1-Cyano-2,3-epithiopropane is a novel plant-derived chemopreventive agent which induces cytoprotective genes that afford resistance against the genotoxic \(\alpha,\beta\)-unsaturated aldehyde acrolein

Michael O. Kelleher, Michael McMahon, Ian M. Eggleston¹, Mark J. Dixon², Keiko Taguchi³, Masayuki Yamamoto⁴, Masayuki Yamamoto³, and John D. Hayes⁵

Biomedical Research Institute, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, Scotland, UK, ¹Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, UK, ²Department of Medical Biochemistry, Tohoku University Graduate School of Medicine, Sendai 980-8577, Japan, ³Center for Tsukuba Advanced Research Alliance, Japan Science and Technology Agency, Tsukuba 305-8577, Japan, ⁴Center for Tsukuba Advanced Research Alliance, Japan Science and Technology Agency, Tsukuba 305-8577, Japan

Email: j.d.hayes@dundee.ac.uk
Fax: +44 1382 669993
Email: j.d.hayes@dundee.ac.uk

Abbreviations: AITC, allyl ITC; ARE, antioxidant response element; CETB, 1-cyano-2,3-epithiobutane; CETP, 1-cyano-2,3-epithiopropionate; CETPent, 1-cyano-2,3-epoxypropionate; CEXPent, 1-cyano-4,5-epoxypropane; ESP, epithionitriles; GST, glutathione-S-transferases (GSTs) and the glutathione-dependent enzymes; GSH, glutathione; GSH, glutathione-S-transferase; ITC, isothiocyanates; Keap1, Kelch-like ECH-associated protein 1; LD₅₀, lethal dose 50 (i.e., a dose of xenobiotic that kills 50% of cells); MEF, mouse embryonic fibroblasts; Nqo1-luciferase, Nqo1-luciferase reporter plasmid; Nrf2, nuclear factor erythroid 2-related factor 2; PKC, protein kinase C; RT-PCR, real-time polymerase chain reaction; SFN, sulforaphane; Sin, sinigrin.

Introduction

Diet and other environmental factors influence profoundly the risk of individuals developing neoplastic disease (1). Regular consumption of cruciferous vegetables shows great potential as a means of inhibiting carcinogenesis (2,3). These plants uniquely contain substantial quantities of glucosinolates, a class of phytochemicals composed of a \(\beta\)-thioglucose group, a sulfonated oxime moiety and a highly variable side chain that is derived from amino acids (4,5). The anticancer properties of cruciferous vegetables are attributed to the presence of glucosinolates (3–5).

A large body of literature suggests that the glucosinolate hydrolysis products, rather than the glucosinolates themselves, are responsible for the chemopreventive effects of cruciferous vegetables (for a recent review, see ref. 6). During consumption of these vegetables, the \(\beta\)-thioglucose enzyme myrosinase (EC 3.2.1.147) is released from specialized plant cells and hydrolyses the \(\beta\)-glucosyl bond in glucosinolates (7). Breakdown of glucosinolates by myrosinase results in the liberation of glucose and the generation of an unstable thiohydroxymate-\(\beta\)-sulfonate that contains a variable amino acid-derived side chain. Once formed, the thiohydroxymate-\(\beta\)-sulfonates readily undergo a ‘Lossen’-type rearrangement to release sulfate and, in so doing, yield a variety of indoles, thiocyanates, isothiocyanates (ITCs) and nitriles (4,7–9). The spectrum of hydrolysis products generated by myrosinase is dependent on the glucosinolate and the reaction conditions. In the case of glucosinolates with an aliphatic side chain, such as glucoraphanin, which is abundant in broccoli (3,10), hydrolysis by myrosinase at pH 7.0 produces 4-methylsulfinylbutyl ITC (also called sulforaphane (SFN)), whereas under acidic conditions, 4-methylsulfinylbutyl nitrile is formed (7). Production of one or other of these alternative glucoraphanin hydrolysis products has significant consequences from a cancer chemoprevention perspective as evidence suggests that SFN, rather than 4-methylsulfinylbutyl nitrile, has anticancer properties (11,12).

