ELSEVIER

Biochemical Pharmacology 64 (2002) 553–564

Biochemical Pharmacology

Commentary

Toxic, halogenated cysteine *S*-conjugates and targeting of mitochondrial enzymes of energy metabolism

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Abstract

Several haloalkenes are metabolized in part to nephrotoxic cysteine *S*-conjugates; for example, trichloroethylene and tetrafluoroethylene are converted to *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC) and *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine (TFEC), respectively. Although DCVC-induced toxicity has been investigated since the 1950s, the toxicity of TFEC and other haloalkene-derived cysteine *S*-conjugates has been studied more recently. Some segments of the US population are exposed to haloalkenes either through drinking water or in the workplace. Therefore, it is important to define the toxicological consequences of such exposures. Most halogenated cysteine *S*-conjugates are metabolized by cysteine *S*-conjugate β-lyases to pyruvate, ammonia, and an α-chloroenethiolate (with DCVC) or an α-difluoroalkylthiolate (with TFEC) that may eliminate halide to give a thioacyl halide, which reacts with ϵ-amino groups of lysine residues in proteins. Nine mammalian pyridoxal 5′-phosphate (PLP)-containing enzymes catalyze cysteine *S*-conjugate β-lyase reactions, including mitochondrial aspartate aminotransferase (mitAspAT), and mitochondrial branched-chain amino acid aminotransferase ($BCAT_m$). Most of the cysteine *S*-conjugate β-lyases are syncatalytically inactivated. TFEC-induced toxicity is associated with covalent modification of several mitochondrial enzymes of energy metabolism. Interestingly, the α-ketoglutarate- and branched-chain α-keto acid dehydrogenase complexes (KGDHC and BCDHC), but not the pyruvate dehydrogenase complex (PDHC), are susceptible to inactivation. mitAspAT and $BCAT_m$ may form metabolons with KGDHC and BCDHC, respectively, but no PLP enzyme is known to associate with PDHC. Consequently, we hypothesize that not only do these metabolons facilitate substrate channeling, but they also facilitate toxicant channeling, thereby promoting the inactivation of proximate mitochondrial enzymes and the induction of mitochondrial dysfunction.

Keywords: *S*-(1,2-Dichlorovinyl)-L-cysteine; Metabolons; Mitochondrial toxicants; Substrate channeling; Tetrachloroethylene; Tetrafluoroethylene; *S*-(1,1,2,2-Tetrafluoroethyl)-L-cysteine; Trichloroethylene

1. Historical

In 1916, it was reported that cattle fed soybean meal extracted with trichloroethylene developed aplastic anemia [1]. The toxic compound present in trichloroethylene-extracted soybean meal was identified as the cysteine *S*-conjugate DCVC [2]. DCVC induces aplastic anemia only in cattle [3], but is nephrotoxic in all experimental animals tested (for reviews, see [4–10]). Cysteine *S*-conjugates are intermediates in the mercapturate pathway, which was discovered over 100 years ago [11]. This pathway is important for the detoxification of exogenous electrophiles

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Abbreviations: AlaAT, alanine aminotransferase; AGATII, alanine:glyoxylate aminotransferase isozyme II; $BCAT_c$ and $BCAT_m$, cytosolic and mitochondrial branched-chain amino acid aminotransferase, respectively; BCDHC, branched-chain α-keto acid dehydrogenase complex; BTC, *S*-(2-benzothiazolyl)-L-cysteine; cytAspAT, cytosolic aspartate aminotransferase; cytGTK, cytosolic glutamine transaminase K; DCVC, *S*-(1,2-dichlorovinyl)-L-cysteine; GST, glutathione transferase; KAT, kynurenine aminotransferase; KGDHC, α-ketoglutarate dehydrogenase complex; mitAspAT, mitochondrial aspartate aminotransferase; mitHSP70, mitochondrial HSP70; PDHC, pyruvate dehydrogenase complex; PDI, protein disulfide isomerase; PLP, pyridoxal 5′-phosphate; TCA, tricarboxylic acid; TFEC, *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine.

0006-2952/02/$ – see front matter
PII: S0006-2952(02)01076-6

and in the elimination of some endogenous electrophiles (e.g. leukotrienes) as follows:

Electrophile → glutathione *S*-conjugate
→ L-cysteinglycine *S*-conjugate
→ L-cysteinyl *S*-conjugate
⇆ *N*-acetyl L-cysteinyl *S*-conjugate(mercapturate)
→ excretion

Most mercapturates are less toxic and more water-soluble than the parent compounds and are readily excreted.

Glutathione *S*-conjugates are formed by the action of GSTs [12]. Haloalkenes and dichloroacetylene undergo GST-catalyzed vinylic substitution (S_NV) reactions, which may be either an addition reaction (e.g. with tetrafluoroethylene, Eq. (1); dichloroacetylene, Eq. (2) or an addition–elimination reaction (e.g. with trichloroethylene, Eq. (3). GSTs also catalyze reactions with epoxide moieties (e.g. in the conversion of leukotriene A_4 to leukotriene C_4) (for a review, see [13]). Glutathione *S*-conjugate formation with haloalkenes and dichloroacetylene is catalyzed by microsomal GST [9] and by cytosolic GSTs [14–16]. The mercapturate pathway is most prominent in liver and kidney, but all of the constituent enzymes are present in most other organs. Thus, many tissues have the capacity to convert glutathione *S*-conjugates to cysteine *S*-conjugates.

$$F_2C{=}CF_2 + GS^- + H^+ \rightarrow F_2C(H)CF_2SG \quad (1)$$

$$ClC{\equiv}CCl + GS^- + H^+ \rightarrow ClC(H){=}C(Cl)SG \quad (2)$$

$$Cl_2C{=}CHCl + GS^- \rightarrow ClC(H){=}C(Cl)SG + Cl^- \quad (3)$$

2. Cysteine *S*-conjugate β-lyases

Although the mercapturate pathway can lead to detoxification, it can also result in bioactivation (i.e. formation of a metabolite that is more toxic than the parent electrophile). Such bioactivation occurs with haloalkenes and dichloroacetylene and is generally brought about by the action of cysteine *S*-conjugate β-lyases on the corresponding halogenated cysteine *S*-conjugates.

