar *Brassica* vegetables, such as cabbage, broccoli, and Brussels sprouts. Glucosinolates are converted to isothiocyanates by the action of the enzyme myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1), which is present at high concentration in *Brassica* plants and which is released when the tissue is disrupted by cutting or chewing (*7*). Gut bacteria also contain myrosinases, and a proportion of ingested glucosinolates is converted to isothiocyanates within the gastrointestinal tract (*7*). More than 100 glucosinolates have been identified in plants, and more than 20 have been found, at various concentrations, in *Brassica* vegetables (*7*, *8*).

The predominant glucosinolate in broccoli is glucoraphanin, which is hydrolyzed to 1-isothiocyanato-4-(methylsulfinyl)-butane (sulforaphane; **Figure 1A**), while that in cabbage and Brussels sprouts is sinigrin, which is converted to allyl isothiocyanate (AITC; **Figure 1B**). In vitro, sulforaphane is a very potent inducer of phase II enzymes, doubling the activity of QR at a concentration of only 0.58 μM in Hepa1c1c7 cells (*9*). Sulforaphane also increases tissue QR and GST activities in vivo. In rats and mice given this compound for 4−5 days at high dose levels (up to 1000 μmol/kg/day), increased phase II enzyme activities were recorded in the liver, lung, mammary gland, pancreas, stomach, small intestine, and colon of the animals (*10*−*14*). AITC is very much less effective in inducing phase II enzymes in vitro, requiring a concentration 14 times that of sulforaphane in order to double QR activity in Hepa1c1c7 cells (*9*). In vivo, however, AITC is a powerful inducer of phase II enzymes, with significant increases in QR and/or GST being recorded in the liver, kidneys, spleen, lungs, urinary bladder, forestomach, glandular stomach, duodenum, jejunum, ileum, cecum, and colon plus rectum of rats at a dose of 200 μmol/kg/day (*15*). The urinary bladder was the organ most susceptible to enzyme induction by AITC, and a dose−response study in rats revealed significant increases in the activity of both QR and GST in the bladder at a dose of only 10 μmol/kg/day (*15*).

The in vivo inductive effects of AITC and sulforaphane have never been directly compared. Furthermore, it is not known

* To whom correspondence should be addressed. Tel:+ 64 7 838 5138. Fax:+ 64 7 838 5012. E-mail: rex.munday@agresearch.co.nz.

10.1021/jf030549s CCC: $27.50
Published on Web 03/12/2004

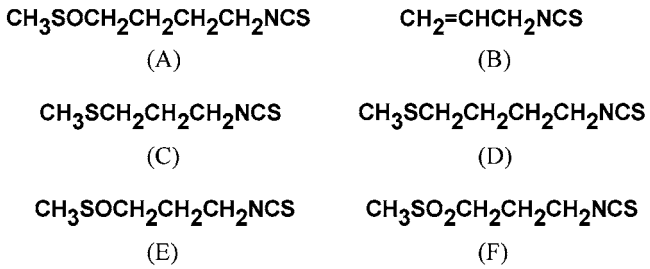

Figure 1. Structures of the compounds evaluated. (**A**) Sulforaphane; (**B**) AITC; (**C**) iberverin; (**D**) erucin; (**E**) iberin; and (**F**) cheirolin.

whether selectivity for enzyme induction in the urinary bladder is a unique characteristic of AITC or whether it is a general property of isothiocyanates. These unknowns have been addressed in the experiments described in the present communication. The effects of chain length and degree of oxidation of the sulfide sulfur in sulforaphane analogues have also been investigated by comparing phase II enzyme induction by the sulfides, 1-isothiocyanato-3-(methylthio)propane (iberverin; **Figure 1C**) and 1-isothiocyanato-4-(methylthio)butane (erucin; **Figure 1D**); the sulfoxides, sulforaphane and 1-isothiocyanato-3-(methylsulfinyl)propane (iberin; **Figure 1E**); and the sulfone, 1-isothiocyanato-3-(methylsulfonyl)propane (cheirolin; **Figure 1F**).

