RESEARCH ARTICLE

Differential response of four human livers to modulation of phase II enzyme systems by the chemopreventive phytochemical phenethyl isothiocyanate

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A principal mechanism of the chemopreventive activity of isothiocyanates is detoxification of the genotoxic metabolites of chemical carcinogens through up-regulation of enzymes such as quinone reductase and the glutathione-*S*-transferases. In this study we report, for the first time, the potential of the aromatic isothiocyanate, phenethyl isothiocyanate (PEITC) to modulate these enzymes in human liver from four donors, in comparison with rat liver. Precision-cut human and rat liver slices were incubated with PEITC at concentrations that can be achieved in plasma following dietary intake. Glutathione-*S*-transferase activity increased in rat slices whereas in human slices activity rose only in three of the four donors. At the protein level, a marked rise in GSTα was seen in one of the human donors whereas much less pronounced elevation was noted in the other three. Quinone reductase activity doubled in rat liver slices incubated with PEITC, and was accompanied by an increase in protein expression. Only in one of the human donors was activity and expression of quinone reductase elevated. These studies illustrate that there are very pronounced differences in the response of human liver to PEITC, indicating that the chemopreventive effect of isothiocyanates may not be manifested in all individuals.

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1 Introduction

Glucosinolates are present in cruciferous vegetables, such as broccoli, cauliflower, and watercress, in substantial amounts resulting in an average daily human intake of about 14.5 mg/day [1]. When these vegetables are disturbed, for example when they are harvested, chopped, or chewed, the enzyme myrosinase (β-thioglucoside glucohydrolase) comes into contact with the glucosinolates and catalyses their breakdown to bioactive molecules such as the isothiocyanates [2]; microbial myrosinase in the human intestine also contributes to the generation of isothiocyanates from glucosinolates [3]. The chemopreventive potential of isothiocyanates has been demonstrated in laboratory studies conducted in animal models of cancer, where isothiocyanates antagonised the carcinogenicity of chemicals [4–6]. Moreover, a number of epidemiological studies have been published showing good inverse relationship between cruciferous vegetable consumption and cancer incidence [4, 6, 7].

One such isothiocyanate is phenethyl isothiocyanate (PEITC), the principal source of which is watercress where it is present as the glucosinolate gluconasturtiin. PEITC afforded protection against chemically induced carcinogenesis induced by nitrosocompounds and polycyclic aromatic hydrocarbons [8–12]. Similarly, exposure to PEITC led

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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; **NQO1,** quinone reductase; **PEITC,** phenethyl isothiocyanate

to a decrease in DNA-adduct level formation in the liver, colon, and prostate of rats treated with the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, a food carcinogen [13], indicating that it modulates favourably the initiation stage of carcinogenesis. A major mechanism of the chemopreventive potential of isothiocyanates is suppression of the availability of the metabolically generated genotoxic intermediates of chemical carcinogens [14]. This is achieved by impairment of their cytochrome P450-catalysed bioactivation and/or stimulation of their detoxification by phase II enzyme systems such as glutathione-*S*-transferases and quinone reductase (NQO1). In studies conducted in our laboratory, PEITC could modulate rat cytochrome P450 and phase II activities in the liver and other tissues following exposure to low doses that simulate human dietary levels of intake [15].

Studies in precision-cut liver slices employing the aliphatic isothiocyanates sulforaphane and erucin indicated that changes in phase II conjugation enzymes observed in rat liver could not be readily extrapolated to humans. In precision-cut rat liver slices, both of these isothiocyanates elevated NQO1 levels and markedly increased enzyme protein levels, whereas in two human livers no increase in activity was observed, and enzyme protein levels rose only moderately [16]. Similarly, when the potential of PEITC to alter hepatic cytochrome P450 composition was evaluated, differences were noted between rat and human [17]. As a result, this study was undertaken to evaluate the ability of the aromatic PEITC to influence the activity and expression of phase II enzyme systems in precision-cut liver slices from four human donors, in comparison with rat, in order to enable its evaluation as a chemopreventive agent in humans.

2 Materials and methods

PEITC [*CAS* 2257-09-02], 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), glutathione reductase, peroxidase-linked anti-rabbit (Sigma, Poole, Dorset, UK), and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (CNBOD) (Fluka, Buchs SG, Switzerland) were all purchased. Antibody to human NQO1 was obtained from Abcam (Cambridge UK), and antibodies to GSTα, GSTπ and GSTμ from Calbiochem (Lutterworth, UK).