Cysteine *S*-conjugate β-lyases contain PLP and catalyze the biotransformation of cysteine *S*-conjugates to aminoacrylate [$CH_2{=}C(NH_2)CO_2H$] and an α-chloroenethiolate (with DCVC) or an α-difluoroalkylthiolate (with TFEC) that may eliminate halide to give a thioacyl halide. The aminoacrylate thus formed undergoes rearrangement to the α-imino acid [$CH_3C(=NH)CO_2H$], which is hydrolyzed to pyruvate and ammonia. The net reaction is shown in Eq. (4)

$$RSCH_2CH(NH_3^+)CO_2^- + H_2O \rightarrow CH_3C(O)CO_2^-(\text{pyruvate}) + NH_4^+ + RSH \quad (4)$$

The toxicity of most halogenated cysteine *S*-conjugates is associated with the formation of a reactive thioacyl halide. For example, 1,2-dichloroethenethiolate formed from DCVC is highly reactive and may give rise to a thioketene (Eq. (5)), which thioacylates macromolecules, particularly lysine residues in proteins (Eq. (6)) [9,17]. The reactive metabolite eliminated from the toxic cysteine *S*-conjugate TFEC yields difluorothionoacetyl fluoride (Eq. (7)) [9], which also thioacylates lysine residues (Eq. (8)) [18,19].

$$[ClC(H){=}C(Cl)SH] \rightarrow ClC(H){=}C{=}S + HCl \quad (5)$$

$$ClC(H){=}C{=}S + H_2NR \rightarrow ClCH_2C(=S)NHR \quad (6)$$

$$F_2C(H)CF_2SH \rightarrow F_2C(H)C(=S)F + HF \quad (7)$$

$$F_2C(H)C(=S)F + H_2NR \rightarrow F_2C(H)C(=S)NHR \quad (8)$$

Cysteine *S*-conjugates derived from bromine-containing fluoroalkenes are more mutagenic than those lacking bromine. The mutagenicity of these cysteine *S*-conjugates may be associated with the formation of a reactive 2,2-difluoro-3-halothiirane following a β-lyase reaction [20–22].

3. Selenocysteine *Se*-conjugate β-lyases

Selenocysteine *Se*-conjugates are β-lyase/transaminase substrates of highly purified rat kidney cytGTK [23]. The compounds are also substrates of multiple cysteine *S*-conjugate β-lyases in human and rat kidney cytosol [23,24]. It was suggested that selenocysteine *Se*-conjugates might be useful as prodrugs to target pharmacologically active selenol compounds to human kidney [24]. A flavin-containing monooxygenase in rat liver microsomes converts *Se*-benzyl-L-selenocysteine and *S*-benzyl-L-cysteine to the corresponding selenoxide and sulfoxide, respectively [25]. The selenoxide of *Se*-benzyl-L-selenocysteine, but not the sulfoxide of *S*-benzyl-L-cysteine, readily undergoes a *syn* elimination to yield aminoacrylate [25]. Selenocysteine *Se*-conjugates may also possibly be bioactivated by L-amino acid oxidase [26].

4. Toxicity of cysteine *S*-conjugates derived from haloalkenes

4.1. Bioactivation by cysteine S-conjugate β-lyases

Trichloroethylene is metabolized in part to DCVC, and, as noted above, this cysteine *S*-conjugate is nephrotoxic. The reactive metabolites generated from toxic cysteine *S*-conjugates by the action of β-lyases are cytotoxic to renal epithelial cells, and their cytotoxicity is associated with covalent modification of macromolecules, depletion of non-protein thiols (such as glutathione), and initiation of lipid peroxidation [27], but other mechanisms may contribute (see below). In the kidney, the S_2 and especially the S_3 regions of the proximal tubules are most susceptible to cysteine *S*-conjugate-induced toxicity [28].

Trichloroethylene is used in industry as a solvent and degreasing agent, and this use may be accompanied by worker exposure. In addition, trichloroethylene and tetrachloroethylene (another haloalkene that is converted to a toxic cysteine *S*-conjugate *in vivo*) are found as contaminants of ground water in certain parts of the US [29]. Tetrafluoroethylene, which is used in industry as a precursor of Teflon™, is nephrotoxic in experimental animals [30], presumably as a result of its conversion to TFEC [18,19]. Moreover, some segments of the general population may be exposed to haloalkenes [31].

Human kidneys contain cysteine *S*-conjugate β-lyase activity [32]. Accordingly, DCVC is toxic to freshly isolated human proximal tubular cells, as measured by lactate dehydrogenase release [33]. An initial report [34] purporting that cardboard workers exposed to high concentrations of trichloroethylene may be at an elevated risk of kidney cancer was questioned initially [35], but other evidence now available supports the contention that workers exposed to high concentrations of trichloroethylene may have an increased incidence of renal carcinomas [36,37]. These cancers are associated with a somatic mutation in the von Hippel-Lindau tumor suppressor gene [36]. In addition, DCVC induces expression of the proto-oncogenes c-*fos* and c-*myc* in LLC-PK1 cells [38,39]. Although there is evidence indicating that long-term exposure to haloalkenes and, thus, to toxic cysteine *S*-conjugates, may induce renal and liver tumor formation, there is strong evidence that many halogenated cysteine *S*-conjugates interfere with mitochondrial energy metabolism (see below). The connection between long-term exposure to haloalkenes, tumor formation, and mitochondrial dysfunction is, however, not clear.

