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Inhibition of Select Mitochondrial Enzymes in PC12 Cells Exposed to *S*-(1,1,2,2-Tetrafluoroethyl)-L-Cysteine

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ABSTRACT. Many halogenated foreign compounds are detoxified by conversion to the corresponding cysteine *S*-conjugate, which is *N*-acetylated and excreted. However, several halogenated cysteine *S*-conjugates [e.g. *S*-(1,1,2,2-tetrafluoroethy)-L-cysteine (TFEC)] are converted to mitochondrial toxicants by cysteine *S*-conjugate β-lyases. In the present work, we showed that TFEC appreciably inactivated highly purified α-ketoglutarate dehydrogenase complex (KGDHC) in the presence of a cysteine *S*-conjugate β-lyase. Incubation of PC12 cells (which contain endogenous cysteine *S*-conjugate β-lyase activity) with TFEC led to a concentration- and time-dependent loss of endogenous KGDHC activity. A 24-hr exposure to 1 mM TFEC decreased KGDHC activity in the cells by 90%. Although treatment with TFEC did not inhibit intrinsic pyruvate dehydrogenase complex (PDHC) activity, it inhibited dichloroacetate/Mg^{2+}-mediated activation/dephosphorylation of PDHC in the PC12 cells by 90%. To determine the selectivity of enzymes targeted by TFEC, several cytosolic and mitochondrial enzymes involved in energy metabolism [malate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, glutamate dehydrogenase, lactate dehydrogenase, cytosolic and mitochondrial aspartate aminotransferases (AspAT)] were also assayed in the PC12 cells exposed to 1 mM TFEC for 24 hr. Of these enzymes, only mitochondrial AspAT, a key enzyme of the malate–aspartate shuttle, was inhibited. The present results demonstrate a selective vulnerability of mitochondrial enzymes to toxic cysteine *S*-conjugates. The data indicate that TFEC may be a useful cellular/mitochondrial toxicant for elucidating the consequences of the diminished mitochondrial function that accompanies numerous neurodegenerative diseases. BIOCHEM PHARMACOL **58**;10: 1557–1565, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. α-ketoglutarate dehydrogenase complex; pyruvate dehydrogenase complex; *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine; cysteine *S*-conjugates; PC12 cells; halogenated xenobiotics

Several halogenated alkenes, such as tetrachloroethylene and trichloroethylene, are in widespread use and are toxic to experimental animals [1, 2], but the mechanisms of their toxicity are not fully determined. Trichloroethylene is often a major chlorinated contaminant of the water table. Because of concerns for human health, the metabolism of halogenated alkenes has been the subject of considerable research (reviewed in Refs. 1–3). The toxicity of halogenated alkenes is due, in part, to formation of the corresponding glutathione *S*-conjugate, which then is converted to the corresponding cysteine *S*-conjugate. Toxic halogenated cysteine *S*-conjugates (e.g. DCVC¶ and TFEC) are substrates of cysteine *S*-conjugate β-lyases. These enzymes catalyze the conversion of the toxic cysteine *S*-conjugates to aminoacrylate [$CH_2{=}C(NH_4^+)CO_2^-$] and "RSH," where the "RSH" is a reactive sulfur-containing fragment. The aminoacrylate is nonenzymatically hydrolyzed to pyruvate and ammonia. Thus, the net reaction is

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

(for reviews, see Refs. 1–4).

Kidney mitochondria are particularly vulnerable to toxic halogenated cysteine *S*-conjugates [5–8]. Although several mechanisms, such as activation of oncogenes, depletion of thiols, oxidative stress, and dissipation of the mitochondrial proton gradient, may contribute to the mitochondrial vulnerability [2, 9–14], a major contributing factor is likely interference with enzymes of energy metabolism. For example, Stonard and Parker [15] showed that DCVC progressively inhibits pyruvate/malate- and αKG-supported respiration in isolated rat liver mitochondria. However, respiration in the presence of succinate (+rotenone) or 3-hydroxybutyrate is unaffected by treatment of the mitochondria with

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¶ *Abbreviations*: αKG, α-ketoglutarate; AspAT, aspartate aminotransferase; cytGTK, cytosolic glutamine transaminase K; DCA, dichloroacetic acid; DCVC, *S*-(1,2-dichlorovinyl)-L-cysteine; DTT, dithiothreitol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GDH, glutamate dehydrogenase; KGDHC, α-ketoglutarate dehydrogenase complex; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; PDHC, pyruvate dehydrogenase complex; TFEC, *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine; PLP, pyridoxal 5′-phosphate; and TCA, tricarboxylic acid.