Male Wistar albino rats (200 g) were obtained from B&K Universal (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. Liver sections from four 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). Pertinent information about the human donors has already been published [17]. The time interval from the moment of donors' death to the beginning of the incubation did not exceed 12 h on all occasions. On receipt, the liver sections were immediately transferred into a sterile container and, after the UW transport solution was carefully decanted, were washed 3–4 times with culture medium.

Rat and human liver slices (250 μm) were prepared from 8 mm cylindrical cores using a Krumdieck tissue slicer (Alabama Research and Development Corporation, Munsford, AL, USA) as previously described [18]. The multiwell plate procedure, using 12-well culture plates, was used to culture the slices. 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 ten rat or human 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, cytosol was isolated by centrifugation (105 000 × *g*; 1 h), and the following assays were performed in the cytosol; NQO1 using MTT [3-(4,-5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide] as substrate [19], glutathione-*S*-transferase activity [20] monitored using as accepting substrates CDNB, DCNB, and CNBOD, glutathione reductase [21] and total glutathione levels [22]. Protein concentration was determined using bovine serum albumin as standard [23]. Finally, in order to determine changes in enzyme protein expression, cytosolic proteins were resolved by electrophoresis and incubated with the primary antibody and the corresponding peroxidase-linked secondary antibody. Immunoblots were quantified by densitometry using the GeneTool software (Syngene Corporation, Cambridge, UK).

Statistical evaluation was carried out by one-way ANOVA followed by the Dunnett's test. Activities are presented as mean ± SD of three pools, each comprising ten slices.

3 Results

Exposure of precision-cut rat liver slices to various concentrations of PEITC for 24 h resulted in modest elevation of glutathione-*S*-transferase activity when CDNB or DCNB was used as substrates to monitor activity (Fig. 1). The increase in activity was seen at the lower PEITC concentrations, and no statistically significant change was evident at the higher concentrations; in fact, activity was suppressed when CDNB was used as substrate. No major changes were observed in the protein levels of GSTα and GSTμ (Fig. 2). Total glutathione levels were not influenced by PEITC, except for a decrease at the highest concentration (50 μM) (Fig. 1). Glutathione reductase was unaffected by the PEITC treatment except for a decline in activity at the highest concentration of the isothiocyanate. A marked increase in NQO1

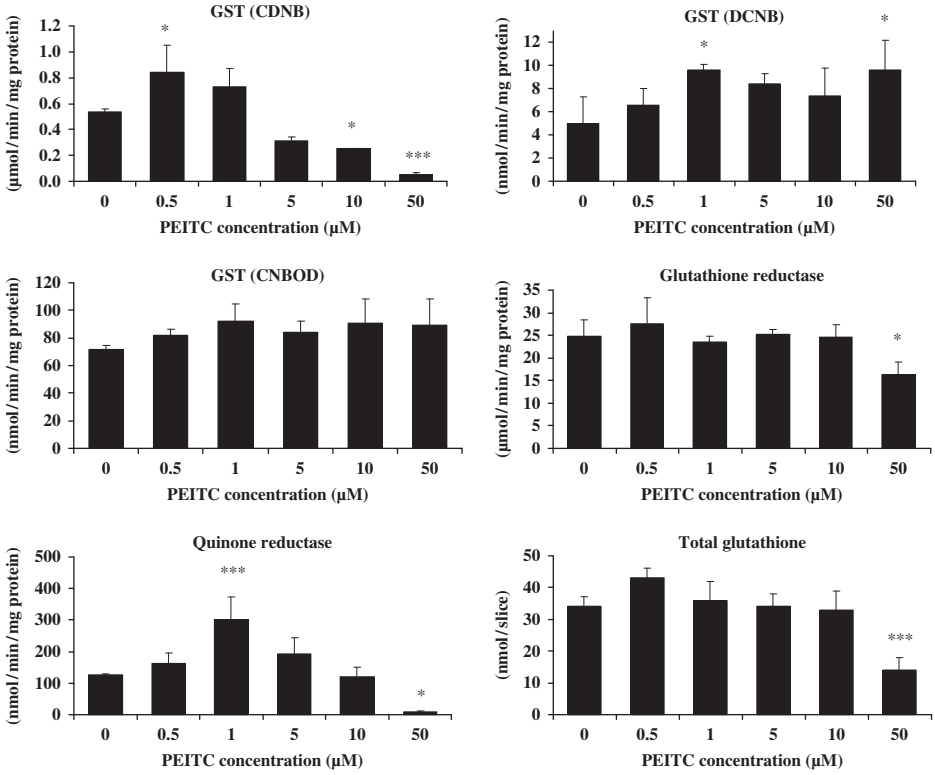

Figure 1. Effect of PEITC on phase II enzyme activities and glutathione levels in precision-cut rat liver slices. Precision-cut liver slices were incubated in culture medium containing PEITC (0.5–50 μM) for 24 h. Values are presented as mean ± SD of three replicates, each containing 10 slices/mL. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