Neurotoxicity may be associated with exposure to some haloalkenes. Trichloroethylene was used formerly as an anesthetic, but its use has largely been discontinued because of reports of neurotoxicity ([40,41] and references cited therein). Trichloroethylene and dichloroacetylene have been detected in some work areas and may pose a risk to exposed workers [42]. As mentioned above, dichloroacetylene, like trichloroethylene, is metabolized to DCVC. However, dichloroacetylene is much more readily converted to DCVC *in vivo* than is trichloroethylene, presumably because it is a much better substrate for GSTs [9,43]. Trichloroethylene can be converted to dichloroacetylene under alkaline conditions, and dichloroacetylene appears to be the causative agent in reports of neurotoxicity in humans exposed to trichloroethylene [44]. A post-mortem study of a severely affected individual exposed to trichloroethylene revealed neuronal degeneration within the brainstem sensory nucleus of the trigeminal nerve and degeneration of axons within its tract [40,41]. Similar damage has been found to occur in laboratory animals exposed to dichloroacetylene [45]. Because dichloroacetylene is such a good GST substrate and the resulting cysteine *S*-conjugate (i.e. DCVC) is toxic, DCVC is likely the causative agent. The brain contains several GSTs [46], including microsomal GST [47,48]. Moreover, cysteine *S*-conjugates that are formed in the liver (a major source of GSTs including microsomal GST) can be released into the circulation and distributed to other organs, including the brain. DCVC, for example, is readily transported across the blood–brain barrier by the L-system amino acid transporter [49].

Interestingly, the pattern of cranial neuropathy observed in severe trichloroethylene-induced neurotoxicity is similar to the lesions that result from activation of orofacial *Herpes simplex* [50]. Therefore, exposure to haloalkene-derived cysteine *S*-conjugates may lead to immune system dysfunction and the activation of a dormant virus.

The discussion above has focused largely on DCVC and TFEC, but cysteine *S*-conjugates formed from many other haloalkenes are also selective nephrotoxicants. 2-Bromo-2-chloro-1,1-difluoroethylene, chlorotrifluoroethylene, 1,1-dichloro-2,2-difluoroethylene, 2-(fluoromethoxy)-1,1,3,3,3-pentafluoro-1-propene (Compound A), hexachloro-1,3-butadiene, hexafluoropropene, tetrachloroethylene, and 1,1,2-trichloro-3,3,3-trifluoro-1-propene are all converted to nephrotoxic cysteine *S*-conjugates, which are bioactivated by β-lyases (for a review, see [13]). Compound A, which is formed from the volatile anesthetic sevoflurane in the anesthesia circuit [51], is of particular interest because of the widespread clinical use of this anesthetic. Compound A and the derived cysteine *S*-conjugates *S*-[2-(fluoromethoxy)-1,1,3,3,3-pentafluoropropyl]-L-cysteine and *S*-[2-(fluoromethoxy)-1,3,3,3-tetrafluoro-1-propenyl]-L-cysteine are nephrotoxic in rats [52–55]. Metabolites attributed to the mercapturic acid pathway and β-lyase-dependent biotransformation of Compound A are excreted in the urine of human subjects anesthetized with sevoflurane and, thereby, exposed to Compound A [56]. This is an important observation because it demonstrates bioactivation of a haloalkene by the β-lyase pathway in humans under actual exposure conditions. Although Compound A is converted to cysteine *S*-conjugates that are metabolized by cysteine *S*-conjugate β-lyase in humans, Compound A-associated nephrotoxicity has not been observed in humans, apparently because of the relatively low cysteine *S*-conjugate β-lyase activity in human kidneys [32,57,58]. Others have questioned whether the β-lyase reaction is the main route for bioactivation of Compound A [59,60].

4.2. Other bioactivation mechanisms

Although β-lyase-dependent biotransformation is the most common bioactivation mechanism for cysteine *S*-conjugates, other routes of bioactivation have been described. The mercapturic acids of some cysteine *S*-conjugates are as nephrotoxic as the cysteine *S*-conjugates themselves [61]. Aminoacylase catalyzes the hydrolysis of many mercapturic acids [62,63]. Hence, the toxicity of these mercapturic acids is attributable to the β-lyase-dependent bioactivation of the

released cysteine *S*-conjugates. Interestingly, the mercapturic acids derived from the cysteine *S*-conjugates of Compound A undergo little aminoacylase-catalyzed hydrolysis [64]; for Compound A, mercapturic acid formation is a true detoxification pathway.

The flavoprotein-dependent monooxygenase catalyzes the sulfoxidation of DCVC to give *S*-(1,2-dichlorovinyl)-L-cysteine *S*-oxide [65], which is nephrotoxic in rats [66]. The role of sulfoxidation in the observed nephrotoxicity of DCVC is unclear. The finding that *S*-(1,1-dichlorovinyl)-α-methyl-L-cysteine, which would be expected to undergo sulfoxidation, is not nephrotoxic may indicate that sulfoxidation does not play a major role in the bioactivation of DCVC *in vivo* [67].

