7-Methylsulfinylheptyl and 8-methylsulfinyloctyl isothiocyanates from watercress are potent inducers of phase II enzymes

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Watercress is an exceptionally rich dietary source of β-phenylethyl isothiocyanate (PEITC). This compound inhibits phase I enzymes, which are responsible for the activation of many carcinogens in animals, and induces phase II enzymes, which are associated with enhanced excretion of carcinogens. In this study, we show that watercress extracts are potent inducers of quinone reductase (QR) in murine hepatoma Hepa 1c1c7 cells, a widely adopted assay for measuring phase II enzyme induction. However, contrary to expectations, this induction was not associated with PEITC (which is rapidly lost to the atmosphere upon tissue disruption due to its volatility) or a naturally occurring PEITC–glutathione conjugate, but with 7-methylsulfinylheptyl and 8-methylsulfinyloctyl isothiocyanates (ITCs). While it was confirmed that PEITC does induce QR (5 μM required for a two-fold induction in QR), 7-methylsulfinylheptanyl and 8-methylsulfinyloctyl ITCs were more potent inducers (0.2 μM and 0.5 μM, respectively, required for a two-fold induction in QR). Thus, while watercress contains three times more phenylethyl glucosinolate than methylsulfinylalkyl glucosinolates, ITCs derived from methylsulfinylalkyl glucosinolates may be more important phase II enzyme inducers than PEITC, having 10- to 25-fold greater potency. Analysis of urine by liquid chromatography–mass spectroscopy (LC-MS) following consumption of watercress demonstrated the presence of N-acetylcysteine conjugates of 7-methylsulfinylheptyl, 8-methylsulfinyloctyl ITCs and PEITC, indicating that these ITCs are taken up by the gut and metabolized in the body. Watercress may have exceptionally good anticarcinogenic potential, as it combines a potent inhibitor of phase I enzymes (PEITC) with at least three inducers of phase II enzymes (PEITC, 7-methylsulfinylheptyl ITC and 8-methylsulfinyloctyl ITC). The study also demonstrates the application of LC-MS for the detection of complex glucosinolate-derived metabolites in plant extracts and urine.

Introduction

Watercress [Rorippa nasturtium-aquaticum (L.) Hayek, syn. Nasturtium officinale R.Br] and other cruciferous salad crops are rich sources of isothiocyanates (ITCs; Figure 1). These compounds are derived from glucosinolates by the action of plant myrosinases (thioglucoside glucohydrolase; EC 3.2.3.1) following tissue disruption, or by hydrolysis in the gut by the action of microbial thioglucosidases (1–3). ITCs have been implicated as chemoprotective agents in reducing the risk of cancer. Mechanistic studies have indicated that, depending upon the specific structure of the ITC, these compounds can act at three stages of carcinogenesis. Firstly, they can prevent carcinogen activation through inhibition of phase I enzymes such as cytochrome P450s (4). Secondly they can induce phase II enzymes such as quinone reductase (QR) [NAD(P)H: (quinone-acceptor) oxidoreductase, EC 1.6.99.2], glutathione S-transferases (GSTs) [EC 2.5.1.18] and UDP-glucuronosyltransferases [EC 2.4.1.17] (5–12), resulting ultimately in the excretion of the potential carcinogens. Thirdly, they can induce apoptosis via activation of the stress-activated protein kinase pathway mediated by sustained activation of JNK 1 (13–15).

The putative anticarcinogenic activity of ITCs is consistent with the results of epidemiological studies, which have suggested a reduction in risk of cancer, particularly of the gastrointestinal tract, through the consumption of cruciferous vegetables (16–19).

ITCs are metabolized in mammals by conjugation with glutathione, followed by conversion via the mercapturic acid pathway to N-acetylcysteine conjugates and excretion in the urine. Thus, the presence of ITC–N-acetylcysteine conjugates in the urine is indicative of uptake and metabolism of ITCs by the body.

