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Intact glucosinolates modulate hepatic cytochrome P450 and phase II conjugation activities and may contribute directly to the chemopreventive activity of cruciferous vegetables

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ABSTRACT

The currently accepted view is that the chemopreventive activity of glucosinolates is exclusively mediated by their degradation products, such as isothiocyanates. In the present study, evidence is presented for the first time that intact glucosinolates can modulate carcinogen-metabolising enzyme systems. The glucosinolates glucoraphanin and glucoerucin were isolated from cruciferous vegetables and incubated with precision-cut rat liver slices. Both glucosinolates elevated the O-dealkylations of methoxy- and ethoxyresorufin, markers for CYP1 activity; supplementation of the incubation medium with myrosinase, the enzyme that converts glucosinolates to their corresponding isothiocyanates, abolished these effects. Moreover, both glucoerucin and glucoraphanin increased the apoprotein levels of microsomal CYP1A1, CYP1A2 and CYP1B1. At higher concentrations, both glucosinolates enhanced quinone reductase activity, whereas glucoraphanin also elevated glutathione S-transferase; in this instance, however, supplementation of the incubation medium with myrosinase exacerbated the inductive effect. Finally, both glucosinolates increased modestly cytosolic quinone reductase, GSTα and GSTμ protein levels, which became more pronounced when myrosinase was added to the incubations with the glucosinolate. It may be inferred that intact glucosinolates can modulate the activity of hepatic carcinogen-metabolising enzyme systems and this is likely to impact on the chemopreventive activity linked to cruciferous vegetable consumption.

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1. Introduction

Strong epidemiological evidence has been published linking the consumption of cruciferous vegetables to lower cancer incidence at a number of sites (Seow et al., 2002; Ambrosone et al., 2004; Hayes et al., 2008; Joseph et al., 2004; Zhao et al., 2007). The chemopreventive activity of these vegetables has been attributed to glucosinolates, a class of sulfur-containing glycosides, which are present at substantial amounts (Hayes et al., 2008; Steinbrecher and Linseisen, 2009). The accepted view is that glucosinolates are not directly responsible for the chemopreventive effects, but their breakdown products such as the isothiocyanates.

When cruciferous vegetables are disturbed, for example during chopping or chewing, the enzyme myrosinase (β-thioglucoside glucohydrolase) comes into contact with the glucosinolate leading to the formation of the isothiocyanate; furthermore, intestinal microbial myrosinase can contribute to the generation of isothiocyanates from their glucosinolate precursors (Getahun and Chung, 1999). Indeed, isothiocyanates have been similarly linked through epidemiological studies to lower cancer incidence (Spitz et al., 2000). Moreover, in laboratory studies isothiocyanates antagonised the carcinogenicity of many classes of chemical carcinogens in animal models, including polycyclic aromatic hydrocarbons and nitrosocompounds which are dietary carcinogens of human relevance (Hecht, 2000; Hecht et al., 2002; Solt et al., 2003; Chung et al., 2000; Fahey et al., 2002; Dinkova-Kostova et al., 2006). Multiple mechanisms have been proposed to underpin the chemopreventive activity of isothiocyanates, influencing both initiation and post-initiation stages of carcinogenesis. One of the most important is modulation of the metabolism of chemical carcinogens so as to limit the availability of their genotoxic metabolites. This can be achieved either through impairment of the cytochrome P450-mediated generation and/or increased detoxification of genotoxic metabolites resulting from up-regulation of enzyme systems such as glutathione S-transferase and quinone reductase. As a matter of fact, isothiocyanates having either an aliphatic or an aromatic side chain could modulate both cytochromes P450 and phase II conjugation systems in the liver and other tissues following oral administration

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to rats at dose levels simulating human dietary intake (Yoxall et al., 2005; Hanlon et al., 2008a; Konsue and Ioannides, 2008). Moreover, in extensive in vitro studies conducted in precision-cut liver and lung slices isothiocyanates could modulate these enzyme systems at concentrations as low as 0.5 μM (Hanlon et al., 2008b, 2009a,b; Konsue and Ioannides, 2010a,b).