MATERIALS AND METHODS

Chemicals. Sulforaphane, iberverin, iberin, cheirolin, and erucin were purchased from LKT Laboratories (St. Paul, MN) and were used without further purification. AITC was from Lancaster Synthesis (Morecambe, U.K.) and was redistilled before use.

Animals and Housing. Female Sprague−Dawley rats (11−12 weeks old) from the Ruakura colony were randomly allocated to treatment groups. The animals were housed in solid-bottomed cages containing a bedding of wood shavings and were allowed free access to food (Laboratory Chow, Sharpes Animal Feeds, Carterton, NZ) and water throughout the experiment. The room temperature was maintained at 21−23°C with a 12 h light/dark cycle.

Dosing and Necropsy. Groups of five rats were dosed by oral intubation with the test compounds, as solutions in soybean oil, each day for 5 days. To facilitate comparison of the various substances, the dose levels were equalized on a molar basis, and each substance was administered at 40 μmol/kg/day. This dose is equivalent to 4.0 mg/kg/day for AITC, 5.9 mg/kg/day for iberverin, 6.5 mg/kg/day for iberin, 6.4 mg/kg/day for erucin, 7.1 mg/kg/day for sulforaphane, and 7.2 mg/kg/day for cheirolin. The volume of solution administered was 2 mL/kg in all cases. Ten control rats were dosed with soybean oil alone.

On the sixth day of the experiment, the rats were killed by carbon dioxide inhalation. The liver was dissected out, and a portion of this organ together with the kidneys, spleen, heart, urinary bladder, and lungs were placed in plastic vials. The gastrointestinal tract was separated into forestomach, glandular stomach, duodenum, jejunum, ileum, cecum, and colon plus rectum. These tissues were cut lengthways, and the contents were washed out with cold running water. They were then gently blotted on absorbent paper and, along with the other organs, stored at −80 °C before analysis.

Enzyme Assays. Tissues were homogenized and centrifuged as described previously (*15*). It should be noted that in these experiments, the phase II enzymes were measured in homogenates of whole gut, as in the method of Posner et al. (*12*), rather than in the scrapings of intestinal mucosa that have been employed by other workers (*10, 14*). In unpublished studies, we have found similar activities of QR and GST in both the mucosa and the nonmucosal portion of rat intestine, and the assay of these enzymes in the whole tissue therefore gives a more accurate estimate of the metabolic potential of the gastrointestinal tract.

QR was assayed at 25 °C by the method of Ernster (*16*) and GST by the method of Habig et al. (*17*), using 1-chloro-2,4-dinitrobenzene as substrate. Enzyme activities were calculated as International Units (IU) per gram of tissue. Statistical significance of the data was tested by two-way analysis of variance followed by Student's *t*-test, using InStat software (GraphPad, San Diego, CA).

Table 1. QR Activities in the Forestomach, Duodenum, and Urinary Bladder of Control Rats and Rats Dosed with Isothiocyanates[a]

	QR activity (IU/g tissue)		
compound	forestomach	duodenum	urinary bladder
none (control)	17.3 ± 1.3^{a}	10.2 ± 0.8^{a}	23.1 ± 2.3^{a}
AITC	$27.5 \pm 2.3^{b,c}$	15.8 ± 1.5^{b}	43.2 ± 4.7^{b}
iberverin	$26.9 \pm 3.8^{b,c}$	$19.1 \pm 2.3^{b,c}$	51.1 ± 5.9^{b}
iberin	$22.9 \pm 2.0^{a,c}$	$18.4 \pm 1.0^{b,c}$	51.3 ± 5.0^{b}
erucin	$22.7 \pm 2.8^{a,c}$	$15.0 \pm 1.7^{b,c}$	39.7 ± 6.3^{b}
sulforaphane	$20.3 \pm 1.5^{a,c}$	22.7 ± 2.1^{b}	44.8 ± 4.4^{b}
cheirolin	18.6 ± 2.5^{a}	$13.4 \pm 0.8^{a,c}$	24.4 ± 2.2^{a}

[a] All compounds were dosed at 40 μmol/kg/day for 5 days. Values shown are the means ± SEM of the results from the five animals in each group. Values with different superscript letters within a column are significantly different at $P < 0.05$.

