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Induction of Detoxication Enzymes in Mice by Naturally Occurring Allyl Nitrile

HIDEJI TANII,*,† TOMOMI HIGASHI,† FUSAE NISHIMURA,† YOSHIHIRO HIGUCHI,§ AND KIYOFUMI SAIJOH†

Departments of Hygiene and Molecular Pharmacology, Kanazawa University School of Medicine, 13-1 Takara-machi, Kanazawa 920-8640, Japan

Little is known about whether glucosinolate-derived nitriles have the ability to increase phase 2 detoxication enzymes and glutathione (GSH) in vivo. In this study, the ability of allyl nitrile, a hydrolysis product of the glucosinolate sinigrin, to increase tissue levels of the phase 2 detoxication enzymes glutathione *S*-transferase and quinone reductase and GSH in a variety of mouse tissues was examined. At the lowest dose level (11.8 mg/kg/day), allyl nitrile showed inductive ability in the stomach and lungs. At 23.6 mg/kg/day, the inductive effect was observed in the stomach, rectum, urinary bladder, and lungs, whereas at 47.2 mg/kg/day, it was recorded in the stomach, rectum, urinary bladder, kidneys, and lungs. These results show that allyl nitrile displays its maximum potency in the stomach and lungs, which is of interest in light of epidemiological studies demonstrating an inverse association between crucifer intake and the incidence of stomach and lung cancers.

KEYWORDS: Allyl nitrile; glutathione; glutathione *S*-transferase; quinone reductase; mice

INTRODUCTION

Epidemiological studies have shown an inverse association between the consumption of cruciferous vegetables and the risk of various cancers (*1*−*3*). The cancer chemopreventive effect of cruciferous vegetables has also been associated with their high levels of glucosinolates (*4*). Food preparation and eating release the plant enzyme myrosinase, which causes hydrolysis of glucosinolates into a number of breakdown products including isothiocyanates, nitriles, and indoles (*4*−*6*). The degradation of glucosinolates is affected by pH. A low pH enhances the formation of nitriles rather than isothiocyanates (*7*). Some of these breakdown products, in particular, isothiocyanates, have been shown to cause induction of phase 2 detoxication enzymes (*8*, *9*). Elevated tissue levels of detoxication enzymes are associated with decreased susceptibility to chemical carcinogenesis (*10*−*12*). These enzymes, including quinone reductase (QR) and glutathione *S*-transferases (GST), promote the conjugation of phase 1 products (principally cytochrome P450) with endogenous ligands such as glutathione (GSH) and glucuronic acid, usually resulting in an increase in water-soluble products.

Relatively few studies have been conducted with regard to glucosinolate-derived nitriles; however, crambene, a hydrolysis product of progoitrin, has been shown to induce GSH in the pancreas and liver at a dose of 30 mg (337 μmol)/kg/day for 6 days (*13*). Crambene also induces hepatic GST and QR at a dose of 50 mg (561 μmol)/kg/day for 7 days (*14*). Sulforaphane nitrile, a hydrolysis product of glucoraphanin, was shown to be a poor inducer of QR in vitro and no inducer in vivo (*15*). The biological activity of other cruciferous nitriles is not known. Of known cruciferous nitriles, we focused on allyl nitrile, a hydrolysis product of glucosinolate sinigrin, which is distributed widely throughout the Cruciferae family (*4*). Allyl nitrile generation has been observed in fermented cabbage (sauerkraut and coleslaw), which tend to be acid (*16*, *17*), and in our previous work in which homogenates of cruciferous vegetables were incubated in water or in buffer at pH 1.09, when nitrile formation would certainly be favored (*18*). Although nitriles are formed in sauerkraut and coleslaw, so are isothiocyanates. The amount of isothiocyanate formed was equal to, or greater than, that of nitrile (*17*), whereas the allyl nitrile content of coleslaw was reported to be $^1/_{10}$ that of allyl isothiocyanate (*16*). If cruciferous vegetables are cooked, whereby myrosinase is inactivated, intact glucosinolates can reach the large intestine, where they can be degraded by the gut flora. Isothiocyanate formation from glucosinolates has been shown both in vivo (*19*, *20*) and in vitro (*21*). In vitro digestion of sinigrin by *Bifidobacterium* sp. showed allyl nitrile to be the major product (*22*), suggesting possible generation in the large intestine under *Bifidobacteria*-preponderant conditions. Accordingly, consumption of cruciferous vegetables containing sinigrin implies that we are repeatedly exposed to allyl nitrile, which, although not toxic when consumed in vegetables (*18*), is a neurotoxicant when administered at high doses (*23*−*25*). It has been shown that the consumption of glucosinolates and their hydrolysis products can result in toxic effects, although no toxic effects on humans have been identified so far (*26*).