In the case of alkyl glucosinolates such as sinigrin (Sin), gluconapin and glucobrassicinapin, all of which contain a terminal double bond in their side chains, their hydrolysis by myrosinase at pH 7 results in the production of 2-propenyl ITC (also called allyl ITC (AITC)), 3-butenyl ITC and 4-pentenyl ITC (2,3). Uniquely, however, these alkyl glucosinolates yield 1-cyano-2,3-epithiopropionate (CETP), 1-cyano-3,4-epithiobutane (CETB) and 1-cyano-4,5-epithiopentane (CETPent) when they are hydrolyzed by myrosinase at pH 4 in the presence of both epithiospecifier protein (ESP) and ferrous ions (7,13,14); these are shown, along with AITC and SFN, in Figure 1. Although it has been known for many years that glucosinolate hydrolysis products include epithionitriles and nitriles (8,9), it is currently not known if these phytochemicals protect against carcinogenesis.

Various mechanisms have been proposed to account for the chemopreventive activities of ITCs produced from glucosinolates. At relatively high concentrations, aromatic ITCs and most aliphatic ITCs stimulate cell cycle arrest and apoptosis (15,16), through a mechanism that involves production of reactive oxygen species, Bax activation, down-regulation of inhibitors of apoptosis proteins and induction of apoptotic peptide activating factor 1 (17,18). At lower doses, ITCs activate NF-E2 p45-related factor 2 (Nrf2), a cap’n’collar basic region leucine zipper transcription factor, causing induction of cytoprotective genes as part of an adaptive response mechanism (15,19). In particular, ITCs increase the detoxification of carcinogens and reactive oxygen species by up-regulating drug-metabolizing enzymes, drug transporters and antioxidant proteins (19–22).

Nrf2 regulates ~150 genes including NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione (GSH) S-transferases (GSTs) and the...
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Fig. 1. Structures of glucosinolate breakdown products and related compounds. (A) CETP; (B) CETB; (C) CETPent; (D) CEXP; (E) butyronitrile; (F) AITC; (G) SFN.

Materials and methods

Chemicals

SFN was purchased from LKT Laboratories (St Paul, MN). All other chemicals were obtained from Sigma-Aldrich (Poole, Dorset, UK). CETP, CETB and CETPent and their related epoxynitriles, 1-cyano-2,3-epoxypropane (CEXP) and 1-cyano-4,5-epoxypentane (CETPent), were synthesized as described by Shofran et al. (36); the purity of each of the products was >95% when examined by nuclear magnetic resonance spectroscopy.

Cell culture and treatment with xenobiotics

Non-transformed rat liver RL-34 epithelial cells were grown in Dulbecco’s modified Eagle’s media supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 50 U/ml penicillin-streptomycin mixture and 2 mM L-glutamine. Mouse embryonic fibroblasts (MEFs) were prepared and grown as described elsewhere (25).

Cytotoxicity testing was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described by Higgins et al. (19).

For gene induction experiments, cells were grown in 60 mm dishes and allowed to reach ~70% confluence before being exposed for between 12 and 24 h to various concentrations of epithionitriles, epoxynitriles, butyronitrile (propyl cyanide) or ITCs, all of which were dissolved in dimethyl sulfoxide to a final concentration of 0.1% (v/v) in the media. Typically, cells were treated with a dose of 50 μmol/l of the epithionitrile, epoxynitrile or butyronitrile for gene induction experiments; this dose was chosen after preliminary experiments using 10, 25, 50 and 100 μmol/l of the various epithionitriles showed that a concentration of 50 mmol/l affected induction of NQO1 in RL-34 cells by all the compounds in this class of phytochemical. The treatment was used at a concentration of 10 μmol/l.

Western blotting

Rodent cells were scraped from dishes in 1 ml phosphate-buffered saline and harvested by centrifugation at 10 000g for 10 min at 4°C, snap frozen and then lysed by thawing in Nonidet P40-containing cell lysis buffer. Protein concentration was determined by the Bradford dye-binding assay, and 25 μg of protein was applied to each electrophoresis well, and proteins were identified using antibodies characterized previously (37–39). The nitrocellulose membranes were routinely stained with Indian ink (Pelikan) prior to incubation with primary antibody to confirm equal loading and transfer of samples.

Quantification of messenger RNA

Total RNA was isolated from RL-34 cells using Trizol® reagent. Following reverse transcription, TaqMan® real-time polymerase chain reaction (RT-PCR) was carried out using the PerkinElmer/ Applied Biosystems (Warrington, Cheshire, UK) Prism Model 7700 Sequence Detector instrument (40). The relative amounts of messenger RNA (mRNA) for NQO1, GSTA3 and GCLM were estimated using assays Rn00568900_ml, Rn00580416_ml and Rn05609000_ml (from Applied Biosystems), respectively, with 18S rRNA as an internal standard.