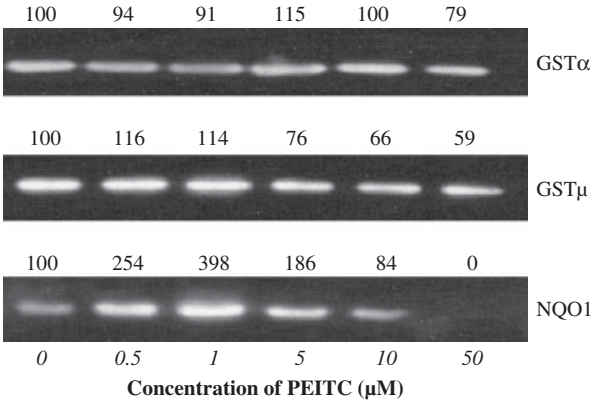

Figure 2. Effect of PEITC on the expression of phase II enzymes in precision-cut rat liver slices. Rat liver slices were incubated in culture medium containing PEITC (0–50 μM) for 24 h. Cytosolic proteins were isolated and equal amounts were loaded on to 10% w/v SDS-PAGE and then transferred electrophoretically to Hybond-P polyvinylidene difluoride membrane. The immunoblot analysis was carried out by exposure to rabbit anti-rat GSTA1-1, GSTM1-1, or NQO1 primary antibodies followed by the appropriate peroxidase-labelled secondary antibody. Each lane was loaded with 30 μg (NQO1) or 15 μg (GSTA1-1 and GSTM1-1) of total protein. Values above blots show percentage levels of optical density of each band relative to control.

activity was observed following exposure of the slices to 1 μM of PEITC, but at the highest concentration of the isothiocyanate a marked decline in activity was noted (Fig. 1). At the protein level, however, NQO1 expression increased markedly, maximal effect being observed at 1 μM of PEITC, but no protein could be detected at the highest concentration of the isothiocyanate (Fig. 2).

When precision-cut liver slices from four human donors were incubated with PEITC and glutathione-*S*-transferase activity was determined using CDNB as substrate an increase in activity was observed in two of the four donors; a decrease in activity was noted in the other two donors, in particular Donor 2 (Fig. 3). However, when the activity was assessed using CNBOD, a rise in activity was evident in three of the donors, at the higher concentrations of PEITC exposure, but once again in Donor 2 activity fell significantly (Fig. 3). Western blot analysis utilising antibodies to GSTα revealed a marked rise in expression in Donor 4, with a much less pronounced increase in Donors 1 and 3, and only a slight increase in Donor 2 (Fig. 4). When antibodies to GSTπ were employed, no consistent major rise in expression could be discerned, but the level of expression declined at the higher PEITC concentrations in all donors, albeit to different extent. Finally, when cytosolic proteins were probed with antibodies to GSTμ, a modest rise in expression could be seen in three of the four donors, but at the lowest concentration of 0.5 μM only, and tended to decrease at higher concentrations (Fig. 4). Glutathione reductase activity was impaired in all donors, but to different extent (Fig. 5). The four human livers responded differentially to PEITC when glutathione levels were determined (Fig. 5). No statistically significant change was seen in Donors 1 and 2,