* Corresponding author (telephone +81 76 265 2211; fax +81 76 234 4232; e-mail taniih@med.kanazawa-u.ac.jp).
† Department of Hygiene.
§ Department of Molecular Pharmacology.

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Table 1. Glutathione *S*-Transferase (GST) Activities in the Stomach, Rectum, Kidneys, and Lungs of Control Mice and Mice Administered Allyl Nitrile at Indicated Doses for 7 Days[a]

dose level (mg/kg/day)	GST activity (μmol of 1-chloro-2,4-dinitrobenzene conjugated/min/mg of protein)			
	stomach	rectum	kidneys	lungs
0 (control)	0.630 ± 0.028	0.527 ± 0.080	0.718 ± 0.098	0.594 ± 0.062
11.8	0.856 ± 0.061[b]	0.614 ± 0.123	0.766 ± 0.148	0.643 ± 0.107
23.6	0.930 ± 0.150[b]	0.714 ± 0.124[b]	0.817 ± 0.130	0.754 ± 0.081[b]
47.2	1.134 ± 0.041[b]	0.700 ± 0.050[b]	0.986 ± 0.038[b]	0.821 ± 0.096[b]

[a] Values represent the means ± SD of four animals in each group. [b] Significantly different from the control at $P < 0.05$.

Table 2. Quinone Reductase (QR) Activities in the Stomach, Small Intestine, Urinary Bladder, Kidneys, and Lungs of Control Mice and Mice Administered Allyl Nitrile at Indicated Doses for 7 Days[a]

dose level (mg/kg/day)	QR activity (μmol of 2,6-dichloroindophenol reduced/min/mg of protein)				
	stomach	small intestine	urinary bladder	kidneys	lungs
0 (control)	1.922 ± 0.029	0.318 ± 0.104	0.380 ± 0.066	0.408 ± 0.053	0.116 ± 0.020
11.8	2.446 ± 0.335[b]	0.394 ± 0.022	0.340 ± 0.090	0.471 ± 0.048	0.155 ± 0.029[b]
23.6	2.674 ± 0.376[b]	0.404 ± 0.055	0.366 ± 0.087	0.442 ± 0.044	0.155 ± 0.023[b]
47.2	3.238 ± 0.187[b]	0.493 ± 0.062[b]	0.529 ± 0.051[b]	0.538 ± 0.035[b]	0.181 ± 0.025[b]

[a] Values represent the means ± SD of four animals in each group. [b] Significantly different from the control at $P < 0.05$.

It is not known whether allyl nitrile induces phase 2 detoxication enzymes and GSH. Elevated GSH levels are known to have a possible chemopreventive effect (*27*). In the present study, we tested the inductive ability of allyl nitrile by measuring the activities of GST and QR and GSH levels in tissues of mice after exposure to subtoxic doses of <47.2 mg/kg/day for 7 days. To study tissue selectivity for induction, measurements were carried out in the stomach, small intestine, colon, rectum, urinary bladder, kidneys, lungs, and liver.

MATERIALS AND METHODS

Chemicals. 2,6-Dichloroindophenol (DCIP), nicotinamide adenine dinucleotide phosphate (reduced), and dicumarol were purchased from Sigma Chemical (St. Louis, MO), allyl nitrile was from Tokyo Kasei Kogyo Co. (Tokyo, Japan), and all other chemicals were from Nakalai Tesque, Inc. (Kyoto, Japan).