It has always been assumed that intact glucosinolates, because of their hydrophilicity, would be unable to reach the bloodstream following oral intake. However, in recent studies it was demonstrated that, at least in rats, glucosinolates such as glucoraphanin, encountered in broccoli and being the precursor of the isothiocyanate sulforaphane, could be absorbed intact following oral intake (Bheemreddy and Jeffery, 2007). Moreover, glucoraphanin could be reduced to another glucosinolate, glucoerucin. These observations prompted us to investigate whether intact glucosinolates have the potential to influence carcinogen-metabolising enzyme systems in the liver, the principal site of carcinogen metabolism.

2. Materials and methods

Sulforaphane and erucin (LKT Laboratories, MN, USA), NADPH, ethoxyresorufin, methoxyresorufin, resorufin, 1-chloro-2,4-dinitrobenzene (CDNB), cytochrome c peroxidase-linked anti-rabbit, anti-mouse and anti-goat antibodies (Sigma Co. Ltd., Poole, Dorset, UK), anti-CYP1A1 (AMS Biotechnology, Abingdon, UK), anti-CYP1A2 (Chemicon International Inc., Hampshire, UK) and anti-CYP1B1 (BD Biochemicals, Oxford, UK) were all purchased. Antibodies to human quinone reductase, lactate dehydrogenase and β-actin were obtained from abcam (Cambridge, UK), and antibodies to GSTα and GSTμ from Calbiochem (Lutterworth, UK).

2.1. Isolation of glucosinolates

Glucoraphanin and glucoerucin were isolated from Brassica oleracea L. var. *acephala sabellica* (Cavolo nero di Toscana) and Eruca sativa ripe seeds, respectively, supplied by SUBA & UNICO (Longiano, Italy), according to a procedure developed at CRA-CIN of Bologna, Italy (Visentin et al., 1992). Seeds were first ground to a fine powder and defatted with hexane. The solvent was removed and the defatted meals were used as starting material. The samples were treated with boiling 70% ethanol in order to quickly deactivate the endogenous enzyme myrosinase. Glucosinolates were extracted using an Ultraturrax homogeniser at medium speed for 15 min. The resulting homogenate was centrifuged at 17,700 × *g* for 30 min. The isolation of the glucosinolates from the extract was carried out by one-step anion exchange chromatography, as previously described (Visentin et al., 1992). Glucosinolate purity was further improved by gel-filtration performed using a XK 26/100 column packed with Sephadex G10 chromatography media (Amersham Biosciences), connected to an FPLC System (Pharmacia). Individual fractions were analysed by HPLC and those containing pure glucosinolates were pooled and freeze-dried (Wagner et al., 2010). Glucosinolates were characterised by ^{1}H and ^{13}C NMR spectrometry and the purity was assayed by HPLC analysis of the desulfo-derivative according to the ISO 9167-1 method (EEC Regulation, 1990). The purities were in the range of 95–99%. As myrosinase was deactivated prior to glucosinolate isolation, the impurities are unlikely to be isothiocyanates. The enzyme myrosinase (β-thioglucoside glucohydrolase, EC.3.2.1.147) was isolated from seeds of Sinapis alba L. as described by Pessina et al. (1990) with some modification. The specific activity of the stock solution used in the present study was about 60 U/mg of soluble protein. The enzymatic activity was about 30 U/ml and the solution was stored at 4 °C in sterile distilled water until use. One myrosinase unit was defined as the amount of enzyme able to hydrolyze 1 μmol sinigrin per min at pH 6.5 and 37 °C.

2.2. Preparation and incubation of precision-cut rat liver slices

Male Wistar albino rats (200–250 g) were obtained from B&K Universal Ltd. (Hull, East Yorkshire, UK). The animals were housed at 22 ± 2 °C, 30–40% relative humidity, in an alternating 12-h light:dark cycle with light onset at 07:00 h. Rat liver slices (200–300 μm) were prepared from 8 mm cylindrical cores using a Krumdieck tissue slicer (Alabama Research and Development Corporation, Munsford, AL, USA) as previously described (Hashemi et al., 1999). The multiwell plate procedure, using 12-well culture plates, was used to culture the slices in the presence of glucosinolates (0–25 μM), isothiocyanates (0–25 μM) or glucosinolates (0–25 μM) plus myrosinase (0.018 U); isothiocyanates were dissolved in DMSO so that the final concentration was 1.5 μl/ml of incubation medium. One slice was placed in each well, in 1.5 ml of culture medium. Slices were incubated under sterile conditions for 24 h on a reciprocating plate shaker housed in a humidified incubator, at a temperature of 37 °C and under an atmosphere of 95% air/5% CO_2. The slices were initially pre-incubated for 30 min in order to slough off any dead cells due to slicing. Three different slice pools, each comprising 10 rat liver slices, were used per concentration.