Of the one hundred or so naturally occurring ITCs, β-phenylethyl isothiocyanate (PEITC) has received considerable attention because of its ability to inhibit cytochrome P450s (i.e. phase I enzymes) and to induce apoptosis. It has also been reported to induce phase II enzymes (11). It reduces the incidence of cancer of the lung and oesophagus induced in rodents by nitrosamines (20–24). Watercress is an exceptionally rich source of PEITC and may have a cancer chemopreventative role in the diet. In a human intervention study, watercress, when fed to smokers, was shown to enhance the metabolism and resultant excretion of the lung carcinogen 4-(methylisothiocyanato)-1-(3-pyridyl)-1-butanone (NKK) either as 4-(methylisothiocyanato)-1-(3-pyridyl)-1-butanol (NNAL) or as 4-(methylisothiocyanato)-1-(3-pyridyl)but-1-yl]-β-ωmga-δ-glucuronic acid (NNAL-Gluc) (25). However, more detailed analysis of urinary metabolites suggested, contrary to expectations, that watercress had no effect on the oxidative metabolism of nicotine by cytochrome P450 enzymes but instead induced UDP-glucuronosyltransferase activity (i.e. a phase II enzyme). While this result is not inconsistent with the possible activity of PEITC, it is not clear whether this phase II induction is due to this compound or to other metabolites of watercress (25).

In this paper, we report the ability of watercress extracts to induce phase II enzymes and provide an analysis of watercress extracts which shows that, in addition to PEITC, 7-methylsul-
finely heptyl ITC and 8-methylsulfinyloctyl ITC are inducers of phase II enzymes. We also demonstrate that N-acetylcysteine conjugates of these ITCs are present in urine following watercress consumption.

Materials and methods

Source of materials and sampling

Three watercress accessions were used: Wc141 is a commercial cultivar obtained from Vitacress Ltd (Andover, UK); Wc138 and Wc7 are wild accessions collected originally from Cornwall, UK, and Menorca, Spain, and obtained from the watercress germplasm collection at Horticultural Research International, Wellesbourne, UK, courtesy of Dr John Walsh. Plants were grown under standard glasshouse conditions (16°C day/12°C night; 16 h photoperiod). Tissue was harvested after 7 weeks, immediately frozen in liquid nitrogen, freeze dried and stored at -20°C until required.

Induction of gainone reductase in murine hepatocyte cell cultures by watercress extracts, PEITC and a PEITC–glutathione conjugate

One hundred milligrams of freeze-dried tissue was hydrated with 2 ml of deionized water and homogenized for 15 s. The hydrated tissue was left at room temperature for 1 h with occasional vortexing. Subsequently, 3 ml of boiling 70% (v/v) methanol was added to the reaction mix and incubated for a further 15 min at 70°C. The mixture was cooled and centrifuged at 3000 × g for 5 min. After centrifugation, 1 ml aliquots were removed, placed in Eppendorf tubes and vacuum centrifuged to 200 µl volumes, giving an equivalent concentration of 100 mg/ml extracts for QR induction assays. Extracts were analysed for the inducibility of QR in murine Hepa 1c1c7 cells as described previously (7,28,29). All QR assays were conducted three times on separate occasions with three separate extracts. In addition to QR assays with watercress extracts, QR assays were undertaken with fractionated extracts (see below for details), commercially purchased PEITC (Sigma) and a synthetic PEITC–glutathione conjugate (see below for details of synthesis), as previously described (7,28,29).

Glucosinolate analysis

Glucosinolates were extracted from freeze-dried tissue, converted to desulfo glucosinolates as previously described (26) and analysed by liquid chromatography–mass spectrometry (LC-MS) with the use of 2-propenyl glucosinolate as an internal standard. Desulfoglucosinolates were separated via a Spherisorb 5 µM ODS2 (4.6 × 250 mm) analytical column, with the use of a gradient from 1% acetonitrile:99% water to 70% acetonitrile:30% water in 19 min followed by a reduction to 1% acetonitrile:99% water in 5 min and a flow rate of 1 ml/min, and detection at 229 nm. Mass spectral data were collected following atmospheric pressure chemical ionization of the eluent. Spray chamber conditions were: gas temperature, 350°C; drying gas, 10.0 l/min; nebulizer pressure, 60 psig; fragmentor voltage, 110 V. Putative glucosinolate degradation products were sought in the mass spectral data through extraction of [M+H+] ions and confirmed by the analysis of fragmentation patterns while varying the fragmentor voltage. Fractionation of QR extracts

Watercress extracts were fractionated with a gradient similar to that described above but without trifluoroacetic acid in the mobile phase. Samples (25 µl) of the original 200 µl of QR extract were injected and fractions were collected each minute for 12 min. The procedure was repeated eight times, eluting eight fractions for each 1 min of sample collected. These fractions were then warmed to 40°C and dried under a stream of air. Once dried, each sample was resuspended in 200 µl of sterile water, the eight fractions for each minute pooled and vacuum condensed to a final volume of 200 µl (the original starting QR volume). Each fraction (1–12) was analysed for the presence of the 7-methylsulfinylheptyl and 8-methylsulfinyloctyl ITCs and other glucosinolate degradation products via LC-MS. The fractions containing these ITCs were thoroughly analysed by LC-MS and UV spectral analysis to identify any other metabolites present.

