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Research Article

The aliphatic isothiocyanates erucin and sulforaphane do not effectively up-regulate NAD(P)H:quinone oxidoreductase (NQO1) in human liver compared with rat

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Isothiocyanate up-regulation of hepatic NAD(P)H:quinone oxidoreductase (NQO1) and glutathione *S*-transferases (GSTs) is an integral mechanism of their chemoprevention. In this paper, for the first time, the potential of the isothiocyanates erucin and sulforaphane to modulate these enzymes was investigated in two human livers and compared to rat liver. Precision-cut liver slices were incubated with erucin or sulforaphane (1 – 50 μM). Both isothiocyanates elevated NQO1 activity in rat slices that was paralleled by a fourfold rise in protein levels. No change in activity was noted in human slices, and only a weak rise in protein levels, < 10% of that in rat, was observed in only one of the human livers, whereas the other was refractive. GST activity, assessed with three substrates, was elevated in rat slices treated with either isothiocyanate, and was accompanied by a rise in GSTα and GSTμ, but not GSTπ, protein levels. A rise in activity and in GSTα and GSTμ protein levels was also noted in one of the human livers. It appears that erucin and sulforaphane elevate GST expression in isoform-specific manner in both rat and human liver, whereas NQO1 is inducible by these compounds only in rat liver and very poorly in human liver.

Keywords: Chemoprevention / Erucin / Isothiocyanates / Quinone reductase / Sulforaphane

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1 Introduction

Isothiocyanates are a class of dietary chemopreventive phytochemicals that are found in abundance in *Brassica* vegetables, where they exist as glucosinolates [1]. The enzyme myrosinase catalyses the breakdown of the glucosinolate to the corresponding isothiocyanate, which is then rapidly absorbed, achieving good bioavailability as for example in the case of sulforaphane [2]. This enzyme is present in the plant and comes into contact with the glucosinolate when the plant tissue is damaged, *e.g.* during harvesting, food preparation and mastication; however, bacterial myrosinase in the intestine also contributes to the generation of isothiocyanates [3].

Although multiple mechanisms are likely to be involved in the chemopreventive activity of isothiocyanates, there is strong evidence to indicate that these compounds are capable of influencing the enzyme systems involved in the metabolism of chemical carcinogens, even at the low dietary levels of intake [4, 5], so as to limit the generation of reactive intermediates that can form DNA adducts. For example, pre-treatment with phenethyl isothiocyanate decreased the unscheduled DNA synthesis in buccal mucosal fragments of hamsters following exposure to *N*-nitrosomethylbenzylamine [6] and formation of benzo[*a*]pyrene-induced DNA and protein adducts in A/J mice [7]. The same isothiocyanate reduced the levels of DNA and protein adducts formed by 2-amino-1-methyl-6-phe-

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Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; **CNBOD,** 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; **DCNB,** 1,2-dichloro-4-nitrobenzene; **EBSS,** Earle's balanced salt solution; **GST,** glutathione *S*-transferase; **NQO1,** NAD(P)H:quinone oxidoreductase

nylimidazo[4,5-*b*]pyridine [8]. Sulforaphane inhibited the genotoxicity and DNA-adduct formation of heterocyclic amines in cell cultures *in vitro* [9, 10]. Finally, isothiocyanates administered to laboratory animals prior to or around the time of treatment with chemical carcinogens, such as azoxymethane, benzo[*a*]pyrenene, 4-methylnitrosamino-1-(3-pyridyl)-1-butanone, dibenz[*a*,*h*]anthracene, 5-methylchrysene and *N*-nitrosomethylbenzylamine have been shown to effectively suppress tumourigenesis at different organ sites [11 – 14]. Collectively the above data illustrate that isothiocyanates can interfere to suppress the initiation stage of carcinogenesis.

Mechanisms established in animal models, almost always rodents, are extrapolated to the human situation to explain the chemopreventive effects of these compounds recognised in epidemiological studies. Such extrapolation makes the crucial assumption that humans respond to isothiocyanate exposure in a similar fashion as animal models. In this paper, we have investigated the effect of isothiocyanates on NAD(P)H:quinone oxidoreductase (NQO1) and glutathione *S*-transferases (GSTs) in human liver, in comparison with rat liver, as this is believed to be a pivotal mechanism through which isothiocyanates anatagonise the carcinogenicity of chemicals; these enzymes enable the cell to detoxify genotoxic intermediates of carcinogens [13, 15]. To achieve this objective, precision-cut liver slices from rats and humans were incubated, under identical conditions, with a range of concentrations of isothiocyanates for 24 h, and the expression of these enzymes at the protein and activity level was determined at the end of this period. Two structurally related dietary isothiocyanates, namely sulforaphane and erucin, both having an aliphatic side-chain were employed in this study. To the best of our knowledge this is the first study to evaluate the modulation of carcinogen-metabolising enzyme systems by isothiocyanates in whole human liver tissue.