Sulfoxidation, however, does play a role in the bioactivation of some mercapturic acids. *S*-(*cis*-3-Chloropropenyl)-*N*-acetyl-L-cysteine and *S*-(*trans*-3-chloropropenyl)-*N*-acetyl-L-cysteine, the mercapturic acids of the soil fumigant 1,3-dichloropropene, are cytotoxic to pig kidney-derived LLC-PK1 cells, and their cytotoxicity is blocked by the flavoprotein-dependent monooxygenase inhibitor methimazole [68], indicating a role of sulfoxidation. The cytotoxicity of these mercapturic acid sulfoxides can be rationalized by a [2,3] sigmatropic rearrangement resulting in the formation of a labile sulfenate ester, which may release acrolein. The acrolein-derived glutathione *S*-conjugate *S*-(3-oxopropyl)glutathione is nephrotoxic in rats [69]. The corresponding mercapturic acid *S*-(3-oxopropyl)-*N*-acetyl-L-cysteine was studied as a surrogate to investigate the mechanism of bioactivation [70]. *S*-(3-Oxopropyl)-*N*-acetyl-L-cysteine is cytotoxic in LLC-PK1 cells, and its cytotoxicity, but not that of *S*-(3-oxopropyl)-*N*-acetyl-L-cysteine *S*-oxide, is blocked by methimazole, suggesting a role for sulfoxidation. The cytotoxicity can be attributed to the sulfoxidation of the mercapturic acid, which undergoes a *retro*-Michael reaction to eliminate the highly cytotoxic acrolein. Support for this mechanism is found in the observation that *S*-(3-oxopropyl)-*N*-acetyl-L-cysteine *S*-oxide undergoes a general-base-catalyzed *retro*-Michael reaction to give acrolein.

Base propenals, which are formed during the degradation of DNA bases, are highly cytotoxic [71]. Other α,β-unsaturated aldehydes (e.g. *trans*-4-hydroxy 2,3-nonenal), which are products of radical reactions and lipid peroxidation, are also highly cytotoxic [72]. Human [72,73] and bovine [74] GSTs catalyze the conjugation of glutathione with cytotoxic alkenals, and *trans*-4-hydroxy 2,3-nonenal is metabolized in part to mercapturates in rats [75]. However, a role for cysteine *S*-conjugate formation in the toxicity of alkenals has not been established.

Methazolamide, which causes ocular toxicity, is metabolized to glutathione *S*-conjugates and cysteine *S*-conjugates [76]. Recently it was shown that the cysteine *S*-conjugate is a substrate of β-lyases in bovine kidney and liver homogenates [77]. This finding may account for the binding of a metabolite to macromolecules [77].

5. Mitochondria as targets for toxic cysteine *S*-conjugates

Much evidence suggests that mitochondria are especially vulnerable to the toxic effects of cysteine *S*-conjugates [78–92]: (a) Stonard and Parker [78] showed that DCVC progressively inhibited pyruvate/malate- and α-ketoglutarate-stimulated respiration in rat liver mitochondria. Moreover, because of the progressive nature of the inhibition, Stonard and Parker suggested that a DCVC metabolite is responsible. (b) The cytotoxicity of *S*-(pentachlorobutadienyl)glutathione in isolated rat renal epithelia is associated with the loss of cellular thiols, the formation of plasma membrane blebs (that coincide with the loss of calcium from the mitochondrial compartment), the inhibition of cellular respiration, and the depletion of cellular ATP concentrations [80]. The toxicity of *S*-(pentachlorobutadienyl)glutathione is blocked by inhibitors of PLP enzymes and of γ-glutamyltranspeptidase, indicating that the toxicity is associated with metabolism of the glutathione *S*-conjugate to the cysteine *S*-conjugate and metabolism of the latter to a reactive intermediate that is toxic to mitochondria [80]. (c) The cysteine *S*-conjugate of hexachlorobutadiene is toxic to renal mitochondria [81–83] and inhibits state 3 mitochondrial respiration in the presence of succinate [83]. (d) Ca^{2+} homeostasis in mitochondria is disrupted by DCVC [84,85]. (e) *S*-(Pentachlorobutadienyl)-L-cysteine uncouples oxidative phosphorylation in isolated mitochondria by dissipating the protein gradient [86]. The same study showed toxicant-induced swelling of the mitochondria in NH_4Cl or NaCl solution. (f) Succinate:ubiquinone reductase in isolated rat renal proximal cells is inhibited by toxic cysteine *S*-conjugates and the concentration of ubiquinol is decreased [87]. (g) Buckberry *et al*. [88] showed that the toxicity of several cysteine *S*-conjugates toward human Chang liver cells followed the rank order of these compounds as substrates of C-S lyase (i.e. cysteine *S*-conjugate β-lyase) activity in the mitochondrial fraction. (h) In more recent work, Chen *et al*. [89] showed that apoptosis is induced in LLC-PK1 cells exposed to 0.5 mM DCVC. After 4 hr, the mitochondrial membrane potential was decreased; after 6 hr, cytochrome *c* was released and caspase 3 activity was detected; and at 8 hr, extensive DNA fragmentation was observed and cellular ATP concentrations began to decline [89]. (i) Several kidney mitochondrial (but not cytosolic) proteins are covalently modified by thioacylation of lysine residues after rats are exposed to nephrotoxic doses of TFEC [90]. Two of the more intensely modified proteins were the heat shock proteins HSP60 and mitHSP70. In addition, mitAspAT was also modified [90]. (j) Administration of TFEC to rats results in a time-dependent loss of renal KGDHC activity (but not PDHC activity) [91]. Intriguingly, the E2k (=E2o) and E3 subunits of KGDHC are labeled by a metabolite of TFEC, but the E2p and E3 subunits of PDHC are not labeled. The E3 subunits (but not