Analysis of headspace volatiles from watercress

Volatiles were collected in the headspace from 0.1 g of rehydrated, freeze-dried leaf material in a 5 ml vial on to a solid-phase micro-extraction (SPME) probe (0.2 mm polydimethylsiloxane; Superloc Inc., Bellefonte, PA, USA) for 5 min and then flash loaded on to an HP5 column. A Hewlett-Packard gas chromatograph–mass spectrometer was used for analysis, with the column being heated to 250°C over a 7 min period.

Analysis of human urine for watercress metabolites

Urine was collected 5 h after consumption of 100 g watercress by a male volunteer. A 1 ml sample was centrifuged at 3000 × g for 5 min and 50 µl of the supernatant was injected directly into an LC-MS system. Analysis of urine was performed using a simple acetonitrile:water + trifluoroacetic acid gradient through a Waters Spherisorb 5 µM ODS2 (4.6 × 250 mm) analytical column. In brief, samples were eluted at a flow rate of 0.75 ml/min and a gradient of 1% acetonitrile:99% water + 0.1% trifluoroacetic acid increasing to 70% acetonitrile over 35 min and then returning to 1% acetonitrile after 38 min, with UV absorbancy measured at 235 nm. Mass spectral data of compounds in the eluent were collected following atmospheric pressure electrospray ionization. Spray chamber conditions were: gas temperature, 350°C; drying gas, 10.0 l/min; nebulizer pressure, 40 psig. ITC metabolites were sought in the mass spectral data through extraction of [M+H+] ions and confirmed by the analysis of fragmentation patterns with varying fragmentor voltages.

Synthesis of a β-phenylethyl–glutathione conjugate [S-(N-β-phenethylthiobromo)glutathione]

[S-(N-β-Phenethylthiobromo)glutathione (PTCG) was synthesized as previously described (27). Mass spectrometry (positive fast atom bombardment; glycerol matrix) gave a prominent peak at 471 [M+H+], the expected mass. Further confirmation of structure and purity was obtained by 1H-NMR (400 MHz, CD3OD).

Results

Glucosinolate variation in watercress accessions

The watercress accessions had similar profiles: the major glucosinolate being β-phenethyl glucosinate, with significant amounts of 7-methylsulfinylheptyl and 8-methylsulfinyloctyl and lower amounts of 7-methylthioheptyl and 8-methylthiooctyl glucosinolates (Figure 2, Table I). The identity of the glucosinolates was confirmed through the presence of the [M+H+] ion and an [M+H+ − 162] fragment ion (corresponding to loss of the glucose moiety) associated with each desulfoglucosinolate peak. There were no significant differences in the concentration of β-phenethyl glucosinate content among accessions (ANOVA, F = 1.71, P = 0.258). However, there were significant differences in the levels of alliphatic glucosinolates (i.e. those derived from methionine) (Table I), with Wc141 having the highest level and Wc7 the
Isothiocyanates in watercress

Fig. 2. Chromatogram of desulfoglucosinolates extracted from watercress. Identity of all compounds was confirmed by mass spectrometry. (1) 2-propenyl (internal standard); (2) 7-methylsulfynylheptyl; (3) 8-methylsulfinylloctyl; (4) 1-indolylmethyl; (5) 4-methoxy-1-indolylmethyl; (6) β-phenylethyl; (7) 7-methylthioheptyl; (8) 8-methylthiooctyl.

Fig. 3. (A) Induction of quinone reductase in murine hepatoma Hepa 1c1c7 cells by watercress extracts: ○, Wc141; □, Wc138; ▼, Wc7. Mean ± standard error of 12 replicates at each concentration. The estimated CD value (i.e. the amount required for a two-fold QR induction) was 0.078, 0.156 and 0.35 mg/ml for Wc141, Wc138 and Wc7, respectively. (B) Induction by fractions 6 (●) and 7 (○) of the fractionated extract which contain 7-methylsulfynylheptyl and 8-methylsulfinylloctyl ITC, respectively. Fraction 5 (▲), which contains PTCG, does not induce QR. Fraction 3 (▲), and all other fractions (data not shown), do not induce QR. Mean ± standard error of eight replicates at each concentration. (C) Induction by PEITC (○) and PTCG (●). Mean ± standard error of 12 replicates at each concentration. The CD value for both compounds was 5 µM.