2 Materials and methods

2.1 Materials

Sulforaphane and erucin (LKT Laboratories, Minnesota, USA), 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), glutathione reductase, peroxidase-linked anti-rabbit and anti-goat antibodies raised in rabbits (Sigma, Poole, Dorset, UK), 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (CNBOD) (Fluka, Buchs SG, Switzerland), 7-benzyloxyquinoline and 7-hydroxyquinoline (BD Biosciences, CA, USA), and Earle's balanced salt solution (EBSS), foetal calf serum, gentamycin and RPMI with L-glutamine culture media (Gibco-BRL Life Technologies, Paisley, Scotland) were all purchased. Twelve-well plates were obtained from Bibby Sterilin (Helena Biosciences, Sunderland, UK). Antibodies were obtained from BD Biochemicals (Oxford, UK), antibodies to human GST P1-1, A1-1 and M1-1 (Calbiochem, Merck, UK) and antibody to human NQO1 (abcam Cambridge, UK) were similarly purchased.

2.2 Preparation and incubation of slices

This study was conducted with the approval of the Ethics committee. Liver sections from two human cadaveric livers that could not be used for transplantation purposes were obtained from the UK Human Tissue Bank (The Innovation Centre, Oxford Street, Leicester, UK). Both donors died following lethal brain injury. Donor 1 was a 26-year-old male smoker who was maintained on ventilation for 4 days prior to death, whereas Donor 2 was a 54-year-old non-smoking female who was maintained on artificial ventilation for 2 days prior to death; both were Caucasian. Donor 1 was a schizophrenic who had taken ecstasy and cannabis in the past and was a binge drinker, whereas Donor 2 did not take any drugs. Liver sections were transported in cold University of Wisconsin (UW) preservation solution on ice. The time interval from the moment of donors' death to the beginning of the incubation did not exceed 12 h on both occasions. On receipt, the liver sections were immediately transferred into a sterile container and, after the UW solution was carefully decanted, were washed 3 – 4 times with culture medium. Male Wistar albino rats (200 – 250 g) were purchased from Bantin and Kingman (B&K) Universal (Hull, 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. Rats were sacrificed by cervical dislocation, and liver was immediately excised and placed in ice-cold EBSS enriched with D-glucose (25 mM) and gassed with 95% O_2/5% CO_2 for 1 h. Tissue cylinders (8 mm in diameter) were cut from the liver lobes with a hand-held 8 mm diameter coring tool (Vitron, Tuscan, USA); tissue was kept submerged in ice-cold EBSS throughout the procedure. Slices were cut (200 – 300 μm thickness) from the tissue core using a pre-chilled Krumdieck tissue slicer (Alabama Research and Development Corporation, AL, USA) [16]. Slices were then transferred into 12-well culture plates, with one slice being placed in each well, in 1.5 mL of culture medium, and maintained at 37°C in an incubator equipped with an orbital shaker (Stuart orbital shaker, Barloword Scientific, Staffordshire, UK), in a humidified atmosphere containing 5% CO_2 (Galaxy B CO_2 incubator, Scientific Laboratory Supplies, Nottingham, UK). Liver slices were initially pre-incubated for 30 min. under these conditions in order to allow the viable tissue to slough the damaged cells. Finally, tissue slices were transferred to a fresh culture medium containing either erucin or sulforaphane or the vehicle DMSO, and were incubated further for 24 h. The final concentration of DMSO was equal in all treatment groups and did not exceed 0.5% v/v. At the end of the incubation period, the slices were removed from the culture medium, briefly rinsed in