Table 1
Mammalian PLP-dependent enzymes with L-cysteine *S*-conjugate β-lyase activity[a,b]

	β-Lyase substrates			Syncatalytic inactivation	Competing transamination	Approximate specific activity[c] (U/mg)	Selected references
	DCVC	TFEC	BTC				
Enzyme (cytosolic)							
Kynureninase (R)	+	ND	+	+	ND	0.25	[98,99]
cytGTK/KAT (R)[d,e]	+	+	−	−	+	0.6–6.4	[100,122]
cytAspAT (R)[f]	+	+	−[g]	+	−	0.04–0.16	[104–108]
AlaAT (P)[f]	+	+	+	+	−	0.004–0.06	[104,105,108]
$BCAT_c$ (H)[f]	+	+	−	+	−	0.3–0.5	
Enzyme (mitochondrial)							
mitAspAT (R)[f]	+	+	+	+	+	0.8–2.3	
$BCAT_m$ (H)[f]	+	+	+	+	−	0.2–0.5	
AGATII (R)	+	+	−	+	ND	0.2	Unpublished
High-M_r β-lyase (R)[h]	+	+	+	−	+	1.0–1.2	[118–120]

[a] A unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol pyruvate/min (usually at 37°), but temperature was not specified in all references cited). ND, not determined. Species abbreviations: R, rat; P, pig; and H, human.

[b] All the enzymes listed except AGATII and the high-M_r β-lyase are homodimers with M_r values for the intact holoenzymes of ∼90,000–110,000. AGATII is a homotetramer ($M_r \sim 210{,}000$). The high-M_r-β-lyase ($M_r > 200{,}000$) of rat kidney homogenates co-purifies with PDI and mitHSP70 [120].

[c] Activity with DCVC and/or TFEC.

[d] cytGTK and KAT have very similar substrate specificities and may be the same enzyme. However, the amino acid sequence obtained by two groups [134,135] specificities is sightly different from that found by another group [136]. Possibly, alternative splicing of a single mRNA occurs [136].

[e] Human liver containes a KAT with strong cysteine *S*-conjugate β-lyase activity [137]. A cysteine *S*-conjugate β-lyase has been highly purified from human kidney [32]. The lyase activity co-purifies with cytGTK. Curiously, the human cytGTK, unlike the rat enzyme, has avtivity with BTC [32].

[f] Manuscripts submitted (footnotes to the text 1 and 2).

[g] Adcock *et al*. reported a value of ∼0.04 μmol/min/mg for BTC as a substrate of pig heart cytAspAT [105], but we were unable to detect BTC-lyase activity with this enzyme.

[h] Probably also present in the cytosolic fraction [119].

the E2 subunits) are identical in KGDHC, PDHC, and BCDHC [93]. (E3 is also a component of the glycine cleavage system [93].) (k) Aconitase [94] and the E3 subunits of BCDHC [92] are also targeted in kidneys of rats given TFEC.

6. Mammalian PLP-dependent cysteine *S*-conjugate β-lyases

In 1965, Colucci and Buyske identified a thiol metabolite of benzothiazolyl 2-sulfonamide in rabbits, rats, and dogs [95]; this was likely the first description in the literature of C-S lyase activity associated with the metabolism of a xenobiotic in mammalian tissues. Later, a mammalian cysteine *S*-conjugate β-lyase was described that cleaved cysteine *S*-conjugates of drugs [96]. A cysteine *S*-conjugate β-lyase from an enteric bacterium (*Eubacterium limosum*) has been characterized [97]. This enzyme catalyzes a β-cystathionase reaction (also a β-lyase scission of a C–S bond). Nine mammalian cysteine *S*-conjugate β-lyases have been identified (Table 1). All are PLP-dependent enzymes, and all but one have well-defined roles in amino acid metabolism. The substrate selectivity of these PLP enzymes apparently allows them to catalyze a non-physiological β-elimination reaction because of the excellent leaving group nature of the substituents on the sulfur of the PLP-cysteine *S*-conjugate aldimine at the active site (see, for example, [98]).

6.1. Cytosolic cysteine S-conjugate β-lyases

Major cysteine *S*-conjugate β-lyases of rat liver and kidney cytosol have been identified as kynureninase [98,99] and cytGTK [100], respectively. Kynureninase, but not cytGTK, is syncatalytically inactivated by the cysteine *S*-conjugate substrate. cytGTK requires an added α-keto acid (e.g. α-keto-γ-methiolbutyrate or phenylpyruvate) to support maximal β-lyase activity [100]. Stevens and colleagues [101] detected cytGTK immunohistochemically in the S_1, S_2, and S_3 regions of the proximal tubules, but not in other regions of the kidney. MacFarlane *et al*. [102] observed immunohistochemical staining and cytGTK mRNA in the S_3 region of the kidney. The presence of cytGTK in the proximal tubules has also been demonstrated by use of selective toxicants that release this enzyme from the tubules [103]. Because cytGTK has relatively strong β-lyase activity, it has been regarded as a possible major contributor to halogenated cysteine *S*-conjugate-induced kidney damage, but this enzyme is unlikely to contribute directly to the mitochondrial dysfunction associated with toxic cysteine *S*-conjugates (see below).

Other mammalian cytosolic PLP-containing enzymes that catalyze a cysteine *S*-conjugate β-lyase reaction include pig heart cytAspAT [104–108], pig heart AlaAT [104,105,108], and human cytosolic $BCAT_c$.[1] All three

[1] Cooper AJL, Bruschi SA, Conway M, Hutson SM. Manuscript submitted for publication.

enzymes are syncatalytically inactivated by the cysteine *S*-conjugate substrate.