DCVC [15]. These results suggest that mitochondrial KGDHC [α-ketoglutarate dehydrogenase (E1k), dihydrolipoyl succinyltransferase (E2k) plus dihydrolipoyl dehydrogenase (E3)] and PDHC [pyruvate dehydrogenase (E1p), dihydrolipoyl transacetylase (E2p) plus E3] are the sites of action of the DCVC metabolite. However, Stonard and Parker [15] did not actually measure the activities of the α-keto acid dehydrogenase complexes in the treated mitochondria. Stonard and Parker [15] suggested that DCVC is not toxic *per se*, but is metabolized within the mitochondria (presumably by a cysteine *S*-conjugate β-lyase) to a noxious product. Later, Lock and Schnellmann [6] showed that several haloalkene cysteine *S*-conjugates inhibit glutathione reductase and E3 in kidney cells.

Although earlier workers (including Stonard and Parker [15]) used DCVC as a model compound to study the mechanism by which toxic cysteine *S*-conjugates are harmful (see historical discussions in Refs. 2–4), some researchers more recently have utilized TFEC as a convenient example of a cysteine *S*-conjugate that is derived from a halogenated xenobiotic and is toxic to mitochondria [16–18]. TFEC is more soluble than DCVC and generally is more active as a substrate of cysteine *S*-conjugate β-lyases (Cooper AJL, unpublished observations). Although the detailed mechanisms differ, the reactive sulfur-containing fragments derived from both DCVC and TFEC are strong thioacylating agents [2, 3].

Cysteine *S*-conjugate β-lyase activity is present in the brain [19], and at least one example is known where a halogenated xenobiotic is thought to be metabolized to a neurotoxic cysteine *S*-conjugate. Dichloroacetylene, a breakdown product of trichloroethylene, is neurotoxic to experimental animals [20, 21] and probably is the breakdown product of trichloroethylene responsible for toxicity to humans [22]. Dichloroacetylene is an exceptionally good substrate of microsomal glutathione *S*-transferase and likely gives rise readily to *S*-(1,2-dichlorovinyl)-glutathione and DCVC *in vivo* [1, 2]. We have a long-standing interest in understanding the metabolic role of the α-keto acid dehydrogenase complexes, particularly in the brain [23–25]. We and others [24–27] also are interested in the mechanisms by which these enzyme activities are altered in neurodegenerative diseases and how these alterations contribute to the pathology. Accordingly, we examined the effects of a toxic cysteine *S*-conjugate (i.e. TFEC) on the mitochondrial dehydrogenase complexes in PC12 cells, a rat-derived pheochromocytoma cell line with many neuronal properties.

MATERIALS AND METHODS

Materials

Crystalline pig heart cytosolic AspAT (270 U/mg protein), rabbit muscle LDH (type XXXIX, 720 U/mg protein), mitochondrial pig heart MDH (900 U/mg protein), beef liver GDH (50 U/mg protein), crystalline rabbit muscle GAPDH (120 U/mg protein), all enzyme substrates (except where noted), cofactors, and β-chloro-L-alanine were obtained from the Sigma Chemical Co.

Highly purified KGDHC (2.3 U/mg protein; 25°) and PDHC (1.7 U/mg protein; 25°), isolated from pig heart according to the method of Stanley and Perham [28] with some modifications as described by Bunik and Follmann [29], were used in some experiments. For comparative purposes, in other experiments, purified commercial (Sigma) preparations of PDHC (2.3 U/mg protein; 37°) were used. Rat kidney cytGTK, a major cytosolic cysteine *S*-conjugate β-lyase of rat kidney, was purified according to the method of Cooper [30]. The preparation had a specific activity of ~5 U/mg protein (transaminase assay with phenylalanine as amino donor and α-keto-γ-methiolbutyrate as α-keto acid acceptor) and ~0.5 U/mg protein (cysteine *S*-conjugate β-lyase assay with TFEC as a substrate). cytGTK was used as a convenient catalyst for the generation of a reactive sulfur-containing fragment from TFEC. The fragment [i.e. $HF_2CC(SH)F_2$] is very reactive and short-lived, losing HF to form the thioacylating agent $HF_2CC(=S)F$ [1, 2]. Protein lysine groups are especially sensitive to thioacylation [1, 2]. Except where noted, a unit of activity (U) represents the amount of enzyme that catalyzes the formation of 1 μmol of product/min at 37°.