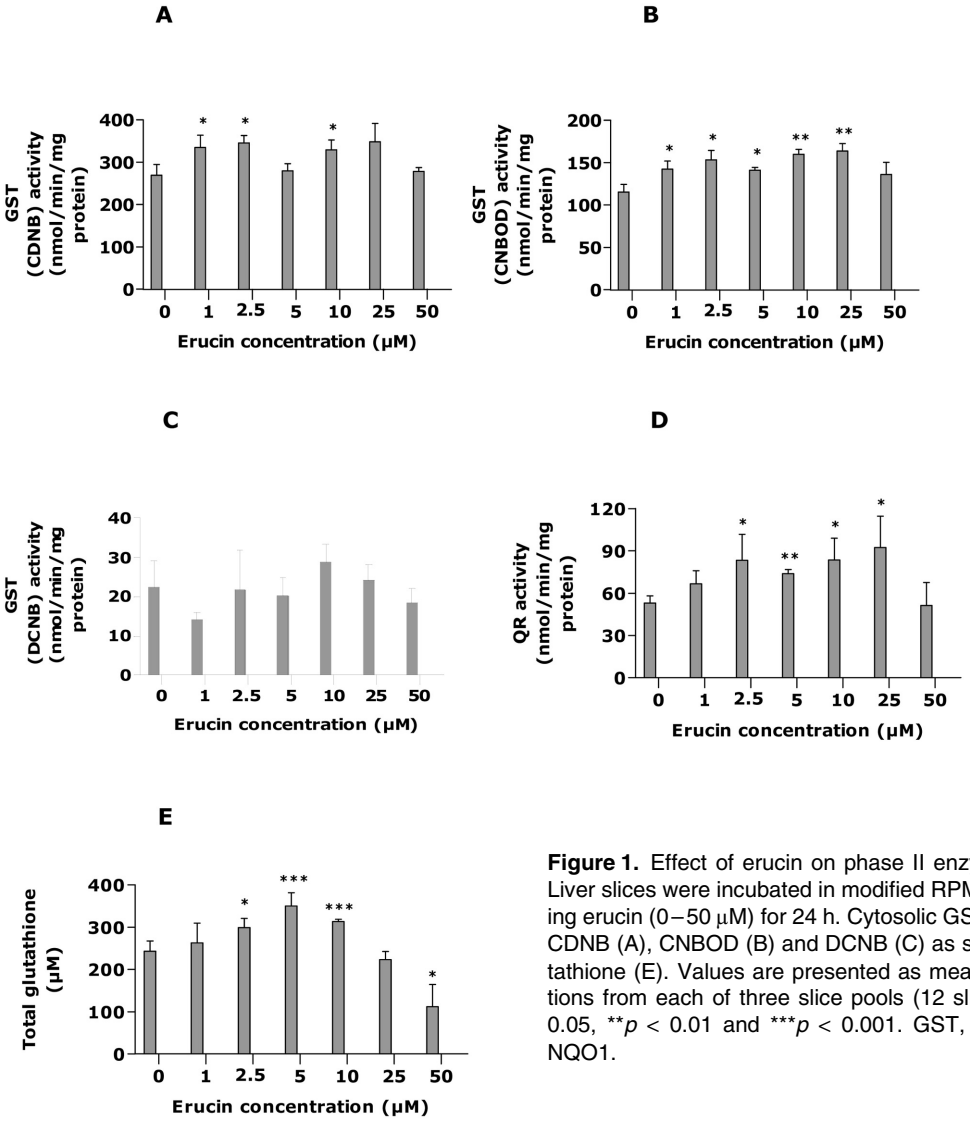

Figure 1. Effect of erucin on phase II enzyme activities in rat liver slices. Liver slices were incubated in modified RPMI 1640 culture medium containing erucin (0–50 μM) for 24 h. Cytosolic GST activity was determined using CDNB (A), CNBOD (B) and DCNB (C) as substrates, QR (D) and total glutathione (E). Values are presented as means ± SD of duplicate determinations from each of three slice pools (12 slices/mL) per concentration. *p< 0.05, **p < 0.01 and ***p < 0.001. GST, glutathione *S*-transferase; QR, NQO1.

KCl (0.154 M) containing Tris (50 mM, pH 7.4) and homogenised in KCl–Tris buffer (12 slices in 1.2 mL). Human liver slices were prepared as with rat liver and pre-incubated in the same way.

Cytosolic fractions were prepared by differential centrifugation of the homogenised slices, and were stored at –80°C until use. The following assays were carried out: NQO1 using MTT [3-(4,–5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide] as substrate [17], GST activity using as accepting substrates CDNB, DCNB [18] and CNBOD [19], and total glutathione [20]. Protein concentration was determined using BSA as standard [21]. Finally, in order to determine changes in enzyme protein expression, hepatic cytosolic proteins were resolved by electrophoresis and incubated with the primary antibody and the corresponding peroxidase-linked secondary antibody. Immunoblots were quantitated by densitometry using the GeneTool software (Syngene Corporation, Cambridge, UK).

LDH release from liver slices into the incubation medium was used as an index of cytotoxicity. The LDH concentration was measured employing a cytotoxicity detection kit ^{plus} (Roche Diagnostics, Mannheim, Germany), using three slices per concentration of isothiocyanate. On completion of a 24-h incubation, the culture medium was aspirated and the tissue slices were each homogenised in 1.5 mL of 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) were used for analysis according to the manufacturer's instructions.

Statistical evaluation was carried out using the Student's *t*-test.