Animals and Treatments. All animal experiments were conducted according to the Guidelines of the Committee on Animal Experimentation of Kanazawa University, Takara-machi Campus. Male ddY mice weighing 26−30 g obtained from Japan SLC Co. (Shizuoka, Japan) were used. They were maintained at 22 ± 2 °C under a 12:12 h light/dark cycle and allowed free access to tap water and laboratory food (CRF-1, Charles River Japan, Inc., Yokohama, Japan).

To study the effect of repeated exposure, groups of four animals were administered subtoxic doses of allyl nitrile (11.8, 23.6, or 47.2 mg/kg) or vehicle-distilled water (control; 4 mL/kg) daily for 7 days by gastric intubation. The subtoxic dose levels were based on our previous reports (*18*, *23*−*25*). On the eighth day, the animals were sacrificed for examination of enzyme activities and GSH levels in their tissues.

Tissue Preparation. After exposure to allyl nitrile, mice were anesthetized with 100 mg/kg sodium pentobarbital and perfused transcardially with 1.15% KCl. The stomach, small intestine, colon, rectum, urinary bladder, kidneys, lungs, and liver were immediately removed and stored at −80 °C until analysis. For enzyme analyses (*28*), a known weight of tissue was immediately homogenized in 4 volumes of buffer (0.15 M KCl and 0.25 M phosphate buffer, pH 7.25). The homogenates were centrifuged at 10000*g* at 4 °C for 20 min, and then the resulting supernatant was centrifuged at 105000*g* at 4 °C for 60 min to obtain a cytosolic fraction. Cytosolic fractions were stored at −80 °C until analyses. Protein concentrations were measured according to the method of Bradford (*29*) using bovine serum albumin as the standard.

Enzyme and GSH Analyses. GST activity was measured according to the spectrophotometric method of Habig et al. (*30*) with 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate. QR activity was measured according to the spectrophotometric method of Ernster (*31*), and GSH concentrations were measured according to the method of Jaeger et al. (*32*).

Statistics. GSH amounts and enzyme activity were expressed as the mean ± standard deviation (SD). Statistical analyses were performed by analysis of variance followed by Fisher's least significant difference test for multiple comparisons. The level of significance was set at $P < 0.05$.

RESULTS

Repeated exposure to allyl nitrile at subtoxic levels did not induce any behavioral abnormalities in the mice, but changes in both detoxication enzyme activities and GSH levels were observed.

No significant differences in GST activity were observed in the small intestine, colon, urinary bladder, or liver of mice given allyl nitrile at any level. However, elevated GST activities were recorded in the stomach at 11.8, 23.6, and 47.2 mg/kg/day, in the rectum and lungs at 23.6 and 47.2 mg/kg/day, and in the kidneys at 47.2 mg/kg/day (**Table 1**).

QR activities in the colon, rectum, and liver showed no change at any dose level. Enhancements of QR activities were observed in the stomach and lungs at 11.8, 23.6, and 47.2 mg/kg/day and in the small intestine, urinary bladder, and kidneys at 47.2 mg/kg/day (**Table 2**).

Allyl nitrile at all dose levels had no effect on GSH levels in the small intestine, colon, kidneys, lungs, and liver. Elevated GSH levels were seen in the stomach at 11.8, 23.6, and 47.2 mg/kg/day and in the rectum and urinary bladder at 23.6 and 47.2 mg/kg/day (**Table 3**).