Table 2. GST Activities in the Forestomach, Duodenum, and Urinary Bladder of Control Rats and Rats Dosed with Isothiocyanates[a]

	GST activity (IU/g tissue)		
compound	forestomach	duodenum	urinary bladder
none (control)	3.51 ± 0.14^{a}	26.6 ± 1.2^{a}	3.49 ± 0.09^{a}
AITC	4.82 ± 0.27^{b}	$29.1 \pm 2.0^{a,b}$	6.56 ± 0.36^{b}
iberverin	$4.15 \pm 0.11^{a,b,c}$	$32.6 \pm 2.0^{a,b}$	5.82 ± 0.21^{b}
iberin	$3.92 \pm 0.17^{a,c}$	$27.4 \pm 2.6^{a,b}$	6.89 ± 0.83^{b}
erucin	$4.47 \pm 0.17^{b,c}$	$29.6 \pm 2.1^{a,b}$	5.92 ± 0.10^{b}
sulforaphane	$4.27 \pm 0.36^{a,b,c}$	35.2 ± 1.6^{b}	8.67 ± 1.78^{b}
cheirolin	$3.70 \pm 0.16^{a,c}$	$29.0 \pm 1.7^{a,b}$	4.52 ± 0.47^{a}

[a] All compounds were dosed at 40 μmol/kg/day for 5 days. Values shown are the means ± SEM of the results from the five animals in each group. Values with different superscript letters within a column are significantly different at $P < 0.05$.

RESULTS

All rats remained in good health throughout the experiment, and there were no significant differences in body weight gain among the different treatment groups. No abnormalities were recorded at necropsy.

No significant differences in QR or GST activity were recorded in the livers, kidneys, spleen, lungs, heart, glandular stomach, jejunum, ileum, cecum, or colon plus rectum of rats receiving any of the test substances. With the exception of cheirolin, however, all of the test compounds caused significant increases in enzyme activity in the forestomach, duodenum, and/or the urinary bladder. QR activities in these tissues are shown in **Table 1**, and GST activities are shown in **Table 2**.

AITC, iberverin, iberin, erucin, and sulforaphane all caused significant increases in the activities of both QR and GST in the urinary bladder of the rats and in QR activity in the duodenum. In the forestomach, significant increases in QR activity were recorded only in rats receiving AITC and iberverin, and GST was induced only in rats dosed with AITC and erucin. Increased duodenal GST was seen only in animals dosed with sulforaphane. In the forestomach, QR activities were significantly higher in rats dosed with AITC than in those receiving iberin, while GST activities in the duodenum of sulforaphane-treated rats were significantly higher than in animals dosed with AITC. In all other cases, there were no statistically significant differences among the various compounds with regard to degree of enzyme induction.

DISCUSSION

In previous experiments with sulforaphane, employing high dose levels of the test compound, increased phase II enzyme activities in a variety of animal tissues were recorded (*10*−*14*). In the present study, using a dose of only 40 μmol/kg/day, induction was limited to the urinary bladder, duodenum, and forestomach. The greatest effect was seen in the urinary bladder, which has not previously been examined in animals dosed with sulforaphane. Among the sulforaphane analogues examined, the length of the carbon chain appears to be of little importance, since no significant differences in inductive activity were recorded between iberverin and erucin or between sulforaphane and iberin. In contrast, the oxidation state of the sulfide sulfur has a pronounced effect on inductive ability, since while all of the sulfides and sulfoxides were active, no effects were recorded with cheirolin, a sulfone.