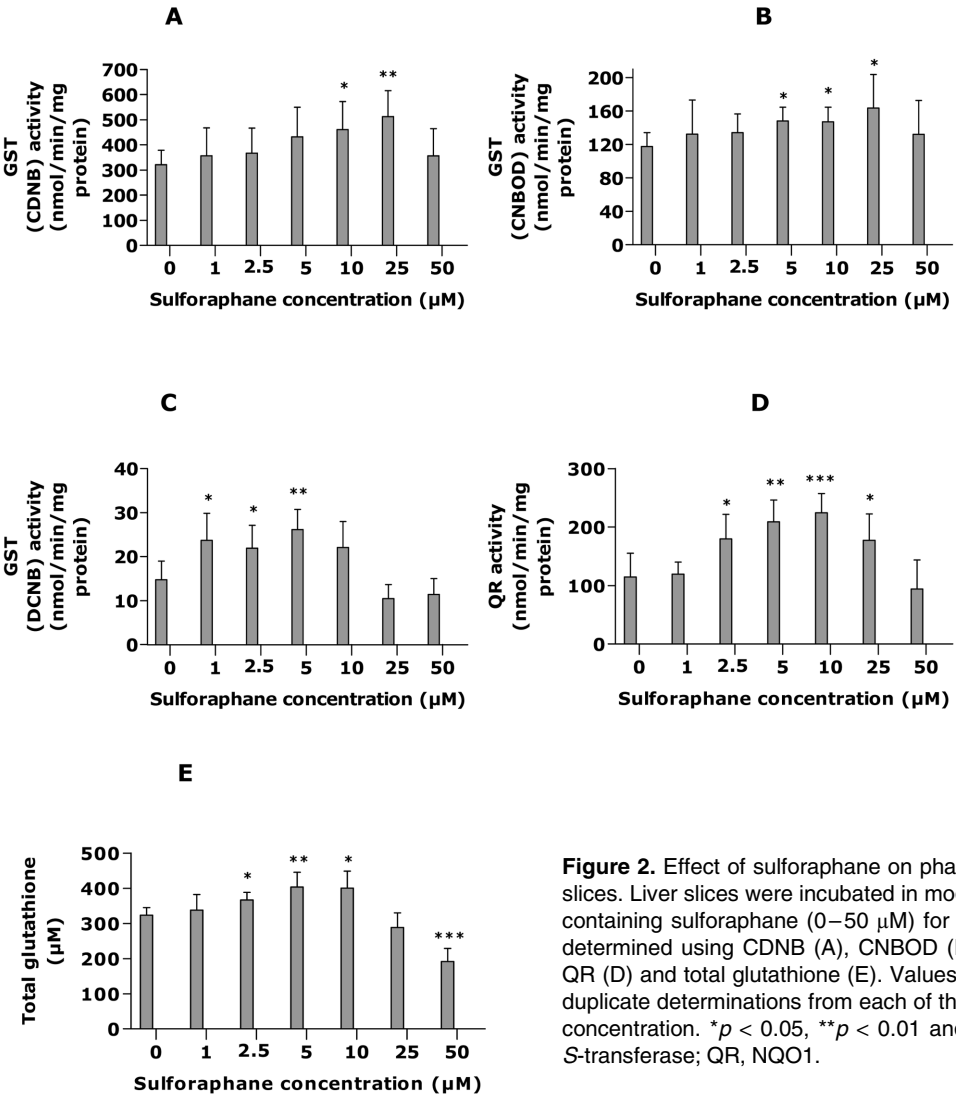

Figure 2. Effect of sulforaphane on phase II enzyme activities in rat liver slices. Liver slices were incubated in modified RPMI 1640 culture medium containing sulforaphane (0–50 μM) for 24 h. Cytosolic GST activity was determined using CDNB (A), CNBOD (B) and DCNB (C) as substrates, QR (D) and total glutathione (E). Values are presented as means ± SD of duplicate determinations from each of three slice pools (12 slices/mL) per concentration. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. GST, glutathione *S*-transferase; QR, NQO1.

3 Results

Incubation of rat liver slices for 24 h with either erucin (Fig. 1) or sulforaphane (Fig. 2) led to a statistically significant rise in NQO1 activity; at the highest concentration used (50 μM) enzyme activity returned to control levels in both cases. Neither compound had any significant effect when human slices from Donor 1 were incubated under identical conditions (Fig. 3); activity was below the detection limit in the case of Donor 2. Western blot analysis established that in rat liver slices the rise in NQO1activity was paralleled by a similar increase in enzyme levels, with sulforaphane being the more potent of the two isothiocyanates, giving rise to nearly a 500% rise in protein levels, twice that of erucin (Fig. 4). When immunological analysis was conducted using human hepatic cytosol, neither compound caused an increase in protein levels in Donor 1, whereas in Donor 2 a very modest increase (30–40%) in protein levels was evident (Fig. 5), markedly lower than observed in rat slices.