2.3. Enzyme assays and immunoblotting

Following incubation, slices were removed from culture media, homogenised and post-mitochondrial supernatants prepared and stored at −80 °C. When required, microsomes were isolated by centrifugation (105,000 × *g* × 1 h). The dealkylations of methoxy- (Burke and Mayer, 1983) and ethoxyresorufin (Burke and Mayer, 1974), were determined in the microsomal fraction. The following determinations were carried out in the cytosolic fraction: quinone reductase (NQO1) using 3-(4,-5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as substrate (Prohaska and Santamaria, 1988), glutathione S-transferase activity (Habig et al., 1974) monitored using CDNB as accepting substrate and total glutathione levels (Akerboom and Sies, 1981). Protein concentration was determined in both cellular subfractions using bovine serum albumin as standard (Bradford, 1976). Finally, in order to monitor whether increase in enzyme activities involves, at least partly, a rise in enzyme availability, Western blot analysis was performed. Hepatic microsomal or cytosolic proteins from pooled slices were loaded on to 10% (w/v) SDS-PAGE and then transferred electrophoretically to Hybond-P polyvinylidene difluoride membrane. The immunoblot analysis of rat proteins was carried out by exposure to the primary antibodies followed by the appropriate peroxidase-labelled secondary antibody. Lactate dehydrogenase (LDH) and β-actin antibodies were used as the housekeeping proteins for the cytosolic and microsomal proteins, respectively, to normalize protein loading. Immunoblots were quantitated by densitometry using the GeneTool software (Syngene Corporation, Cambridge, UK), with the control band designated as 100%. Each immunoblot was reproduced on a different occasion.

2.4. Glucosinolate cytotoxicity

LDH release from liver slices into the incubation medium was used as an index of cytotoxicity and was determined employing a cytotoxicity detection kitplus (Roche Diagnostics, Mannheim, Germany). On completion of a 24-h incubation, the culture medium was aspirated and the tissue slices were each homogenised in 1.5 ml of phosphate-buffered saline (PBS), pH 7.4. The media and homogenates were centrifuged at 2000 × *g* × 5 min at 4 °C using a bench centrifuge. Duplicate aliquots (0.1 ml) of each triplicate incubation were used for analysis according to the manufacturer's instructions.

2.5. Statistical analysis

Enzyme activities are presented as mean ± standard deviation of 3 pools, each comprising 10 slices. Statistical evaluation was carried out by one-way ANOVA followed by the Dunnett's test.

3. Results

3.1. Effect of glucosinolates on CYP1 activity and expression

Incubation of rat liver slices with sulforaphane had no effect on the dealkylation of ethoxyresorufin or methoxyresorufin but, in contrast, both activities were up-regulated when the slices were exposed to glucoraphanin, the precursor of sulforaphane; however, this effect was abolished or decreased when myrosinase was added to the incubation mixture with the glucosinolate (Fig. 1). At the apoprotein level, glucoraphanin up-regulated CYP1A1, CYP1A2 and, to a lesser extent, CYP1B1; addition of myrosinase had no major impact on the glucoraphanin-induced changes (Fig. 2). Similarly, erucin did not influence the O-deethylation of ethoxyresorufin and enhanced methoxyresorufin O-demethylase at only one concentration; glucoerucin up-regulated both activities, the effect being more pronounced in the case of ethoxyresorufin; the effect, however, was not observed when the incubation system was supplemented with myrosinase (Fig. 3). Both erucin and glucoerucin elevated the apoprotein levels of CYP1A1, CYP1A2 and CYP1B1 (Fig. 4). Supplementation of the incubation system with myrosinase had no major influence on the glucoerucin-mediated increase of CYP1A1 and CYP1B1, but the effect on CYP1A2 was attenuated.

3.2. Effect of glucosinolates on phase II enzyme activity and expression, and on glutathione levels

The phase II activities glutathione S-transferase and quinone reductase were both increased following exposure of the liver slices to sulforaphane and erucin (Figs. 5 and 6). Their glucosinolate pre-

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