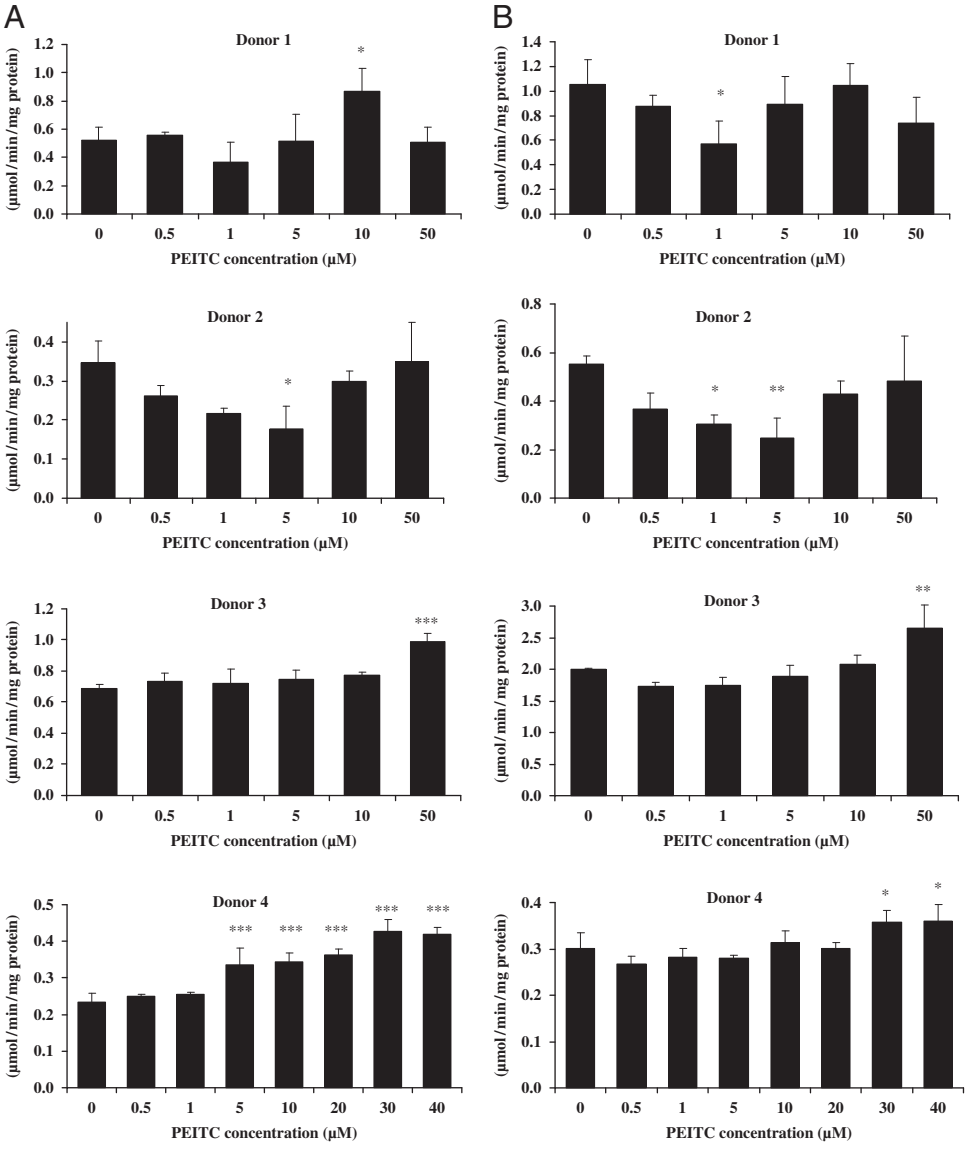

Figure 3. Glutathione-*S*-transferase activity in precision-cut human liver slices exposed to PEITC. Human liver slices from four donors were incubated in culture medium supplemented with PEITC (0.5–50 μM in Donors 1, 2 and 3, and 0.5–40 μM in Donor 4) for 24 h. Glutathione-*S*-transferase activity was determined using CNBOD (A) and CDNB (B) as the accepting substrates. Values are presented as mean ± SD of three replicates, each containing 10 slices/mL. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

but an increase was noted in Donor 3, at the lower concentrations of PEITC, and in Donor 4, but at the higher PEITC concentrations only. When NQO1 was monitored, a modest significant rise was seen in Donor 4 at a PEITC concentration of 20 μM, but in the other donors either there was no effect or activity was declined (Fig. 6). Only in Donor 4 was there a very marked rise in NQO1 protein expression, whereas in all other donor livers' expression dropped markedly (Fig. 6).

4 Discussion

Detoxification of the genotoxic metabolites of chemical carcinogens is believed to be an important mechanism of the chemoprevention of isothiocyanates [2]. Isothiocyanates enhance the conjugation of electrophiles with glutathione, a major defensive cellular mechanism, by stimulating the activity of cytosolic glutathione-*S*-transferases, and enhance NQO1, an enzyme that converts quinones to hydroquinones thus preventing them from undergoing redox cycling, in both liver and lung [16, 24–26]. Similarly, when PEITC was administered to rats at low doses hepatic glutathione-*S*-transferase and NQO1 activities were up-regulated [15]. These findings have been confirmed in the current study using precision-cut rat liver slices, and highlight the usefulness of this *in vitro* system in assessing the potential of these chemicals to modulate carcinogen-metabolising enzyme systems.