Cytosolic GST activity was determined using CNBOD, CDNB or DCNB as accepting substrates. When CDNB or CNBOD served as substrates, both isothiocyanates caused a modest, but statistically significant, increase in activity, with erucin being more effective (Figs. 1 and 2); when DCNB was employed, only sulforaphane elevated GST activity. When human slices from Donor 1 were incubated with the isothiocyanates, neither compound had an effect on activity when CDNB or CNBOD were used to monitor activity, but both compounds caused an increase when the slices emanated from Donor 2 (Fig. 3). GST activity could not be detected in either human liver sample when DCNB was used as substrate. At the protein level, following incubation of rat slices, both isothiocyanate elevated moderately GSTα and GSTμ, but there was no effect on GSTπ (Fig. 4).

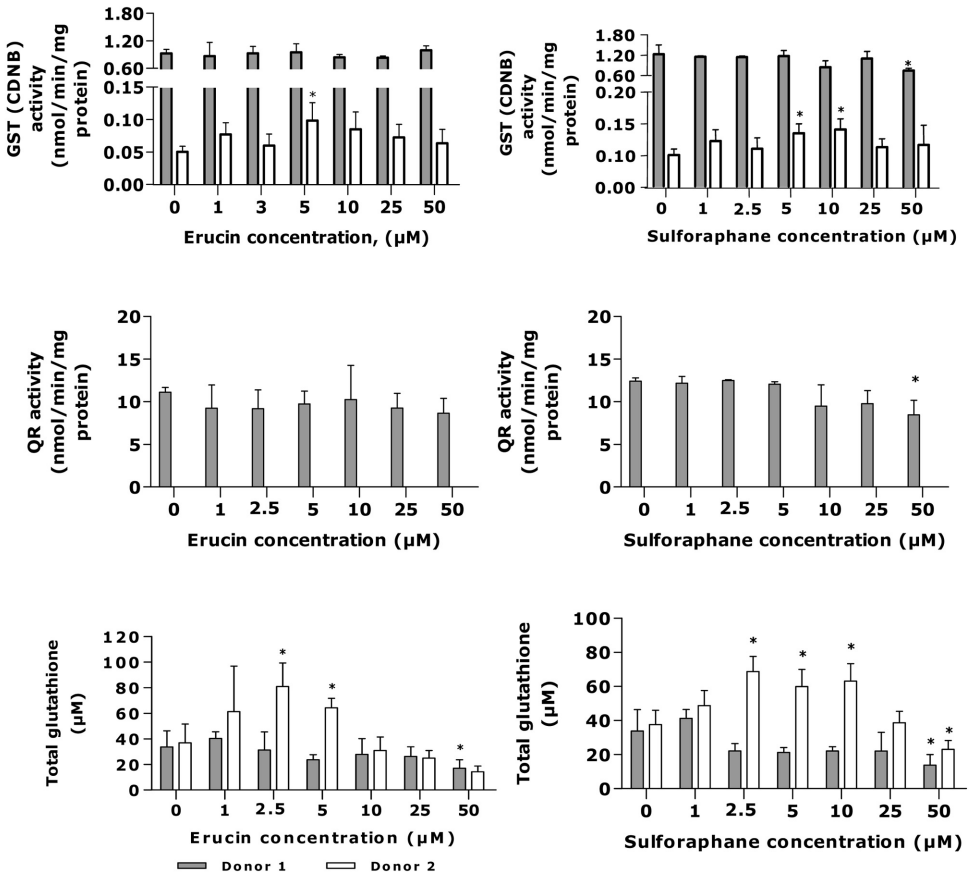

Figure 3. Effect of erucin and sulforaphane on phase II enzyme activities in human liver slices. Liver slices from two human donors were incubated in modified RPMI 1640 culture medium containing either erucin or sulforaphane (0–50 μM) for 24 h. The activities of GST, with CDNB or CNBOD as substrate, QR and total glutathione content were determined in the cytosolic fraction. Values are presented as means ± SD of duplicate determinations from each of three slice pools (12 slices/mL) per concentration. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. GST, glutathione *S*-transferase; QR, NQO1.

When human hepatic cytosol was used, a similar picture emerged with Donor 2 when slices were incubated with erucin, but in the case of Donor 1 no change in enzyme protein levels was evident (Fig. 5). Sulforaphane increased expression of GSTα and GSTμ in Donor 2, but no consistent, concentration dependent, rise was seen in GSTπ. Once again no response was seen in the liver from Donor 1.