With AITC, iberverin, iberin, and erucin, the degree of induction of QR in the rat tissues was generally greater than that of GST. With sulforaphane, however, the opposite was true. The relative inductive potency of AITC is consistent with the results of earlier studies (*15*), but the effect of sulforaphane on QR and GST activities appears to be tissue-dependent. The extent of GST induction by sulforaphane in the glandular stomach (*10*) and mammary gland (*11*) was greater than that of QR, while QR was preferentially induced in the liver (*10*, *11*, *14*), small intestine (*10*), lung (*10*), pancreas (*14*), and colon (*14*). The reason for such variations is not presently known, and more studies on the relative effects of isothiocyanates on different phase II enzymes are required.

The relative inductive activities of the various isothiocyanates in rat tissues are not in accord with those determined in cells in vitro. In isolated cells, sulforaphane was far more active than AITC (*9*), but the two compounds were of similar activity in vivo, in terms of both degree of enzyme induction and site of effect. Furthermore, sulforaphane and cheirolin were more active than erucin, iberin, and iberverin in vitro (*10*), but in rats, erucin, iberin, and iberverin were of similar activity to sulforaphane, while cheirolin was very much less active. The results with erucin are in accord with earlier observations (*10*), but the other derivatives have not previously been compared. In vitro, there is a clear correlation between the inductive ability of an isothiocyanate and the degree to which it accumulates within cells (*18*), and this is likely also to be true in vivo. However, many more factors control cellular xenobiotic levels in vivo than in vitro, including rate of uptake from the gut, rate of metabolism, and rate of excretion, and it would appear that these factors override the differences seen among these compounds in vitro.

Metabolism may play an important part in determining inductive activity in vivo. The sulfide group of erucin is extensively oxidized in rats, forming sulforaphane, while sulforaphane is partly reduced to erucin (*19*). Because these compounds are interconverted in vivo, similar degrees of induction are to be expected. Aliphatic sulfides are oxidized to sulfoxides mainly via the hepatic flavin-containing monooxygenase (*20*), while sulfoxides are reduced by gut bacteria (*21*, *22*) or via an hepatic thioredoxin-dependent system or aldehyde dehydrogenase in the presence of an electron donor (*23*). While no information on the metabolism of iberverin and iberin is available, interconversion of these substances in a manner analogous to that of sulforaphane and erucin would account for their similar inductive action in vivo. The lack of effect of cheirolin on phase II enzyme activities may be due to the fact that sulfones, being polar compounds, are readily eliminated from the body (*24*). Conversion of cheirolin to iberin or iberverin is not possible, since sulfones are not reduced to sulfoxides or sulfides in vivo, and when administered to animals are excreted unchanged (*25*, *26*).

The disposition of these compounds in vivo may account for their selectivity toward the urinary bladder. Isothiocyanates are rapidly conjugated with glutathione in animals (*27*). The glutathione moiety is subsequently degraded to N-acetylcysteine, and the N-acetylcysteine conjugates are excreted via the kidney (*19*, *28*). Within the urinary bladder, however, the conjugates dissociate, and the isothiocyanate so formed is reabsorbed through the bladder epithelium (*28*). In this way, the thiol conjugate acts as a "delivery system" for isothiocyanates to the urinary bladder, leading to selective concentration in this organ (*29*).

In animal studies designed to investigate possible mechanisms whereby natural products have a positive effect on human health, it is important to relate the dose levels administered experimentally to the amounts that are consumed by humans as part of their diet.