In studies employing the aliphatic isothiocyanates erucin and sulforaphane, precision-cut human liver slices did not respond in the same way as rat liver slices; for example, both isothiocyanates elevated NQO1 activity and expression in rat slices whereas activity was unaffected in liver slices from two

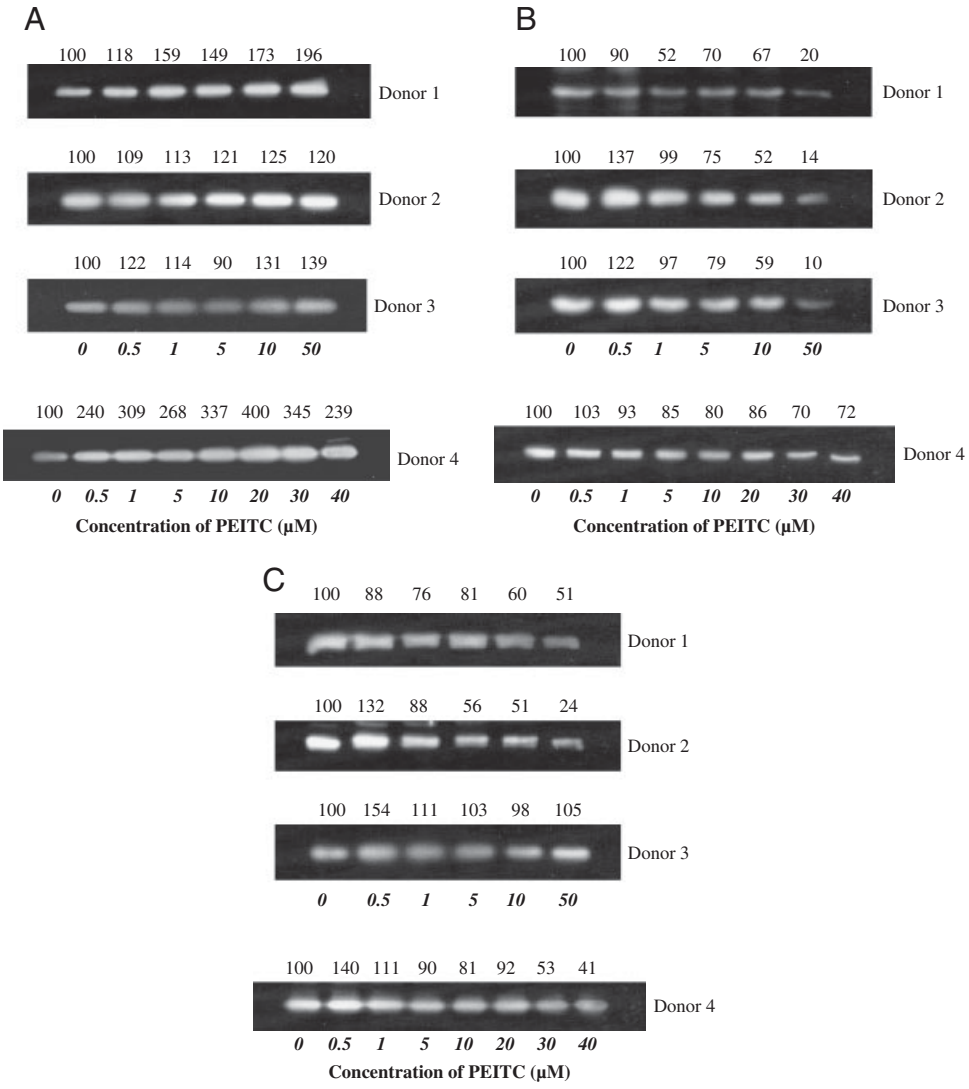

Figure 4. GSTα (A), GSTπ (B) and GSTμ (C) protein levels in precision-cut human liver slices exposed to PEITC. Human liver slices from four donors were incubated in medium supplemented with PEITC (0.5–50 μM in Donors 1, 2 and 3, and 0.5–40 μM in Donor 4) for 24 h. Cytosolic proteins were isolated and equal amounts (15 μg) of protein were loaded on to 10% w/v SDS-PAGE and then transferred electrophoretically to Hybond-P polyvinylidene difluoride membrane. The immunoblot analysis was carried out by exposure to either rabbit anti-human GSTA1-1, GSTM1-1 or GSTP1-1 primary antibody followed by peroxidase-labelled anti-rabbit IgG. Values above blots show percentage levels of optical density of each band relative to control.

human donors 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 [16]. These studies demonstrate that the effect of isothiocyanates on conjugation systems in human liver may be different to rat liver with consequences in its chemopreventive activity. As a result, this study was undertaken to evaluate the potential of PEITC to modulate detoxification enzymes in human precision-cut liver slices from four donors.