Exposure of rat liver slices to erucin or sulforaphane elevated the levels of cytosolic glutathione; at the highest concentration (50 μM); however, a marked decrease in levels was observed (Figs. 1 and 2). A very similar picture emerged with the human liver from Donor 2 (Fig. 3). In human liver from Donor 1, however, no increase in glutathione concentration was seen following exposure to either isothiocyanate, but a decrease was still manifested at the 50 μM concentration (Fig. 3).

Finally, no increased leakage of LDH was observed when rat liver slices were incubated with concentrations of erucin or sulforaphane up to 25 μM, but a statistically significant rise was noted at 50 μM that increased further when the concentration was raised to 100 μM (Fig. 6).

4 Discussion

A major mechanism through which isothiocyanates are believed to exert their chemopreventive effect is by limiting the availability of the reactive intermediates of carcinogenic compounds that would otherwise interact covalently with DNA to form adducts. This is achieved by suppressing their generation, through impairment of cytochrome P450-mediated activation, and by encouraging their detoxication by up-regulating protective enzyme systems such as NQO1 and GSTs [13]. However, at low levels of exposure, which mimic dietary levels of intake, it appears detoxication of the reactive intermediates is the dominant mechanism, at least in rat [4]. It is assumed that similar mechanisms are operative in humans exposed to these compounds.

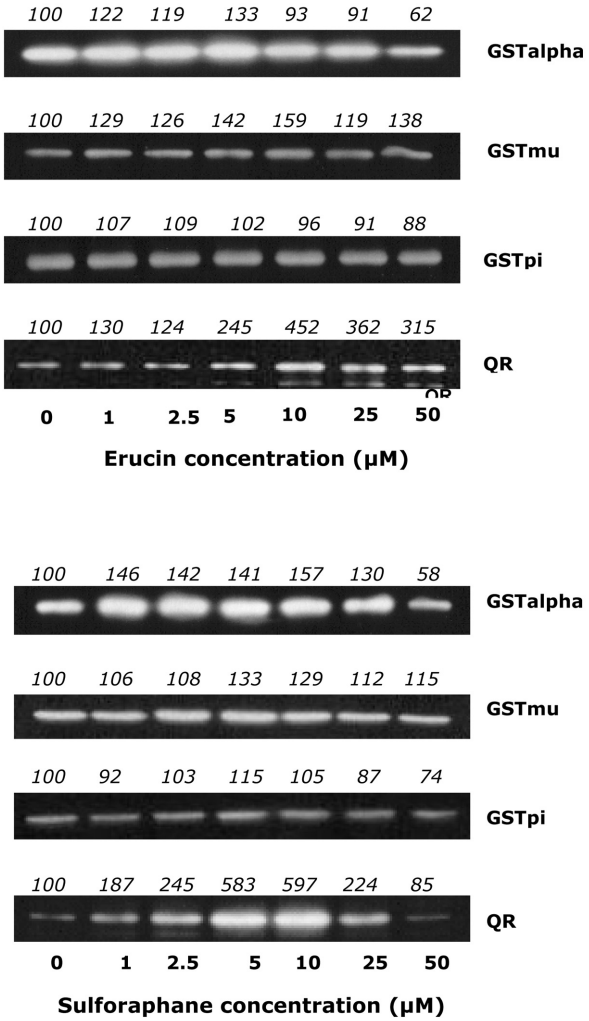

Figure 4. Modulation of rat hepatic phase II enzyme expression by treatment with erucin and sulforaphane. Liver slices were incubated in modified RPMI 1640 culture medium containing either erucin or sulforaphane (0 – 50 μM) for 24 h. Hepatic cytosolic proteins were resolved by 10% w/v SDS-PAGE and then transferred electrophoretically to Hybond-P polyvinylidene difluoride membranes. Immunoblot analysis was carried out using anti-rat antibodies to phase II enzymes, followed by the appropriate peroxidase-labelled secondary antibody. Each lane was loaded with 10 (GSTα and GSTμ) or 40 μg (GSTπ and QR) of total protein. Molecular markers were run concurrently. The values in italics show OD percentage of each band relative to control. GST, glutathione *S*-transferase; QR, NQO1.

The use of precision-cut tissue slices allows for the first time an investigation of the effects of isothiocyanates on carcinogen-metabolising enzyme systems in human tissue, in comparison to rat, following exposure to these compounds under identical conditions. The many advantages of precision-cut tissue slices over other *in vitro* systems such as cell lines and subcellular fractions have been highlighted in a number of reviews [22, 23]. We have already established that both rat and human liver slices can be used to evaluate the potential of chemicals to up-regulate the activities of enzyme systems such as the cytochromes P450 and GSTs [24 – 26].