Measurement of GSH

This was carried out by the method of Tietze (41).

Nqo1-luciferase reporter construct

The luciferase reporter plasmid P ( (i.e. primed) with phytochemicals when compared with that at the beginning of the experiment. The MTT data were normalized and analyzed using non-linear regression to calculate the mean LD50 values and 95% confidence intervals. Significant differences between the LD50 of Nrf2+/− MEFs or Nrf2−/− MEFs that had been pre-treated (i.e. primed) with phytochemicals when compared with Nrf2−/− MEFs, which was used at a concentration of 10 μmol/l.
had not been ‘primed’, was determined by two-way analysis of variance. The statistical evaluation of data is indicated as follows: P > 0.05, not significant; P = 0.05−0.01, *; P = 0.01−0.001, ** or §§; P < 0.001, *** or §§§ (with an asterisk indicating a significant increase and the symbol § indicating a significant decrease).

Results

Toxicity of epithionitriles and ITCs to cells

We tested whether epithionitriles and other phytochemicals were toxic to either RL-34 cells or Nrf2−/− MEFs. Using the MTT assay, we found that CETP, CETB and CETPent had LD50 values of 770, 510 and 530 μmol/l, respectively, in RL-34 cells and 540, 380 and 430 μmol/l in wild-type MEFs. In contrast, AITC and SFN gave LD50 values of 200 and 85 μmol/l, respectively, in RL-34 cells and 115 and 55 μmol/l in wild-type MEFs.

Epithionitriles can increase expression of cytoprotective genes

RL-34 cells were treated with various epithionitriles, their related epoxides or butyronitrile (propyl cyanide) for 24 h before immunoblotting was undertaken; SFN was used as a positive control. Figure 2A shows that the level of NQO1 protein was increased ~8-, 4- and 3-fold upon 24 h treatment with CETP, CETB and CETPent, respectively. Western blotting also revealed that NQO1 protein was increased ~2-fold by treatment with 50 μM CEXP. However, not by CEXB or CEXPent. NQO1 protein was not induced by butyronitrile, suggesting that the cyanide moiety in CETP and CEXP is not responsible for gene induction. Western blotting for GCLM and GST class Alpha 3 (GSTA3) revealed that the levels of these subunits in RL-34 cells were increased between 2- and 3-fold by treatment with CETP, CETB or CETPent. Immunoblotting for GCL catalytic subunit showed that the level of the protein remained essentially unchanged following treatment with epithionitriles, epoxynitriles or SFN (data not shown).

Both CETP and AITC are generated as breakdown products of Sin (32). We therefore wished to determine if CETP was as potent an inducing agent as AITC. Immunoblotting of RL-34 cells that had been treated with increasing amounts of CETP or AITC revealed that they were equally effective at inducing NQO1 protein (Figure 2B). In contrast, Sin had no significant effect on the expression of NQO1.