Studies conducted largely in rats revealed that the most sensitive hepatic enzyme system to isothiocyanates is NQO1 [15, 16, 24–26] and, in concordance, in these studies activity was more than doubled, and Western blotting indicated that exposure of rat slices to as little as 1 μM of PEITC quadrupled protein expression, the most marked effect observed in the enzyme systems monitored; activity was impaired, however, at the highest concentration used. As at the 50 μM concentration PEITC displays toxicity, at least in rat slices [17], the decline activity may not be related to the isothiocyanate treatment; a contributory factor in the toxicity of PEITC may be the decreased glutathione levels compromising the cellular defence mechanisms. However, when the human livers were exposed to PEITC in only one of the four livers, namely Donor 4, was there up-regulation of this enzyme, accompanied by a doubling in enzyme activity; no rise in activity was evident in the remaining livers and, in fact, protein expression diminished. The differential response in Donor 4 cannot be ascribed to low initial NQO1 activity as this was higher than that of Donor 2, where no change in activity was evident following treatment with PEITC. Clearly, these studies point out that individual differences occur in the modulation of NQO1 by PEITC. The Ah receptor, which controls the up-regulation of this enzyme [27, 28], is polymorphically expressed in humans, although the impact of the various alleles on its function is not entirely clear [29, 30]. The heterogeneity in response