Both human and rat liver slices were incubated with the isothiocyanates for 24 h, this being the period required to achieve maximal induction [24]. In the studies with the rat slices, erucin and sulforaphane behaved in a similar way, indicating that the oxidation state of sulphur has little impact on the ability of these isothiocyanates to modulate carcinogen-metabolising systems. Both compounds up-regulated NQO1 activity, an enzyme that facilitates the detoxication of quinones, such as those generated by the oxidation of polycyclic aromatic hydrocarbons [27], and concords with observations made *in vivo* [4, 5]. Moreover, the enzyme protein levels were markedly elevated. However, when human liver slices from two different donors were used, no increase in activity was noted and only in one of the two human livers studied was a weak rise in protein levels noted, in marked contrast to the observations made in rat. These findings suggest that human NQO1 activity may be resistant to induction by these aliphatic isothiocyanates. Collectively, these data point to an important species difference between rat and human with implications for the chemopreventive activity of theses isothiocyanates, as it would imply that chemopreventive potential may be less pronounced in human compared with rat. It is, however, conceivable that the rate of metabolism of isothiocyanates by rat and human liver slices differs substantially, so that effective concentrations during incubation are different, and this possibility merits investigation. In addition, the presence of a polymorphism in the up-regulation of this enzyme in humans cannot be discounted. A more definitive conclusion would require the analysis of more human liver samples.

GST activity was monitored using three substrates. CDNB is a substrate for a number of the cytosolic transferases, whereas DCNB is a substrate associated with the μ-family and CNBOD has been reported to be selective for the α-class [19, 28]. Both compounds stimulated this activity in rat liver when CDNB and CNBOD served as substrates, but when DCNB was used only sulforaphane stimulated activity, implying a rise in the μ- and α-class enzymes; immunological studies revealed modest rises in the protein levels of these classes, but not in the π-class. When rats were treated *in vivo* with low doses of erucin, no increase in activity was achieved but at the protein level it up-regulated the levels of GSTα and GSTμ, whereas GSTπ was unaffected [5], the latter in agreement with the current studies in precision-cut slices; it is relevant to point out that following oral administration of sulforaphane to rats, at the dietary dose of 0.5 mg/kg, the peak plasma concentrations achieved were <0.3 μM which is below the lowest concentration employed in the present study [2]. When human liver slices were investigated, GST activity was not detect-