We thank Dr. James L. Stevens (W. Alton Jones Cell Science Center) for the gift of TFEC. NMR, elemental analyses, and HPLC analysis [16] are consistent with the pure product. A stock solution of 40 mM TFEC in 50 mM Tris–HCl (pH 8.0) was stored frozen at −20°.

Cell Culture

PC12 cells were cultured as described previously [31]. Briefly, cells were maintained at 37° in 6-well culture plates containing Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% (v/v) fetal bovine serum (Sigma) and 5% (v/v) donor horse serum (Life Technology) in a humidified 37° incubator with 10% CO_2. The cultures were split when they reached confluency. Cells were treated with stock TFEC solutions (final concentrations of 0.001 to 1.0 mM). The final concentration of Tris in control and TFEC-treated cells was always 1.25 mM. Each well in the culture plate was estimated to contain 2–3 × 10^6 cells (~0.1 mg protein). The cells were harvested in scraping buffer [10 mM sodium phosphate buffer (pH 7.2) and 150 mM NaCl] and centrifuged at 300 *g* for 5 min. The cells in the pellet were lysed by treatment with 100 μL of "lysate buffer" containing 50 mM Tris–HCl (pH 7.2), 1 mM DTT, 0. 2 mM EGTA, 50 μM leupeptin, and 0.4% (v/v) Triton X-100. Enzyme activities in the cell lysate were determined as described below.

Enzyme Assays

GDH, GAPDH, LDH, total (i.e. mitochondrial plus cytosolic) AspAT, and total MDH activities in the PC12 homogenates were assayed at 30° in a 0.2-mL reaction

mixture containing 100 mM potassium phosphate buffer (pH 7.4) and substrates as indicated: *AspAT* (10 mM αKG, 80 mM l-aspartate, 0.2 mM NADH, and 2 U of pig heart MDH); *MDH* (10 mM oxaloacetate and 0.2 mM NADH); *GDH* (10 mM αKG, 50 mM ammonium chloride, 0.2 mM NADH, and 0.1 mM ADP); *LDH* (0.2 mM NADH and 5 mM pyruvate); *GAPDH* (1 mM NAD^+, 10 mM DTT, 12.5 mM sodium arsenate, and 1 mM glyceraldehyde 3-phosphate). The reaction was initiated by the addition of 2–10 μL of the cell homogenate (except in the case of MDH, where the cell homogenate was diluted 10-fold with distilled water before assay). To ensure that nonenzymatic breakdown of oxaloacetate to pyruvate in the MDH-assay mixture was minimal, solid oxaloacetic acid was added to the stock reaction mixture immediately before it was pipetted into the wells. To distinguish between the cytosolic and mitochondrial forms of AspAT, 20 μL of homogenate was diluted 1:1 in 100 mM potassium phosphate buffer (pH 7.0) containing 8 mM αKG. The activity of AspAT in this diluted homogenate was measured immediately before and immediately after heating at 65° for 15 min. Mitochondrial AspAT activity is destroyed by this treatment, whereas the cytosolic isoform is stable [32]. For each enzyme assay, two controls were used: (i) a reaction mixture lacking tissue homogenate; and (ii) a reaction mixture lacking α-keto acid substrate (or glyceraldehyde 3-phosphate in the case of GAPDH). Changes in NADH absorbance at 340 nm ($\epsilon = 6.23 \times 10^3$) were monitored in a SpectraMax 96-well plate analyzer (Molecular Devices).

To measure cysteine *S*-conjugate β-lyase activity, an aliquot (10 μL) of the cell homogenate was incubated in a reaction mixture (25 μL) containing 100 mM potassium phosphate buffer (pH 7.4), 0.05 mM PLP, and 10 mM TFEC. After incubation at 37° for 1 hr, 25 μL of 5 mM 2,4-dinitrophenylhydrazine in 2 M HCl was added. After a further 10 min, 150 μL of 1 M NaOH was added, and the absorbance at 430 nm was determined relative to a blank to which homogenate was added after the addition of 2,4-dinitrophenylhydrazine reagent. The molar extinction coefficient of pyruvate 2,4-dinitrophenyl-hydrazone under these conditions is ~15,000.