Epithionitriles stimulate GSH synthesis

Many cancer chemopreventive agents are capable of interacting with intracellular thiols (19,43). We therefore wished to establish whether epithionitriles influence intracellular GSH levels. Table I shows that treatment of RL-34 cells with CETP causes depletion of intracellular GSH to ~50% of its normal homeostatic level after 2–4 h of exposure to the xenobiotic. This acute depletion was followed by re-synthesis of the antioxidant tri-pestide that allowed recovery of the intracellular GSH levels by 12 h of exposure to CETP. Thereafter, the intracellular content of GSH continued to increase such that at the 18 and 24 h time points, it was 1.8- and 2.0-fold higher, respectively, than the normal homeostatic levels. Treatment of RL-34 cells with CETB and CET-
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Nrf2 (27,44–46). Furthermore, the Cys-151 residue in Keap1 is also necessary for SFN to overcome repression of Nrf2 (27,46,47). To test whether CETP induces ARE-driven gene expression by inactivating Keap1, we performed transient knock-in experiments using mKeap1 and zKeap1a; these were chosen because while Cys-151, Cys-273 and Cys-288 are important in mKeap1, the zebrafish protein contains only two of these three cysteines [zKeap1a contains Cys-127 and Cys-264 that are the equivalent of Cys-151 and Cys-288 in mKeap1, but it lacks the equivalent of Cys-273 in the mouse protein (42)]. Initial experiments showed that the plasmid for the zKeap1a with 20 upstream nucleotides, called pcDNA3.1/20V5HisBzKeap1a, provided closely comparable amounts of protein as the pcDNA3.1/V5HisCmKeap1 expression vector, the zebrafish pcDNA3.1/20V5HisBzKeap1 expression vector or an empty pcDNA3.1 vector, each along with Pnqo1/Luc and pRL-TK Renilla, demonstrated that knock-in of either Keap1 protein substantially repressed reporter gene activity in a dose-dependent fashion. As shown in Figure 5B, reintroduction of mKeap1 into MEFs, or knock-in of zKeap1a, reduced luciferase activity to ~20% of the activity observed when Keap1−/− MEFs were transfected with the empty pcDNA3.1 expression vector along with Pnqo1/Luc. When Keap1−/− MEFs that had been co-transfected with an expression vector for either mKeap1 or zKeap1a along with the Pnqo1/Luc reporter construct were treated with 50 µM CETP or 10 µM SFN, luciferase reporter activity increased to the level observed when the mutant fibroblasts were transfected with an empty pcDNA3.1 expression vector along with the reporter construct (Figure 5C). Together, these results suggest that CETP affects induction of Nqo1 by antagonizing the negative regulation of Nrf2 by Keap1 and that this process probably does not involve Cys-273.

I-Cyano-2,3-epithionitrile affords protection against electrophiles

To test the hypothesis that induction of gene expression and up-regulation of GSH synthesis produced by CETP confers resistance against electrophiles, MTT cytotoxicity testing was carried out. We examined acrolein in this context because it is a genotoxic α,β-unsaturated aldehyde that is present in the environment, as a combustion product, and is also generated endogenously from the oxidation of threonine by myeloperoxidase (33–35,48). Additionally, we wished to examine whether CETP and AITC might confer comparable levels of resistance to xenobiotics since they are produced as alternative Sin breakdown products (36). Table II shows that pre-treatment of Nrf2−/− MEFs with 15 µM CETP for 24 h increased resistance to subsequent challenge with acrolein 2.4-fold. In contrast, Nrf2−/− MEFs were twice as sensitive to the reactive aldehyde as Nrf2+/− MEFs, and pre-treatment of the mutant fibroblasts with 15 µM CETP conferred no protection. Similar experiments using 15 µM AITC or 3 µM SFN revealed that these ITCs also conferred resistance on Nrf2−/− MEFs, but not Nrf2−/− MEFs, to acrolein.

Discussion

Cruciferous vegetables appear to possess anticancer properties, and this is attributed to the fact that they contain glucosinolates which when hydrolyzed are capable of inducing cytoprotective genes and/or stimulating apoptosis (6). Among the glucosinolate breakdown products, ITCs have attracted substantial attention as phytochemicals with potential chemopreventive activity. However, in addition to forming products, ITCs have been inferred that ESP activity is undesirable from a cancer chemoprevention perspective because its production diminishes the yield of ITCs...
Table I. Chemopreventive effects of epithionitriles

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Duration of exposure</th>
<th>Fold change</th>
<th>GSH/mg protein (nmol change)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>12 h</td>
<td>8 h</td>
</tr>
<tr>
<td>DMSO (0.1%)</td>
<td>88.4 ± 4.2</td>
<td>103.3 ± 9.8</td>
<td>91.2 ± 2.4</td>
</tr>
<tr>
<td>CETB (50 μmol/l)</td>
<td>103.3 ± 9.8</td>
<td>94.8 ± 2.9</td>
<td>91.2 ± 2.4</td>
</tr>
<tr>
<td>CETP (50 μmol/l)</td>
<td>103.3 ± 9.8</td>
<td>91.2 ± 2.4</td>
<td>88.1 ± 4.2</td>
</tr>
<tr>
<td>SFN (10 μmol/l)</td>
<td>103.3 ± 9.8</td>
<td>91.2 ± 2.4</td>
<td>88.1 ± 4.2</td>
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</table>

The levels of GSH in RL-34 cells were measured by the method of Tietze (41) at the various time points following treatment with epithionitriles, epoxynitriles or butyronitrile as indicated. Statistically significant differences were determined by using wild-type and knockout MEFs, we demonstrated that priming with CETP could protect against acrolein and that the inducible resistance was dependent on Nrf2. Although we only challenged the fibroblasts with acrolein, it seems likely that CETP will confer pleiotropic drug resistance because we have found previously that priming Nrf2<sup>−/+</sup> MEFs for 24 h with 3 μM SFN induces an adaptive response that affords protection against ITCs, reactive carbonyl-containing compounds, epoxides, peroxydes, redox-cycling agents, heavy metal salts and nitrogen mustards (19). Furthermore, inducible drug resistance observed following priming with SFN was accompanied by a marked overproduction of GSH (19), and this was also observed when MEFs were treated with CETP.