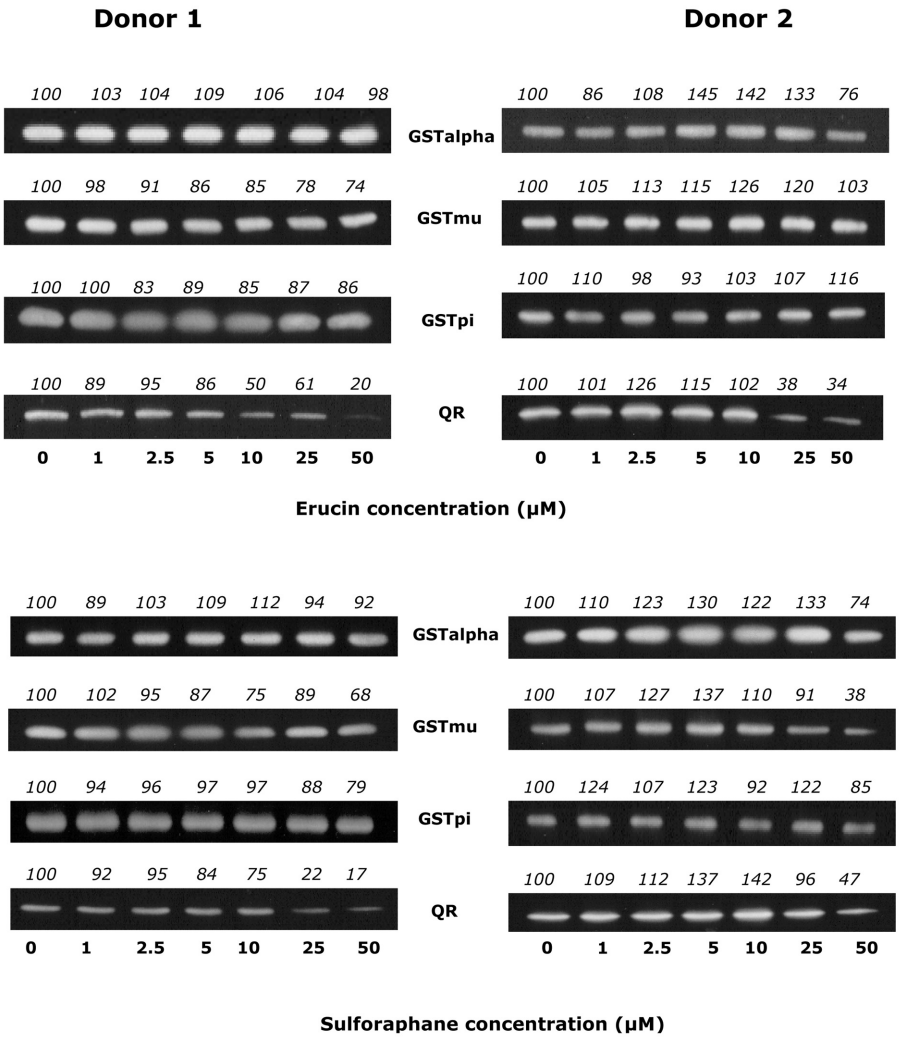

Figure 5. Modulation of hepatic phase II enzyme expression by erucin and sulforaphane in human liver slices. Liver slices from two human livers were incubated in modified RPMI 1640 culture medium containing either erucin or sulforaphane (0–50 μM) for 24 h. Hepatic cytosolic proteins were resolved by 10% w/v SDS-PAGE and then transferred electrophoretically to Hybond-P polyvinylidene difluoride membranes. Immunoblot analysis was carried out using either rat antibodies to phase II enzymes, followed by the appropriate peroxidase-labelled secondary antibody. Each lane was loaded with 10 (GSTs) or 40 μg (QR) of total protein. Molecular markers were run concurrently. The values in italics show percentage of OD of each band relative to control. GST, glutathione *S*-transferase; QR, NQO1.

able when DCNB was used as substrate; even in rat slices the lowest activity was obtained with this substrate. It is pertinent to emphasise that incubation of liver slices for 24 h leads to the loss of GST activity, particularly when assessed using DCNB [29], such that it may drop below the levels of detection. The response seen in the two human livers following incubation with the two isothiocyanates differed. Neither compound modulated GST activity, whatever the substrate used, or following Western blot analysis when studies were performed with liver slices from Donor 1. In the liver from Donor 2, however, both isothiocyanates elevated GST activity moderately, accompanied by similar rises in the levels of immunologically determined GSTα and GSTμ with no similar concentration-dependent changes in GSTπ, in keeping with observations made in rat liver slices. The varied response obtained in the two human livers may be attributed to either genetic differences or to factors linked to the time of liver removal relative to the time of death and/or transport of the tissue. We believe that the latter is unlikely as, in fact, the basal hepatic GST activity in Donor 1 was much higher than that of Donor 2. Sex, age and medical history may be also contributors to the difference in response. Clearly, more human livers need to be analysed for a clearer picture to emerge. Similarly, both compounds increased total glutathione levels in rat liver but only in human liver from Donor 2. The ability of isothio-

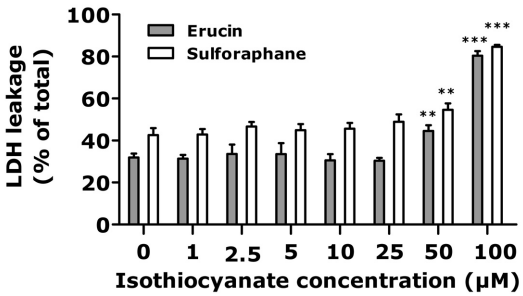

Figure 6. LDH leakage from rat liver slices incubated with erucin or sulforaphane. LDH leakage was determined in liver slices incubated in modified RPMI 1640 medium containing either erucin or sulforaphane (0 – 100 μM) for 24 h. LDH release is expressed as percentage of total LDH. Values are presented as means ± SD of duplicate measurements from each of three slices per concentration. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

cyanates to enhance glutathione synthesis in animals has already been documented with sulforaphane [30], but this is the first time that this has been demonstrated with erucin.

A general observation was that at the highest isothiocyanate concentration used (50 μM), the increases in activity of the various enzymes observed in rat liver at lower concentrations were no longer evident. It is conceivable that this may be the result of toxicity to the slices, which is supported by the observation that at this and also at higher concentrations an increased leakage of lactate dehydrogenase was seen. It is likely that this toxicity is related to the depletion of glutathione that occurs at this concentration, presumably the consequence of utilisation of the tripeptide in the metabolism of the isothiocyanate, this being their principal pathway of metabolism [31]. Of importance is the observation that such depletion of glutathione was also evident in human liver slices following incubation with 50 μM of either isothiocyanate.

In conclusion, it was demonstrated that erucin and sulforaphane elevate GST expression in isoform-specific manner in both rat and human liver. However, NQO1, either at the activity or protein level, is inducible by these compounds only in rat, and very poorly, or not at all, in human liver.

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The authors have declared no conflict of interest.

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