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Up-regulation of cytochrome P450 and phase II enzyme systems in rat precision-cut rat lung slices by the intact glucosinolates, glucoraphanin and glucoerucin

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ABSTRACT

It is believed that the chemopreventive activity of cruciferous vegetables in the lung and other tissues is exclusively the result of exposure to degradation products of glucosinolates, such as the isothiocyanates, and that the parent glucosinolates make no contribution. In the present study, evidence is presented for the first time that, in rat lung, the intact glucosinolates, glucoraphanin and glucoerucin, can modulate carcinogen-metabolising enzyme systems. The glucosinolates were isolated from cruciferous vegetables and incubated (1–25 μM) with precision-cut rat lung slices for 24 h. Both glucosinolates, at concentrations as low as 1 μM, up-regulated the *O*-deethylation of ethoxyresorufin and the apoprotein levels of CYP1A1 and CYP1B1; supplementation of the incubation medium with myrosinase, the enzyme that converts glucosinolates to their corresponding isothiocyanates, abolished the rise in ethoxyresorufin *O*-deethylase activity. In contrast, neither glucosinolate, at the concentrations studied, influenced quinone reductase activity in the lung slices, but addition of myrosinase to the glucosinolate incubations led to a marked rise in activity. Glutathione *S*-transferase activity, monitored using 1-chloro-2,4-dinitrobenzene as the accepting substrate, was elevated in lung slices exposed to glucoraphanin. GSTα protein levels were increased by glucoraphanin and, to a much lesser extent, glucoerucin. It may be concluded that intact glucosinolates can modulate the activity of pulmonary carcinogen-metabolising enzyme systems, and can thus contribute to the documented chemopreventive activity of cruciferous vegetables in the lung.

1. Introduction

Despite the enormous effort expended at discouraging the use of tobacco, lung cancer remains worldwide a leading cause of death [1]. Consequently, additional approaches for reducing lung cancer incidence must be considered, one of which is to employ dietary habits that protect against this disease.

In many epidemiological studies, consumption of cruciferous vegetables has been linked to low cancer incidence at a number of sites including lung [2–7]. The chemopreventive effect of cruciferous vegetables has been attributed to the presence of glucosinolates, which are found in abundance in these vegetables. It is believed that glucosinolates are not bioactive *per se* but following exposure to the enzyme myrosinase (β-thioglucoside glucohydrolase), which comes into contact with these compounds during the harvesting, chopping and mastication of these vegetables, they generate metabolites such as isothiocyanates that are responsible for the chemopreventive activity. Moreover, microbial myrosinase activity in the human intestine appears to contribute to the release of isothiocyanates from their glucosinolate precursors. Indeed, in rodents, isothiocyanates afforded protection against chemical carcinogens, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and polycyclic aromatic hydrocarbons, attenuating tumorigenesis in a number of tissues including lung [8–12]. Moreover, the malignant progression of lung adenomas induced by a mixture of benzo[a]pyrene and NNK was inhibited by isothiocyanates [13].

A number of mechanisms, affecting both initiation and post-initiation stages of cancer appear to contribute to the chemopreventive activity of isothiocyanates. One of the most important is modulation of the metabolism of chemical carcinogens leading to impaired generation of genotoxic metabolites. This can be achieved either through impairment of the cytochrome P450-mediated generation and/or increased detoxification of these metabolites. Indeed, isothiocyanates, having either an aliphatic or an aromatic side chain, could modulate both cytochromes P450 and phase II

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conjugation systems in the liver and other tissues following oral administration to rats at dose levels simulating human dietary intake [14–16]. Moreover, in extensive *in vitro* studies performed using precision-cut rat liver and lung slices isothiocyanates could modulate these enzyme systems at concentrations as low as 0.5 μM [17–21].

The view has prevailed that the chemopreventive activity of glucosinolates is exclusively mediated by myrosinase-generated metabolites such as isothiocyanates; 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 [22]; moreover, glucoraphanin could be metabolically reduced to another glucosinolate, glucoerucin. These observations prompted us to investigate whether intact glucosinolates had the potential to influence carcinogen-metabolising enzyme systems in the lung, a major target tissue for cruciferous vegetables and isothiocyanates. These studies were conducted *in vitro* utilising precision-cut lung slices [23,24]. The principal advantages of this *in vitro* system are that it maintains tissue architecture and cell–cell communication, and allows the facile use of a wide range of concentrations of chemicals, that would otherwise necessitate large animal numbers for commensurate *in vivo* studies [25]. This *in vitro* system is particularly suited to the lung, a heterogeneous tissue composed of many different cell types that differ markedly in their carcinogen-metabolising enzyme profile [26]. Studies emanating from our laboratory have established that precision-cut slices are an appropriate system for use in evaluating the potential of xenobiotics to modulate cytochrome P450 and phase II activities in the lung [18,27].

2. Materials and methods

Sulforaphane and erucin (LKT Laboratories, MN, USA), NADPH, ethoxyresorufin, 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) 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).

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 [28]. 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 [24]. 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 GL were pooled and freeze-dried [29]. 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 [30]. The purities were in the range of 95–99%. The enzyme myrosinase (β-thioglucoside glucohydrolase, E.C.3.2.1.147) was isolated from seeds of *Sinapis alba* L. as described by Pessina et al. [31] 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 minute of at pH 6.5 and 37 °C.

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:12-h dark cycle with light onset at 07:00 h. Rat lung slices (400–600 μm) were prepared from 8 mm cylindrical cores using a Krumdieck tissue slicer (Alabama Research and Development Corporation, Munsford, AL, USA) as previously described [23]. The multiwell plate procedure, using 12-well culture plates, was used to culture the slices in the presence of glucosinolate (0–25 μM), isothiocyanate (0–25 μM) or glucosinolate (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 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 lung slices, were used per concentration.

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 dealkylation of ethoxyresorufin [32] was determined in the microsomal fraction. The following determinations were carried out in the cytosolic fraction: quinone reductase (NQO1) using MTT [3-(4,-5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide] as substrate [33], glutathione *S*-transferase activity [34] monitored using CDNB as accepting substrate, and total glutathione levels [35]. Protein concentration was determined in both cellular subfractions using bovine serum albumin as standard [36]. Finally, in order to monitor changes in enzyme protein expression, Western blot analysis was performed. Hepatic microsomal or cytosolic proteins 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 and β-actin were used as the housekeeping proteins for the cytosolic and microsomal proteins respectively. Immunoblots were quantitated by densitometry using the GeneTool software (Syngene Corporation, Cambridge, UK), with the control band designated as 100%.

In order to evaluate the viability of lung slices in the presence of glucoerucin or glucoraphanin, lactate dehydrogenase (LDH) release from the slices into the incubation medium was used as an index of cytotoxicity, and was determined employing a cytotoxicity detection kit^{plus} (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* for 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.

Results are presented as Mean ± Standard Deviation of 3 pools, each comprising 10 slices. Statistical evaluation was