During this study, an important question that we felt ought to be addressed is whether the formation of epithionitriles from glucosinolates, rather than ITCs, is desirable from a chemopreventive perspective. In the case of Sin, the combined activities of myrosinase and ESP result in the formation of CETP, whereas myrosinase activity alone produces AITC. As we have found that CETP has a similar potency as AITC at increasing the level of NQO1 in RL-34 cells, it could be argued that ESP makes little impact on the effect that Sin breakdown products have on the induction of cytoprotective genes. We also found that CETP conferred similar levels of resistance against acrolein as AITC. Clearly, the conclusion that CETP and AITC are equally effective chemopreventive agents in vivo predicates on both phytochemicals possessing similar pharmacokinetic properties, and this is an issue that requires further study. It should also be recognized that AITC can have genotoxic effects, at least in Salmonella typhimurium (50), and therefore, it might even be desirable to hydrolyze Sin to CETP rather than AITC.

In the present study, we have found that CETB and CETPent can increase NQO1 at the mRNA and protein levels. As the relative abilities of CETB and CETPent to serve as inducing agents have not been compared with those of 3-butenyl ITC and 4-pentenyl ITC, it is not possible to comment whether the presence of ESP diminishes the activity of gluconapin and glucobrassicanapin hydrolysis products to up-regulate cytoprotective genes. Furthermore, it is not known whether these epithionitriles have pharmacokinetic properties that are comparable with 3-butenyl ITC and 4-pentenyl ITC.

During the early stage of this project, we performed MTT assays to determine whether CETP was toxic to RL-34 cells or MEFs. These analyses showed that the epithionitrile had an LD<sub>50</sub> in RL-34 epithelial cells of 770 μmol/l and an LD<sub>50</sub> in Nrf2<sup>−/+</sup> MEFs of 540 μmol/l. We therefore conclude that it is unlikely that this phytochemical contributes to chemoprevention by causing apoptosis. In the light of these findings for CETP, it is possibly surprising that CETB is
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Mechanism of gene induction by epithionitriles

We performed reporter gene assay experiments using the $P_{-1016}nqo1-Luc$ construct along with a mutant plasmid to demonstrate that induction of gene expression by CETP, CETB and CETPent was dependent on the presence of an intact ARE. Xenobiotics that induce ARE-driven gene expression include Michael reaction acceptors, hydroperoxides, ITCs, vicinal dimericarbons, organosulfurs and heavy metal salts (53), which all share the characteristic that they can react with protein thiol. These compounds appear to induce ARE-driven gene expression by CETP, CETB and CETPent was carried out in Keap$^{+/+}$ and Keap$^{−/−}$ MEFs following treatment with 0.1% dimethyl sulfoxide (DMSO), 10 μM SFN or 50 μM CETP. (B) The relative amounts of Nqo1 mRNA in Keap$^{+/+}$ and Keap$^{−/−}$ MEFs under basal and inducible conditions were determined by TaqMan RT-PCR. The results obtained are expressed as fold increases in mRNA relative to that observed in Keap$^{+/+}$ MEFs treated with 0.1% DMSO.

Moderately toxic to rats: when administered by gavage as a single dose of 125 mg/kg of body weight, CETB has been reported to produce acute nephrotoxic injury and diuresis along with necrosis of renal epithelial cells (51); when administered by intra-peritoneal injection as a single dose of 200 mg/kg of body weight, CETB can cause mild to moderate periacinar necrosis and congestion in the liver (52). It is therefore possible that high doses of CETB could stimulate apoptosis, autophagy or necrosis, but we found no evidence for this in the present study. The bioavailability and safety of CETP are obviously topics that warrant future investigation.