Table I. Glucosinolate composition of watercress accessions

<table>
<thead>
<tr>
<th>Side-chain structure</th>
<th>Glucosinolates (µmol/g dry wt, mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wc7</td>
</tr>
<tr>
<td>6-Methylsulfinylhexyl</td>
<td>0.2 ± 0.00</td>
</tr>
<tr>
<td>7-Methylsulfynylheptyl</td>
<td>3.9 ± 0.09</td>
</tr>
<tr>
<td>8-Methylsulfinyloctyl</td>
<td>2.1 ± 0.05</td>
</tr>
<tr>
<td>7-Methylthioheptyl</td>
<td>1.8 ± 0.14</td>
</tr>
<tr>
<td>8-Methylthiooctyl</td>
<td>0.8 ± 0.06</td>
</tr>
<tr>
<td>Total aliphatic</td>
<td>8.7 ± 0.32</td>
</tr>
<tr>
<td>β-Phenylethyl</td>
<td>23.7 ± 0.64</td>
</tr>
<tr>
<td>Indolyl</td>
<td>0.7 ± 0.02</td>
</tr>
</tbody>
</table>

Values within rows that have different letters are significantly different (ANOVA, P < 0.01). Indolyl glucosinolates represent the sum of 3-indolylmethyl and 4-methoxy-3-indolylmethyl glucosinolates. The mean ratio of phenylethyl glucosinolate to methylsulfinylalkyl glucosinolates was 3.1.

The mean ratio ± standard error of phenylethyl glucosinolate to methylsulfinylalkyl glucosinolates was 3.1 ± 0.47. An additional sample of watercress, which was purchased from a local grocery store and eaten before collection of urine, had a similar glucosinolate content.

Induction of quinone reductase by watercress extracts

Watercress extracts induced quinone reductase in murine hepatoma Hepa 1c1c7 cells (Figure 3). There were significant differences between accessions; Wc141 was the most potent inducer and Wc7 the least potent inducer. The CD (i.e. the concentration required for a two-fold enzyme induction) values for the three accessions (Figure 3) were negatively correlated with the aliphatic glucosinolate content (linear regression; r² = 0.92).

Glucosinolate metabolites in watercress QR extracts

To identify the QR inducer(s), watercress extracts were analysed by LC-MS. PEITC was not detected. However, analysis of headspace volatiles by gas chromatography-mass spectrometry collected during sample preparation indicated that large quantities of this volatile ITC were produced following hydrolysis but were lost to the atmosphere and not incorporated into the aqueous extract. A similar fate was observed for 7-methylthioheptyl and 8-methylthiooctyl ITCs. The major glucosinolate products in the aqueous extracts were the non-volatile ITCs derived from 7-methylsulfynylheptyl and 8-methylsulfinylloctyl glucosinolates. The presence of these was confirmed by LC-MS; the ITCs having a [M+H+] ion and a
major [M+H+ – 64] fragment ion (corresponding to loss of the terminal methylsulfinyl moiety).

Further analysis of the QR extract identified PTCG in the aqueous extract. This probably forms spontaneously following tissue disruption and serves to stabilize the volatile ITC in solution. The identity of this conjugate was confirmed by comparison of its mass spectrum (Figure 4) and UV absorbance spectrum with those of synthetic PTCG. The ratio of the molecular ion [M+H+] to fragmentation ions, including [M+H+ – 129] (produced by loss of C5H8NO3) and [M+H+ – 163] (produced by loss of PEITC), were identical to those of the synthetic standard, as was the UV spectrum. The relative amounts of [M+H+] + 1 and [M+H+] + 2 ions were consistent with the predicted isotopic occurrence of 13C, 15N and 33S. With the use of a standard concentration curve obtained with synthetic PTCG, the content in the extract was estimated to be 100 pmol. In an extract equivalent to 0.625 mg/ml of To confirm that PEITC and methylsulfinylalkyl ITCs were ingested following consumption of watercress, a urine sample was collected 5 h after the consumption of 100 g of fresh watercress. The N-acetylcysteine conjugates of β-phenylethyl (APCC), 7-methylsulfinylheptyl (AMSHC), 8-methylsulfinyl-octyl (AMSOC), 7-methylthioheptyl and 8-methylthiooctyl ITCs were detected initially by the presence of the [M+H+] ion and then confirmed through the analysis of fragmentation products. All conjugates produced similar mass spectra, with prominent [M+H+], [M+Na+] and [M+K+] ions and a major fragmentation ion of [M+H+ – 163] due to the loss of the N-acetylcysteine residue at the sulfhydryl bond (Figure 5). The (M+H+)+1 and (M+H+)+2 ions also corresponded to the predicted relative isotopic abundances of 13C, 15N and 33S. The fragmentation pattern was confirmed via the analysis of synthetic APCC.