KGDHC and PDHC activities in the PC12 homogenates, and PDHC in the commercial preparation, were determined as described previously [24, 33], except that the volume of the reaction mixture was 0.2 mL. PDHC exists in active (dephosphorylated) and inactive (phosphorylated) forms. Total (i.e. dephosphorylated) PDHC activity was obtained in parallel cell cultures by the removal of the culture medium and treatment of the cells with scraping buffer containing 5 mM DCA, 10 mM $MgCl_2$, and 1 mM $CaCl_2$ for 30 min at 37° prior to harvesting. Then the cells were harvested as described above, except that the lysate buffer also contained 5 mM DCA, 10 mM $MgCl_2$, and 1 mM $CaCl_2$. DCA inhibits PDHC kinase activity and has been used to activate PDHC fully in cell cultures [34]. Mg^{2+} activates PDHC phosphatase [28].

In studies of the inhibitory effects of TFEC/cytGTK on purified KGDHC and PDHC, the activities were measured as specified in the legends to Tables 1 and 2. In a separate experiment, the effects of TFEC/cytGTK on several isolated enzymes of energy metabolism other than KGDHC and PDHC were also investigated. The highly purified commercial enzyme [cytosolic AspAT (405 mU), LDH (216 mU), mitochondrial MDH (180 mU), GDH (125 mU), or GAPDH (60 mU)] was added to a 100-μL incubation mixture containing 100 mM potassium phosphate buffer (pH 7.4), and where indicated, 12 μL of cytGTK (~3 mU of lyase activity) and 5 mM TFEC. In incubation mixtures containing GAPDH, 10 mM DTT also was included. In incubation mixtures containing AspAT, 1 mM αKG was added. Four incubations were carried out: (i) no addition, (ii) addition of TFEC, (iii) addition of cytGTK, and (iv) addition of TFEC/cytGTK. Aliquots (2–5 μL) were withdrawn and assayed for enzyme activity immediately as described above (N = 3–6). After incubation at 37° for 1 hr, the activities were determined again.

Measurement of PLP in Cytosolic AspAT Inactivated by Either TFEC or β-Chloro-L-Alanine

Highly purified pig heart cytosolic AspAT (1.5 mg) was incubated at 37° for 2 hr in a reaction mixture (0.2 mL) containing 100 mM potassium phosphate buffer (pH 7.4), 1 mM αKG, and the following additions: (i) none (control), (ii) 5 mM TFEC, or (iii) 5 mM β-chloro-L-alanine. The reaction mixture was brought to pH 11 with 1 M NaOH to denature the enzyme [35], and PLP was removed by filtration through a Millipore Microcentrifuge filter. PLP in the filtrate was determined by the method of Likos *et al.* [35]. Then the filtrate was brought to pH 13 with 1 M NaOH and boiled for 4 hr, and PLP was determined again.

Protein Determination

Protein was measured by the Bradford method according to the directions of the manufacturer (Bio-Rad Laboratories) adapted to a microplate reader. Bovine serum albumin was used as a protein standard.

Data Analysis

The activity of each enzyme in each cell lysate was determined in triplicate. Enzyme activities were determined for 5 or 6 different cell lysates on one day. In a separate experiment carried out on another day, enzyme activities were determined in triplicate for an additional 5 or 6 cell lysates. No significant differences were noted among any of the enzyme activities measured between the two sets of experiments. Therefore, the data for the two experiments were combined, and the values for enzyme activities in the PC12 cells are reported as means ± SEM, N = 10–12, where each value is the mean of a triplicate determination. Single comparisons to a control value were determined by using Student's *t*-test. A *P* value of ≤0.05 was considered

significant. Multiple comparisons were analyzed as described in the legends to the figures.

RESULTS

Effects of TFEC and/or cytGTK on Purified KGDHC and PDHC

Table 1 shows that KGDHC was somewhat unstable at 37° in the incubation buffer used ($k = 0.019 \pm 0.008$ min^{-1}). The enzyme was stabilized in the presence of 2 mM αKG ($k = 0.007 \pm 0.002$ min^{-1}). The addition of TFEC had a small destabilizing effect on the KGDHC activity in the absence of αKG ($k = 0.034 \pm 0.003$ min^{-1}) or in the presence of αKG ($k = 0.022 \pm 0.001$ min^{-1}). The presence of cytGTK had no effect on the stability of KGDHC in the incubation buffer. On the other hand, the presence of both cytGTK and TFEC (whether or not αKG was present) resulted in a 6-fold increase in the rate of loss of KGDHC activity compared with KGDHC in the presence of TFEC alone. The activity of cytGTK/TFEC-treated KGDHC was not restored upon 100-fold dilution of the inactivated enzyme, followed by a 10-min incubation in the reaction medium (data not shown). Thus, the inactivation observed was essentially irreversible. In contrast to KGDHC, the combined addition of TFEC and cytGTK did not stimulate the time-dependent inactivation of PDHC relative to control, either with commercial PDHC (data not shown) or with freshly purified PDHC (Table 2).