Fig. 4. Induction of Nqo1 expression by CETP is only observed in wild-type MEFs, not Keap1-null MEFs. (A) Western blotting for Nqo1 protein was carried out in Keap$^{+/+}$ and Keap$^{−/−}$ MEFs following treatment with 0.1% dimethyl sulfoxide (DMSO), 10 μM SFN or 50 μM CETP. (B) The relative amounts of Nqo1 mRNA in Keap$^{+/+}$ and Keap$^{−/−}$ MEFs under basal and inducible conditions were determined by TaqMan RT-PCR. The results obtained are expressed as fold increases in mRNA relative to that observed in Keap$^{+/+}$ MEFs treated with 0.1% DMSO.

Gene induction by epithionitriles occurs through an ARE and requires Nrf2. (A) RL-34 cells were transfected with $P_{-1016}nqo1-Luc$, $P_{-1016}nqo1-T-435Gmut-Luc$ or empty pGL3-Basic vector, each along with pRL-TK Renilla, and allowed to recover for 24 h before treatment for 24 h with vehicle [0.1% by volume dimethyl sulfoxide (DMSO)], alone, 50 μM CETP, 50 μM CETB, 50 μM CETPent, 50 μM CEXP, 50 μM CEXB, 50 μM CEXPent, 10 μM SFN or 50 μM butyronitrile (But). Following correction for transfection efficiency, based on Renilla activity, the fold activation of luciferase activity was calculated from duplicate results and is shown as the mean ± SD from three independent experiments compared with that obtained from the DMSO vehicle control. Data obtained using $P_{-1016}nqo1-Luc$ are in grey-shaded bars, and those from the mutant $P_{-1016}nqo1-T-435Gmut-Luc$ are in black-shaded bars, and those from the mutant $P_{-1016}nqo1-T-435Gmut-Luc$ are in grey-shaded bars. (B) RL-34 cells were treated with the specified xenobiotic for 120 min before the level of Nrf2 protein was determined by western blotting. Lane 1 contains Nrf2 protein generated from ectopic Renilla, and allowed to recover for 24 h before treatment for 24 h with various doses of CETP prepared in DMSO to a final concentration of 0.1% (v/v). Lysates from these cells were prepared and luciferase activity measured. Following correction for transfection efficiency, luciferase activity relative to that obtained from DMSO-treated Nrf2$^{+/+}$ MEFs was calculated from duplicate results and is shown as the mean fold increase ± SD from three independent experiments.

One model that has been proposed to explain antagonism of Keap1 activity by inducing agents is that these compounds stimulate the formation of an intermolecular disulfide bridge between Cys-273 and Cys-288 in the two subunits of a Keap1 dimer, and that oxidation and degradation. In this respect, the 3-membered thiirane ring of the epithionitriles appears to be a prerequisite for gene induction by these phytochemicals. We found that CETP, CETB and CETPent are all inducers, but butyronitrile, a simple aliphatic nitrile that is related to CETP, failed to activate ARE-driven gene expression. We therefore conclude that it is the epithio moiety of these phytochemicals, rather than the nitrile group, that is responsible for interaction with Keap1 and stabilization of Nrf2.

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Epoxynitriles induce ARE-driven gene expression

Although it is recognized that trans-stilbene oxide can induce AKR7A1, GSTA5 and GSTP1 in rat liver (39), little is known about the ability of epoxides to induce cytoprotective genes. During the present study, we found that CEXP, CEXB and CEXPent up-regulate NQO1 modestly in RL-34 cells and Nr2f2+/− MEFS. These compounds were not as potent as CETP, CETB and CETPent at inducing gene expression, perhaps reflecting the less favorable match in hard/soft reactivity (55) associated with the nucelophilic opening of an epoxide by cysteine residues of Keap1 compared with the opening of a thiirane by BTB-Kelch protein on the thiirane ring, with concomitant alkylation and ring opening. Modification of one, or both, of these thiols presumably prevents Keap1 from binding Nrf2 through both its high-affinity Glu-Thr-Gly-Glu (ETGE) and its low-affinity Asp-Leu-Gly (DLG) motif that are situated between amino acids 79-82 and amino acids 29-31, respectively, in the transcription factor (31). As a consequence of the Keap1 dimer failing to immobilize Nrf2 through an interaction with both ETGE and DLG motifs, Cul3/Rbx1 is unable to ubiquitinate the transcription factor; under such circumstances, the Keap1–Nrf2 complex can be considered to exist in a ‘stalled’ state, thereby allowing the cap’n’ collar basic region leucine zipper factor to accumulate (24).