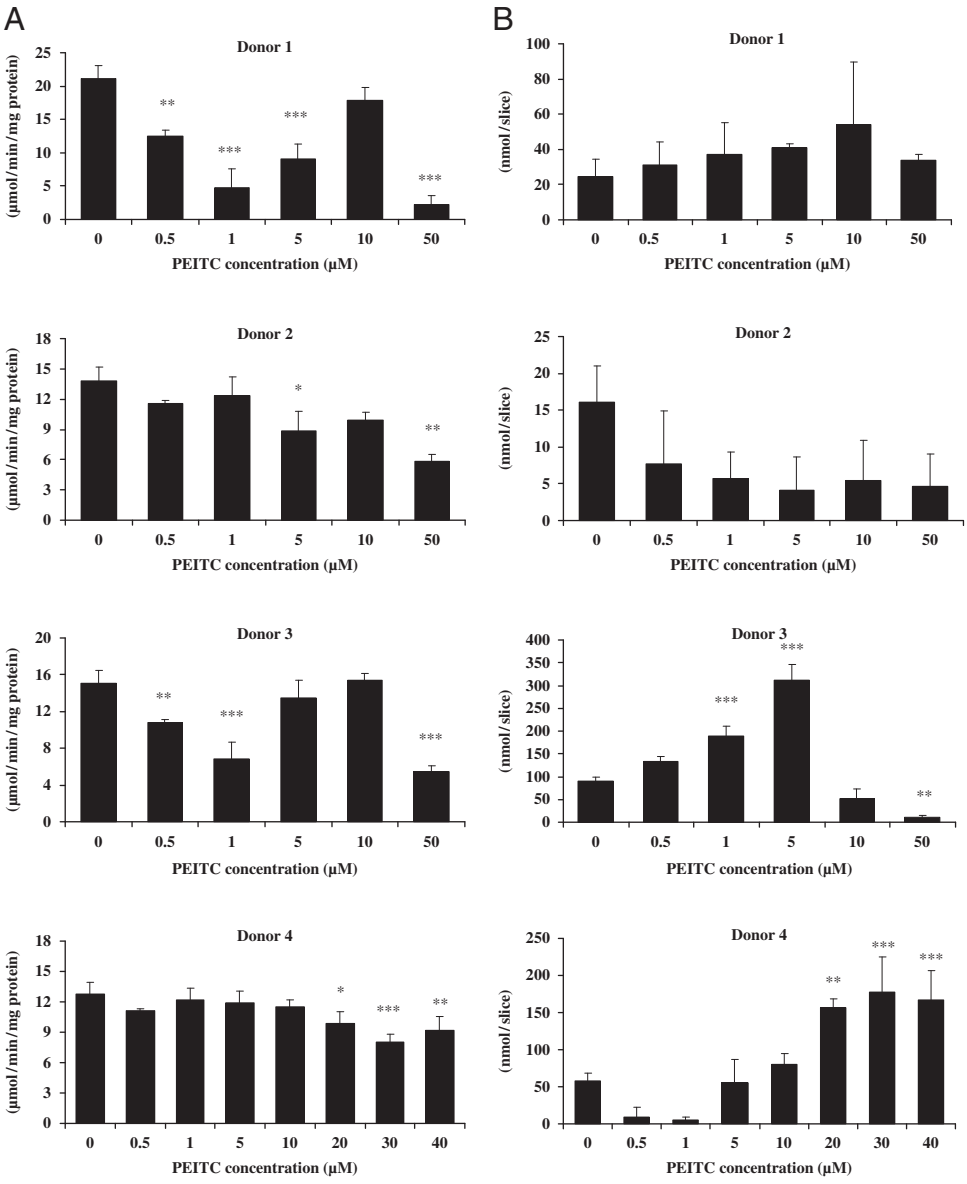

Figure 5. Effect of PEITC on glutathione reductase (A) and glutathione levels (B) in precision-cut human liver slices. Human liver slices from four donors were incubated in culture medium supplemented with PEITC (0.5–50 μM in Donors 1, 2 and 3, and 0.5–40 μM in Donor 4) for 24 h. Values are presented as mean ± SD of three replicates, each containing 10 slices/mL. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

among the four donors may also be associated with individual polymorphisms. Three well-defined alleles of NQO1 have been reported, namely NQO1∗1, ∗2, and ∗3 representing a functional allele, a non-functional allele, and diminished NQO1 activity allele, respectively [31]. In Donor 2, a remarkable decline in protein levels was observed at all PEITC concentrations indicating down-regulation of the NQO1 expression by PEITC whereas the activity remained unchanged. It is possible that the immunodetectable protein in Donor 2 is of the diminished NQO1 activity allele, and is supported by the fact that this donor displayed relatively low activity compared to the others. Hence changes in protein expression do not translate into detectable changes in activity.

Glutathione-*S*-transferase activity was determined using three substrates; CDNB is a substrate metabolised by a number of GST enzymes, including the μ- and π-classes, whereas CNBOD appears to be selectively catalysed by GSTα [32], and DCNB is a substrate associated with the μ-family [33]. In rat slices, activity was elevated by the PEITC treatment when CDNB and DCNB were used as substrates. In human liver slices, however, a differential response was obtained when CNBOD served as substrate, in that a rise in the activity was noted in three of the four donors; in Donor 2 activity was impaired. The expression of GSTα rose markedly in Donor 4 and to a much lesser extent in Donors 1 and 3; as GSTα is also regulated by the Ah receptor, it is not surprising that the Donor 4 liver exhibited such a marked increase, since it also displayed a marked increase in NQO1, which is regulated by the same receptor. When CDNB was used, a rise in activity was seen in two of the donors, but a decrease in activity was observed in the remaining two.