In The Netherlands, the average human intake of glucosinolates is reported to be only 8 mg/day (*30*), which, assuming an average molecular weight of 450 for the glucosinolates and a body weight of 70 kg, equates to 0.25 μmol/kg/day. In Canada and the United States, the estimated average glucosinolate consumption is 0.5 and 0.6 μmol/kg/day, respectively (*30*). In the United Kingdom, the average daily intake of glucosinolates is 1.6 μmol/kg/day, while individuals with a high intake of *Brassica* vegetables may consume 9.5 μmol/kg/day (*31*). In Japan, the average intake is approximately 4 μmol/kg/day (*30*) while the intake in Singapore may be as high as 11 μmol/kg/day (*32*). These figures are based on the glucosinolate content of the raw vegetables. If the vegetables are cooked, up to 30% of the glucosinolate may be lost (*33*). Glucosinolates per se are not inducers of phase II enzymes (*34*), and activity is gained only after conversion to isothiocyanates. The degree of conversion of glucosinolates to isothiocyanates depends on the preparation and subsequent treatment of the foodstuff. Maceration of vegetables before consumption, thus releasing tissue myrosinase, will permit substantial conversion of glucosinolates to isothiocyanates. However, myrosinase is inhibited by heating and in cooked, unmacerated vegetables, hydrolysis of glucosinolates to isothiocyanates will be dependent on the action of intestinal thioglucosidases, which degrade only 10−20% of *Brassica*-derived glucosinolates during their passage through the gut (*35*, *36*). In Western countries, therefore, in which cooked *Brassica* vegetables are the norm, the maximum intake of isothiocyanates by humans would be of the order of 1.3 μmol/kg/day. In Asia, however, intakes may be much higher. For example, daikon (Japanese white radish) is grated before use and often eaten raw. With a daily intake of 100 g of this vegetable, an isothiocyanate intake as high as 5.6 μmol/kg is feasible (*37*).

In a dose−response experiment with AITC (*15*), significant increases in bladder QR and GST were recorded at a dose of 10 μmol/kg/day. In the present study, sulforaphane, iberin, iberverin, and erucin were found to be as effective as AITC at a dose of 40 μmol/kg/day, and if the dose−response curves are similar, any one of these compounds, or a combination thereof, would similarly induce bladder phase II enzymes at a dose of 10 μmol/kg/day. This dose approaches that which could be achieved through a high human intake of *Brassica* vegetables. In this context, it should be noted that in most experiments on enzyme induction by isothiocyanates, the test compounds have

been administered over only a few days, whereas humans regularly consume *Brassica* vegetables, often on a daily basis, throughout the whole of their lives. There is evidence (*15*) that activities of QR and GST in the rat bladder are influenced by duration of dosing. With the former enzyme, activities increased to a maximum after 15 daily doses, after which a plateau was reached. In contrast, bladder activities of GST continued to increase with continued dosing over the whole of the 21 day duration of this experiment. Further studies on the long-term effects of isothiocyanates are required.

The selectivity of isothiocyanates for phase II enzyme induction in the urinary bladder is of particular significance in view of recent epidemiological studies showing a significant decrease in the incidence of cancer in this organ in individuals with a high intake of *Brassica* vegetables (*38*). Furthermore, the high intake of *Brassica* vegetables in Japan and Singapore may contribute to the very low incidence of bladder cancer seen in these countries (*39*, *40*).

The fact that AITC and the sulfide and sulfoxide isothiocyanates all increased phase II enzyme activity in the urinary bladder suggests that this may be a common property of isothiocyanates. Studies with other saturated and unsaturated aliphatic and aromatic isothiocyanates are in progress in order to further explore the generality of induction in the bladder and establish structure−activity relationships for this effect. In this context, the recent report that low levels of benzyl isothiocyanate decrease the incidence of dysplasia, papilloma, and carcinoma in the urinary bladder of rats dosed with the known bladder carcinogen, N-butyl-N-(4-hydroxybutyl)nitrosamine (*41*), is of interest. Our preliminary studies with benzyl isothiocyanate indicate that this compound is a potent inducer of phase II enzymes in the rat bladder, and such induction may contribute to its chemoprotective effect.

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Received for review July 30, 2003. Revised manuscript received January 12, 2004. Accepted January 20, 2004. This work was supported by a grant from the Waikato Medical Research Foundation.

JF030549S