6.2. Mitochondrial cysteine S-conjugate β-lyases

The findings mentioned above that (a) mitochondria are especially vulnerable to toxic cysteine *S*-conjugates, and (b) thioacylating metabolites derived from TFEC covalently modify mitochondrial (but not cytosolic) proteins indicate that cysteine *S*-conjugates are transported into mitochondria and that mitochondria contain substantial cysteine *S*-conjugate β-lyase activity. In support of this concept, two groups have detected cysteine *S*-conjugate β-lyase activity in fractionated rat kidney mitochondria. Stevens *et al*. [109] reported mitochondrial cysteine *S*-conjugate β-lyase activity (DCVC as substrate) in the matrix. Lash *et al*. [110] detected cysteine *S*-conjugate β-lyase activity (DCVC and BTC as substrates) in the outer mitochondrial membrane. Possibly, multiple cysteine *S*-conjugate β-lyases are distributed in the matrix and outer membrane.

Which cysteine *S*-conjugate β-lyases contribute to mitochondrial toxicity? Although, as noted above, purified cytGTK exhibits appreciable cysteine *S*-conjugate β-lyase activity, it may not be the major enzyme involved in cysteine *S*-conjugate-induced mitochondrial toxicity in rat kidney. High (non-physiological) levels of α-keto acid cytGTK substrates (α-keto-γ-methiolbutyrate or phenylpyruvate) are required to potentiate the toxic effects of DCVC in kidney preparations [111]. Moreover, fractionation studies of rat kidney showed that most of the GTK activity (as measured by the L-phenylalanine:α-keto-γ-methiolbutyrate transaminase activity associated with GTK) is located in the cytosol [112] and that the fraction containing GTK activity in the kidney mitochondria exhibits little cysteine *S*-conjugate β-lyase activity [113].

We have found recently that human $BCAT_m$ (see footnote 1), rat liver mitAspAT,[2] and rat kidney mitochondrial AGATII[3] possess cysteine *S*-conjugate β-lyase activity. Each of these aminotransferases is syncatalytically inactivated during turnover of the cysteine *S*-conjugates. mitAspAT is present in almost all mammalian tissues in high amounts [114]. $BCAT_m$ is present in most rat tissues with the notable exception of liver [115]; the enzyme is, however, present in human liver [116]. In the rat, AGATII is most active in kidney and to a lesser extent in liver [117]. All three enzymes are present in the kidney mitochondrial matrix and presumably contribute to the total mitochondrial cysteine *S*-conjugate β-lyase activity.

6.3. An unusual mitochondrial cysteine S-conjugate β-lyase

When samples of rat kidney homogenates were subjected to non-denaturing polyacrylamide gel electrophoresis and stained for cysteine *S*-conjugate β-lyase activity, a low-M_r protein ($M_r \sim 95{,}000$), as expected for cytGTK, was detected [118–120]. In addition, an intense protein band with a high-M_r (~350,000) was also detected [118–120]. This high-M_r protein also exhibited weak methionine:phenylpyruvate transaminase activity [118]. Activity staining of rat homogenates showed that the high-M_r cysteine *S*-conjugate β-lyase is present in kidney and to a lesser extent in liver; no activity was detected in brain [118,119]. The enzyme is active with DCVC, TFEC, and BTC as substrates [120]. In contrast, cytGTK is active with DCVC and TFEC, but not with BTC [120–122]. The purified high-M_r cysteine *S*-conjugate β-lyase (but not cytGTK) catalyzes a β-lyase reaction with leukotriene E_4 and 5′-*S*-cysteinyldopamine [119] and requires an added α-keto acid or PLP for maximal activity. In recent work, we showed that at least two proteins co-purified with the rat kidney high-M_r cysteine *S*-conjugate β-lyase. N-Terminal analysis revealed that the smaller protein was mature PDI ($M_r \sim 54{,}200$) from which the 42-amino acid endoplasmic reticulum signal peptide had been removed [120]. Internal sequencing revealed that the larger protein was mitHSP70. Because of its high reactivity, it is unlikely that the reactive metabolite eliminated from TFEC by cysteine *S*-conjugate β-lyases will travel far before thioacylating a nucleophile. Therefore, the finding that mitHSP70 is a component of a high-M_r cysteine *S*-conjugate β-lyase may provide an explanation for the previous findings that kidney mitHSP70 is thioacylated in rats given TFEC [90].

Precedent exists for the association of an enzyme within a complex that contains HSP70. For example, some catalytically competent brain glutamate decarboxylase is found in association with HSC70 (the constitutively expressed member of the HSP70 family) [123]. In another example, HSP70 binds to partially unfolded mitAspAT as it refolds *in vitro* from the acid-denatured state [124] or during the synthesis of the precursor protein in cell-free extracts [125,126]. These complexes are formed between the apoenzyme and HSP70 and are, therefore, not enzymatically active. However, mitAspAT is ''sticky'', and a fraction of the enzyme is often found within the inner mitochondrial membrane.[4] Moreover, HSP70 is also found in association with the translocon in the inner mitochondrial membrane. Because HSP70 is probably involved in the translocation of mitAspAT into the matrix [124–126], a fraction of the enzyme might remain bound

[2] Cooper AJL, Bruschi SA, Iriate A, Martinez-Carrion M. Manuscript submitted for publication.

[3] Cooper AJL. Unpublished observation.

[4] Dr. Ana Iriarte, University of Missouri-Kansas City, personal communication. Cited with permission.

either directly or indirectly to the translocon machinery or to mitHSP70 after it folds, acquires the PLP cofactor, and becomes active. Therefore, the possibility exists that the PLP component of the high-M_r lyase is mitAspAT (or a closely related enzyme). Work is ongoing to identify the catalytic component of the high-M_r cysteine *S*-conjugate β-lyase.