Discussion

Watercress is a rich source of PEITC, which has been shown to inhibit cytochrome P450 enzymes responsible for the activation of carcinogens (4,20–24). A dietary intervention study provided evidence that watercress was able to increase
the glucuronidation of nicotine metabolites in humans (25), suggesting the induction of phase II enzymes. In the current study, we confirmed that watercress extracts could induce phase II enzymes through the use of the widely adopted murine hepatoma Hepa 1c1c7 cell culture assay. Analysis of the watercress extracts demonstrated that 7-methylsulfinylheptyl ITC and 8-methylsulfinyloctyl ITC are potent phase II enzyme inducers. These ITCs, and their glucosinolate precursors, have been overlooked in watercress because of the predominant amounts of β-phenethyl glucosinolate. Our study, however, suggests that they are important dietary components. The inducing potencies of 7-methylsulfinylheptyl and 8-methylsulfinyloctyl ITCs were similar to that previously reported for 4-methylsulfinylbutyl ITC (sulforaphane) (7,31) and considerably greater than that for PEITC (this study). The relative QR-inducing potential of three watercress accessions correlated with their aliphatic glucosinolate content.

PEITC was not found in our extracts, because of its high volatility. Many other ITCs, such as alkenyl and methylthioalkyl ITCs, are also volatile, and would not be found in significant amounts in aqueous extracts of crucifers. Low levels of PTGC were present in the extract, but at a level insufficient for induction. While conjugates between exogenous and endogenous compounds with glutathione have frequently been described in mammals, this conjugate is the first naturally occurring conjugate between a plant secondary metabolite and glutathione to be described in plant extracts. The assays demonstrated an equivalent ability of PEITC and PTGC to induce phase II enzymes. It is not known whether this is due to the conjugate dissociating when in association with the murine Hepa 1c1c7 cells, resulting in PEITC being taken up into cells, or whether the conjugate itself has the ability to be taken up into cells and induce phase II enzymes.

To confirm that these ITCs are taken up and metabolized following watercress consumption, urine was analysed in order to detect the specific ITC–N-acetylcysteine conjugates by LC-MS. Previous studies have detected ITC–N-acetylcysteine conjugates in urine by cyclocondensation reactions with 1,2-benzenedithiol to yield 1,3-benzodithiole, which can be detected spectroscopically (32,33). While this technique is useful, it does not enable the structure of the specific ITC conjugate to be determined. This may be important in dietary studies in which the majority of crucifers consist of mixtures of glucosinolates, as is found in watercress. In the current study, we demonstrate that through the use of LC-MS and selective ion monitoring, individual ITC–N-acetylcysteine conjugates can be detected with minimal sample preparation. The range of conjugates reflected the glucosinolate content of watercress (Table I and Figure 5). This method will enable studies on the rate of excretion of N-acetylcysteine conjugates with different ITCs as part of studies on in vivo phase I and II enzyme inhibition and induction.

Consumption of watercress thus involves the ingestion of at least two structurally diverse groups of glucosinolate metabolites with biological activity: (i) PEITC, derived from phenylalanine, acts by inhibiting phase I enzymes (cytochrome P450s) (4) and inducing phase II enzymes and (ii) long-chain methylsulfinylalkyl ITCs, derived from methionine, are potent phase II enzyme inducers. While watercress contains 3.1 times as much phenethyl glucosinolate as methylsulfinylalkyl glucosinolates, the ITCs from the latter are 10–25 times more potent inducers of phase II enzymes. It is conceivable that these structurally different ITCs may exhibit synergistic interactions as chemoprotective compounds in the diet.

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