Besides modification of Keap1, it has been proposed that certain inducing agents, such as tert-butyl hydroquinone and phorbol 12-myristate 13-acetate, stimulate phosphorylation of Nrf2 at Ser-40 by protein kinase C (PKC) and that this in turn allows release of the cap’n’ collar basic region leucine zipper factor from Keap1 (reviewed in ref. 54). If this hypothesis was applicable to epoxynitriles, it would have been predicted that knock-in of mKeap1 or zKeap1 into Keap1−/− MEFS would not repress ARE-driven gene expression in the presence of CETP because Nrf2 would be phosphorylated at Ser-40. Our data show that knock-in of either mouse or zebrafish Keap1 proteins into Keap1−/− MEFS inhibited Nrf2 activity between 40% and 60% at a dose of 100 μM CETP (Figure 5). There are several interpretations for this observation, but it seems likely that Keap1, rather than PKC, is the major regulator of Nrf2. It would be interesting to examine whether similar knock-in experiments in the presence of phorbol 12-myristate 13-acetate would still lead to repression of Nrf2 or whether knock-down of the PKC isozyme responsible for phosphorylating Nrf2 would block induction of ARE-driven gene expression by tert-butyl hydroquinone, phorbol 12-myristate 13-acetate or CETP. Clearly, further work is required to help elucidate what contribution PKC makes to activation of Nrf2 and regulation of the ARE gene battery.
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\begin{array}{lcccccc}
\text{‘Priming’ with phytochemical} & \text{No} & \text{Yes} & \text{Fold change} & \text{No} & \text{Yes} & \text{Fold change} \\
\hline
\text{LD}_{50} \text{ for acrolein (µmol/l)} & \text{Mean (95% CI)} & \text{Mean (95% CI)} & \text{Mean (95% CI)} & \text{Mean (95% CI)} & \text{Mean (95% CI)} & \text{Mean (95% CI)} \\
\text{CETP (15 µmol/l)} & 46 (38–53) & 110 (107–114)**** & 2.42 & 24 (20–29)$^\text{§§}$ & 0.54 & 24 (20–29)$^\text{§§}$ & 0.54 \\
\text{SFN (3 µmol/l)} & 48 (43–51) & 120 (112–128)**** & 2.53 & 24 (23–25)$^\text{§§}$ & 0.51 & 25 (23–27)$^\text{§§}$ & 0.53 \\
\text{AITC (15 µmol/l)} & 46 (40–52) & 109 (99–119)**** & 2.38 & 25 (23–27)$^\text{§§}$ & 0.55 & 25 (23–27)$^\text{§§}$ & 0.54 \\
\end{array}
\]

\(Nrf2^{+/+}\) and \(Nrf2^{-/-}\) MEFs (1.32 × 10^6 cells) were seeded into 96-well plates and allowed to adhere for 24 h before they were pre-treated with 15 µM CETP, 3 µM SFN or 15 µM AITC for a further 24 h; treatment with 0.1% (v/v) dimethyl sulfoxide (DMSO) was used as a negative control. Thereafter, the fibroblasts were exposed for 24 h to increasing concentrations of acrolein (dissolved in ethanol to give a final concentration of 0.5% by volume) in fresh culture medium. Cell viability was measured by the MTT assay. The fold change in survival was calculated using 0.1% DMSO-treated \(Nrf2^{+/+}\) fibroblasts as the reference. Statistically significant increases in survival are indicated with an asterisk and significant decreases in survival are indicated by the symbol ‡, with \(P\) values <0.05 (**), <0.01 (**‡) or <0.001 (**‡‡).

Concluding comments

In this paper, we show for the first time that naturally occurring epiphenithionitriles induce ARE-driven gene expression in a rodent hepatocyte cell line and that CETP can confer resistance against acrolein. Such phytochemicals can therefore be considered to represent a previously unrecognized class of cancer chemopreventive blocking agent. From our data showing that CETP has similar cytoprotective effects as AITC, it can be inferred that the presence of ESP in plants such as cabbage, that have high levels of Sin, is not necessarily undesirable; this conclusion depends on the bioavailability of the two hydrolysis products, and further work is required to address this issue. Our finding that CETB and CETPent were relatively poor inducing agents, however, suggests that in plants with high levels of glucovanil and glucobrassicanapin, the presence of ESP may diminish the anti-cancer properties of the vegetables.

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References


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