Precedent also exists for the presence of PDI in an enzyme complex. PDI is a component of prolyl hydroxylase (an $\alpha_2\beta_2$ tetramer; $M_r \sim 250{,}000$) and of microsomal triacylglycerol transfer protein (MTP) [127,128]. In mammals, PDI is also thought to regulate the modulation of liver *S*-adenosylmethionine synthetase by glutathione [129] and to participate in peptide binding, cell adhesion, and, perhaps, chaperoning [130]. In addition, PDI is a membrane-associated thyroid-hormone binding protein that strongly binds T3 [131]. In the cell, PDI is thought to be predominantly present in the endoplasmic reticulum. Recent work has shown that PDI is also present in the mitochondrial outer membrane [132,133]. Therefore, a portion of cellular PDI and mitHSP70 may be present in the same compartment. Further work is necessary, however, to determine the functional significance of PDI and the high-M_r cysteine *S*-conjugate β-lyase.

7. Summary of known mammalian cysteine *S*-conjugate β-lyases

A summary is provided in Table 1. Because different authors have used different assay conditions and different β-lyase substrates, it is not possible to compare directly the specific activities of these enzymes. For example, all of the cysteine *S*-conjugate β-lyases listed in Table 1 accept TFEC and DCVC as substrates, but only some of the enzymes accept BTC. Comparisons are also complicated because of different susceptibilities to syncatalytic inactivation. For example, cytGTK is not inactivated, but $BCAT_c$, $BCAT_m$, and mitAspAT are inactivated on the average after ~40, ~200, and ~2900 turnover events per monomer, respectively, by TFEC (see footnotes 1 and 2). If long incubation times are used for end-point assays of total cysteine *S*-conjugate β-lyase activity in tissue homogenates, a disproportionately high percentage of the activity would be assigned to enzymes that are not inactivated or are inactivated slowly. Nevertheless, Table 1 highlights the prevalence of β-lyase activity among PLP-containing enzymes, particularly aminotransferases. By comparing the specific activities of TFEC lyase and aspartate-α-ketoglutarate transamination reactions of crude rat kidney mitochondria with those of highly purified rat mitAspAT, we estimate that under the conditions of our assay about 18% of the TFEC β-lyase activity of rat kidney mitochondria can be accounted for by mitAspAT (see footnote 2).

8. Mechanism of the syncatalytic inactivation of PLP enzymes by cysteine *S*-conjugates

It has long been known that cytAspAT is syncatalytically inactivated by β-lyase substrates, such as β-chloro-L-alanine and L-serine-*O*-sulfate. Originally, inactivation was attributed to attack by the aminoacrylate intermediate on a crucial amino acid residue [138]. However, evidence from Metzler's group [139,140] showed that inactivation of pig heart cytAspAT and bacterial glutamate decarboxylase by L-serine-*O*-sulfate is accompanied by formation of a PLP-pyruvate aldol product. We showed that the PLP cofactor of pig heart cytAspAT is destroyed after inactivation with TFEC [141]. This finding is strong, but indirect, evidence that aminoacrylate formed from the β-lyase reaction on TFEC also inactivates cytAspAT by forming a PLP-pyruvate aldol product [141]. However, additional mechanisms are also possible for inactivation by TFEC and DCVC, but not by BTC. Presumably, inactivation of enzymes such as kynureninase [98] and $BCAT_m$ (see footnote 2) by BTC, which does not form a reactive sulfur-containing metabolite, is due solely to interactions involving aminoacrylate formed from the cysteine *S*-conjugate at the active site. On the other hand, for cysteine *S*-conjugates such as TFEC and DCVC, where a reactive, thioacylating metabolite is generated, inactivation may involve both addition of aminoacrylate to PLP (or other active site nucleophiles) and thioacylation of susceptible lysine residues. Each subunit of rat mitAspAT is inactivated on the average after about 3900 turnovers in the presence of β-chloro-L-alanine (a β-lyase substrate), but only after 2700 turnovers in the presence of TFEC or DCVC (see footnote 2). Possibly, mitAspAT is inactivated by both aminoacrylate attack on the PLP cofactor (or other active site nucleophile) and thioacylation of a critical lysine residue. In this regard, it is interesting, as noted above, that mitAspAT is modified by a thioacylating metabolite in the kidneys of rats given TFEC [90]. Because mitAspAT is a critical component of the malate:aspartate shuttle for the transport of reducing equivalents across the mitochondrial membrane [142], its inhibition might contribute to overall energy impairment and mitochondrial dysfunction (see also [104,105]).

9. TFEC and toxicant channeling in mitochondrial enzyme complexes

As noted above, the E2k and E3 subunits of kidney KGDHC are thioacylated, but not the E2p and E3 subunits of PDHC, after administration of TFEC to rats [91]. KGDHC activity, but not PDHC activity, is also decreased in the kidneys of TFEC-treated rats. We have noted that KGDHC is strongly inhibited in PC12 cells exposed to 1 mM TFEC, but PDHC is not directly inhibited [141]. It has been suggested that KGDHC, but not PDHC, may be in close proximity to a cysteine *S*-conjugate β-lyase [94].

On the other hand, there is some evidence that the E3 subunits are more tightly bound to E2p subunits in PDHC than they are to the E2k subunits of KGDHC [143,144]. Moreover, PDHC is resistant to inactivation in the presence of TFEC and cytGTK (a source of thioacylating fragments) [141]. Thus, Cooper and colleagues [141] suggested that PDHC might be more inherently resistant to inactivation by thioacylation than KGDHC.