DISCUSSION

The present study shows that allyl nitrile at subtoxic doses has the ability to increase GST and QR activities and tissue GSH in mice. Moreover, this inductive effect appears to be tissue

Table 3. Glutathione (GSH) Levels in the Stomach, Rectum, and Urinary Bladder of Control Mice and Mice Administered Allyl Nitrile at the Indicated Doses for 7 Days[a]

dose level (mg/kg/day)	GSH level (μmol/g of tissue)		
	stomach	rectum	urinary bladder
0 (control)	2.00 ± 0.29	3.39 ± 0.25	2.82 ± 0.14
11.8	2.39 ± 0.34[b]	3.26 ± 0.15	3.09 ± 0.26
23.6	2.40 ± 0.13[b]	4.02 ± 0.50[b]	3.24 ± 0.23[b]
47.2	2.61 ± 0.08[b]	4.06 ± 0.31[b]	3.23 ± 0.31[b]

[a] Values represent the means ± SD of four animals in each group. [b] Significantly different from the control at $P < 0.05$.

dependent. Of eight tissues tested, allyl nitrile displayed its maximum potency in the stomach at the lowest level [11.8 mg (176 μmol)/kg/day], inducing GST and QR activities and GSH levels. Allyl nitrile at this level also enhanced QR activity in the lungs. The inductive effect of allyl nitrile at 23.6 mg (352 μmol) and 47.2 mg (704 μmol)/kg/day was observed in the stomach, small intestine, rectum, urinary bladder, kidneys, and lungs. These results suggest involvement of allyl nitrile in the chemopreventive effect of cruciferous vegetables.

Allyl nitrile displayed its inductive effect in several tissues, especially the stomach and lungs. This result is associated with epidemiological studies demonstrating inverse associations between crucifer intake and the incidence of stomach, lung, urinary bladder, colon, pancreas, prostate, thyroid, and skin cancers. Cohort studies have shown inverse associations between total cruciferous vegetable intake and the risk for stomach cancer; between intake of cabbage, cauliflower, or broccoli and the risk for lung cancer; and between broccoli intake and the risk for all cancers (*3*). Allyl nitrile is a breakdown product of the glucosinolate sinigrin, which is abundant in cruciferous vegetables. We previously showed that of various cruciferous vegetables, allyl nitrile generation was observed in eight, including cabbage, cauliflower, and broccoli (*18*).

Allyl isothiocyanate, another breakdown product of sinigrin, is an inducer of phase 2 enzymes. Allyl isothiocyanate increases QR and/or GST in the liver, kidneys, spleen, lungs, urinary bladder, forestomach, grandular stomach, duodenum, ileum, cecum, and colon plus the rectum of rats at a dose of 200 μmol/kg/day (*33*), QR and GST in the bladder at a dose of 10 μmol/kg/day (*33*), and QR and/or GST in the forestomach, duodenum, and bladder at a dose of 40 μmol/kg/day (*34*). It appears that the isothiocyanate is the more potent inducer compared with allyl nitrile, on a molar basis, and its activity is expressed in a wider range of tissues.

It is important to relate the dose levels employed with those that could be consumed by humans. In our previous study (*18*), it is noted that the daily dietary intake of allyl nitrile by the Japanese was at least 0.12 μmol/kg of body weight, although no information on the daily intake is available for other ethnic groups. As for isothiocyanates, in Western countries the maximum intake of them by humans is estimated to be of the order of 1.3 μmol/kg/day (*34*), whereas in Asia an isothiocyanate intake as high as 5.6 μmol/kg is feasible (*35*). Allyl nitrile appears to be less consumed than allyl isothiocyanate. Although the lowest dose of allyl nitrile employed in the present study (176 μmol/kg/day) is >1400 times the possible human intake mentioned above, it is evident that the nitrile at subtoxic levels has the ability to induce phase 2 detoxication enzymes and GSH. It is not known whether allyl nitrile displays its inductive potency at much lower levels, and, therefore, more studies on the dose−response with the nitrile are needed.

The inductive ability of allyl nitrile could be related to its metabolism in the body. β,γ-Unsaturated allyl nitrile ($CH_2{=}CHCH_2CN$) undergoes α-hydroxylation, leading to cyanohydrin formation and CN^- release; it is likely that the epoxide of allyl nitrile is also formed, although not leading to CN^- release (*36*). This epoxide meets the electrophilicity required for inducers of GST and QR (*37*).

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