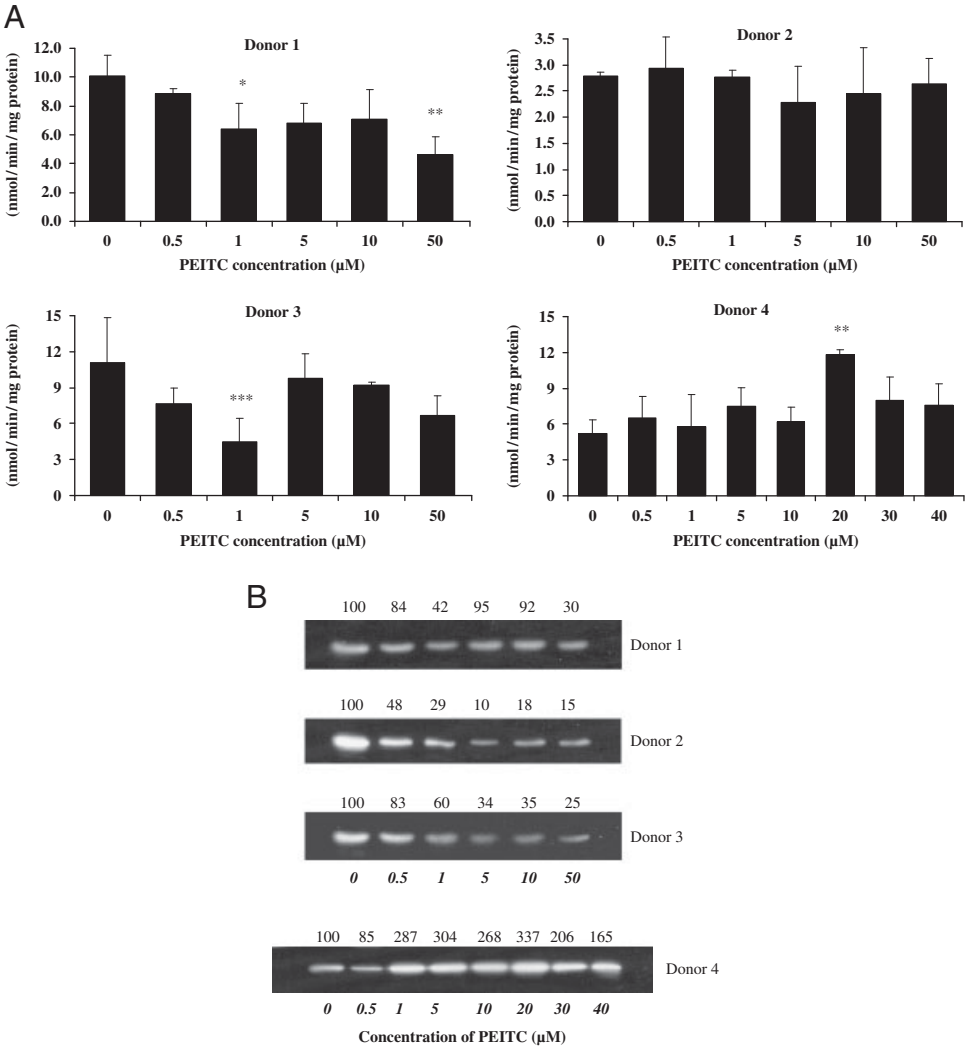

Figure 6. Effect of PEITC on NQO1 activity (A) and expression (B) in precision-cut human liver slices. Human liver slices from four donors were incubated in culture medium supplemented with PEITC (0.5–50 μM in Donors 1, 2 and 3, and 0.5–40 μM in Donor 4) for 24 h. NQO1 values are presented as mean ± SD of three replicates, each containing 10 slices/mL. For the immunoblot analysis, cytosolic proteins were isolated and equal amounts (30 μg) of protein were loaded on to 10% w/v SDS-PAGE and then transferred electrophoretically to Hybond-P polyvinylidene difluoride membrane. The immunoblot analysis was carried out by exposure to rabbit anti-human NQO1 primary antibody followed by peroxidase-labelled anti-rabbit IgG. Values above blots show percentage levels of optical density of each band relative to control. $^{*}p < 0.05$.

GSTπ expression tended to decrease in all the four livers and, similarly, in the case of GSTμ, except that at the lowest concentration of PEITC a modest rise in expression could be seen in three of the four livers. These studies indicate that the effects of PEITC on human hepatic glutathione-*S*-transferase are isoform dependent, and concur with our previous studies using the isothiocyanates erucin and sulforaphane [16].

In rat liver slices total glutathione levels dropped at the highest concentrations presumably because of utilisation, since glutathione conjugation is the principal pathway of the metabolism of isothiocyanates [14, 34]. A similar observation has been made with other isothiocyanates incubated with slices under the same conditions [16]. In two of the human livers, however, total glutathione content was markedly elevated following exposure to PEITC, but at different concentrations of the isothiocyanate indicating a marked difference in response.

The differential response of the four human livers to PEITC cannot be ascribed to the condition of the liver at the time of processing. All livers were delivered 8–12 h following removal from the donors and, moreover, in most cases basal activities did not differ markedly among the four liver samples so as to denote deterioration of the sample. It is possible, however, that the metabolism of PEITC in the four human livers may differ significantly so that the effective concentration of the isothiocyanate may vary, and this may account partly for the difference in response. It is noteworthy that the liver from Donor 4 was the most responsive in the up-regulation of the two enzymes studied, NQO1 and glutathione-*S*-transferase, both at the activity and protein levels, as well as in the increase in glutathione levels, and this possibility deserves further investigation.

It is relevant to point out that the effects of PEITC on the various enzyme systems studies were observed at concen-

trations as low as 0.5 μM, a concentration which is attainable after dietary level of intake. We have recently reported that following a single oral dose of PEITC to rats, at a dose level simulating human dietary intake, the plasma C_{max} values attained were about 2 μM [35]. Thus changes in glutathione-*S*-transferase and NQO1 activities that may impact on carcinogen metabolism can occur at dietary levels of intake, and may contribute to the reported chemopreventive effect of this isothiocyanate.

In conclusion, these studies describe for the first time the ability of an aromatic isothiocyanate to modulate the detoxification enzyme systems glutathione-*S*-transferase and NQO1, as well as glutathione levels in human tissue; these effects were observed at concentrations that are attainable at the low dietary levels of intake. However, although only based on four samples, very pronounced differences were observed in the response of human livers to PEITC indicating that the chemopreventive effect of isothiocyanates may not be manifested in all individuals exposed to this phytochemical.

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The authors have declared no conflict of interest.

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