Our recent finding that mitAspAT has strong cysteine *S*-conjugate β-lyase activity (Table 1) raises the possibility that this enzyme contributes to the inactivation of KGDHC. Much evidence suggests that enzymes of the tricarboxylic acid (TCA) cycle and ancillary enzymes are arranged in supramolecular complexes (metabolons) that facilitate substrate channeling [145–149]. For example, mitAspAT is thought to form a metabolon with KGDHC [145–147]. We propose that not only is it possible for metabolites to be channeled, but it may also be possible for toxicants to be channeled through supramolecular complexes. This concept could explain in part why KGDHC is susceptible to inactivation from a reactive metabolite of TFEC in rat kidney and PC12 cells, but PDHC is not (Fig. 1). This concept might also explain why aconitase and BCDHC are also susceptible to inactivation by TFEC metabolites. Aconitase is also thought to be part of a metabolon that involves KGDHC and mitAspAT [149]. As noted above, $BCAT_m$ has cysteine *S*-conjugate β-lyase activity. This enzyme may form a metabolon with BCDHC facilitating transfer of branched-chain α-keto acids to the dehydrogenase complex. As with mitAspAT/KGDHC, such a supramolecular complex might facilitate toxicant channeling from $BCAT_m$ to BCDHC subunits. The resistance of PDHC to thioacylation in rat kidney *in vivo* and the resistance of PDHC to inactivation in rat kidney and PC12 cells may be due in part to its different arrangement of E3 subunits, but also because PDHC does not form a metabolon that includes a PLP-containing enzyme.

10. Conclusion

Nine mammalian cysteine *S*-conjugate β-lyases are currently known (Table 1), and no doubt the list will grow in the future. The toxicity of cysteine *S*-conjugates may be attributable to at least two mechanisms: syncatalytic inactivation of β-lyases may lead to loss of crucial PLP-enzyme activity. For example, incubation of PC12 cells with 1 mM TFEC leads to a time-dependent loss of mitAspAT activity [141], which could lead to disruption of the malate:aspartate shuttle and compromised energy metabolism. The BCATs are intimately involved in the metabolism of the branched-chain aminotransferases. Moreover, the brain is almost unique among tissues in possessing both BCAT isozymes [115]. It has been suggested that the possession of both isozymes may be important for the cycling of branched-chain amino acids in the brain and may play a role in excitatory amino acid metabolism [150]. As a result, cysteine *S*-conjugate-induced inhibition of BCATs may lead to brain dysfunction or developmental CNS alterations, or both. Second, metabolism may be compromised by the selective loss of key mitochondrial TCA enzyme activities, such as KGDHC and aconitase, in part brought about by close juxtapositioning of bioactivating aminotransferases. Finally, because many PLP enzymes possess

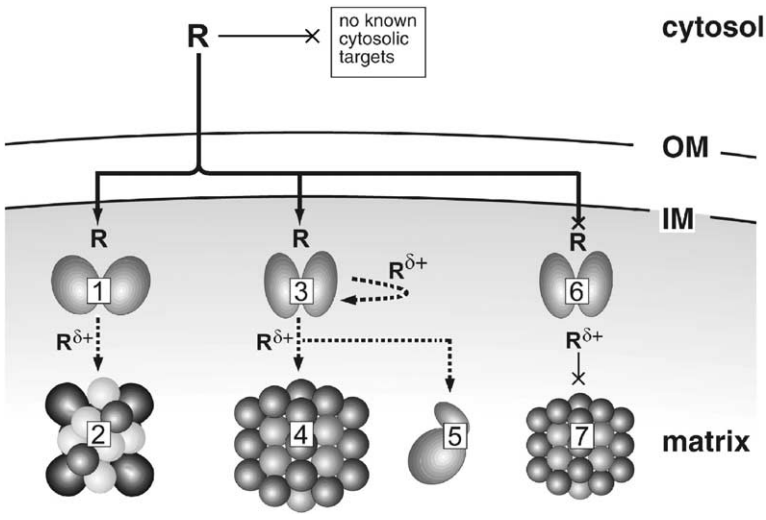

Fig. 1. Model for toxicant channeling in mitochondria *in vivo*. Transport of the pro-toxicant TFEC (R) into the mitochondria is accomplished by an unknown transporter. Once within the mitochondria, TFEC is converted to a toxicant ($R^{\delta+}$, a thioacylating agent) by the action of cysteine *S*-conjugate β-lyases. The mitochondrial cysteine *S*-conjugate β-lyases include the homodimeric $BCAT_m$ (1) and the homodimeric mitAspAT (3). The close juxtapositioning of $BCAT_m$ and mitAspAT to mitochondrial enzymes of energy metabolism results in channeling of the toxicant to BCDHC (2) and KGDHC (4)/aconitase (5), respectively, resulting in their inactivation. On the other hand, PDHC (7) is not known to be associated with any aminotransferase/cysteine *S*-conjugate β-lyase (6), and is not inactivated by direct thioacylation. The curved arrow represents "self-thioacylation" of mitAspAT. OM, outer membrane; IM, inner membrane.

cysteine *S*-conjugate β-lyase activity and many of these enzymes, most notably mitAspAT, are widespread in the body, the potential may exist for cysteine *S*-conjugate-induced cytotoxicity in many tissues.

Acknowledgments

Work from the authors' laboratories cited in the text was supported in part by NIH Grants ES08421 and AG14930 (A.J.L.C.), R29 GM5196 and ES07033 (S.A.B.), and ES03127 (M.W.A.).

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