Effect of TFEC Plus Cysteine S-Conjugate β-Lyase on the Activities of Selected, Purified Enzymes of Energy Metabolism

Several key enzymes of energy metabolism, in addition to KGDHC and PDHC, were tested for their sensitivity toward inactivation by the reactive fragment metabolized from TFEC. Cysteine *S*-conjugate β-lyase activity is present in the mitochondria and cytosol of various tissues [4, 16, 19, 36, 37]. To determine whether TFEC treatment results in impairment of both cytosolic and mitochondrial enzymes of energy metabolism, highly purified enzymes representative of both compartments were studied. GAPDH and LDH are abundantly expressed cytosolic enzymes that are important in intermediary metabolism. Both MDH and AspAT occur in cytosolic and mitochondrial isoforms and are important components of the malate–aspartate shuttle for the transport of reducing equivalents across the mitochondrial membrane. Additionally, mitochondrial MDH is a component of the TCA cycle. GDH is a mitochondrial enzyme involved in glutamate metabolism.

TABLE 1. Rate constants for inactivation of highly purified KGDHC in the presence of TFEC and/or cytGTK

	k (min^{-1})	
Addition	(− cytGTK)	(+ cytGTK)
None	0.019 ± 0.008	0.018 ± 0.003
TFEC	0.034 ± 0.003	0.193 ± 0.010*
αKG	0.007 ± 0.002	ND†
TFEC + αKG	0.022 ± 0.001	0.116 ± 0.005*

Highly purified KGDHC (0.6 mg/mL) was incubated at 37° in 100 mM potassium phosphate buffer (pH 7.6) containing 0.1 mM thiamin pyrophosphate (TPP), 0.05 mM $MgCl_2$, and the indicated additions. The final concentrations of the compounds added were 2 mM αKG, 1 mM TFEC, and 0.6 mg/mL of cytGTK. At intervals over a 1-hr period, aliquots (10 μL) were withdrawn, and KGDHC activity was assayed. The assay mixture (0.6 mL) contained 100 mM potassium phosphate buffer (pH 7.0), 0.1 mM DTT, 1 mM TPP, 1 mM $MgCl_2$, 2 mM αKG, 0.05 mM CoA, and 2.5 mM NAD^+. The initial rates of formation of NADH were determined at 25° in a Uvicon spectrophotometer (Kontron Instruments, GmbH) with a 5-sec delay after mixing. The activity (A_t) was plotted as a function of time (t). Initial KGDHC activity (A_0) was 2.3 U/mg (100%). Slopes of the plots of $\ln(A_t/A_0)$ versus t were computed using MathCAD 2.09© (MathSoft Inc.) with a built-in routine for computing slopes by the least-squares method. Each value represents the mean ± SEM of at least three independent measurements.

*$P < 0.05$, compared to − cytGTK value.

†Not determined.

TABLE 2. Rate constants for inactivation of highly purified PDHC in the presence of TFEC and/or cytGTK

	k (min^{-1})	
Addition	(− cytGTK)	(+ cytGTK)
None	0.031 ± 0.007	0.032 ± 0.003
TFEC	0.034 ± 0.002	0.027 ± 0.002

Purified PDHC (0.56 mg/mL) was incubated at 37° in 100 mM potassium phosphate buffer (pH 7.6) containing 0.1 mM thiamin pyrophosphate, (TPP), 0.05 mM $MgCl_2$, and 1 mM TFEC with or without 0.6 mg/mL of cytGTK. At intervals over a 30-min time period, aliquots (20 μL) were withdrawn, and PDHC activity was assayed. The assay mixtures contained 100 mM potassium phosphate buffer (pH 7.6), 0.1 mM DTT, 1 mM TPP, 1 mM $MgCl_2$, 2 mM sodium pyruvate, 0.05 mM CoA, and 2.5 mM NAD^+. The initial rates of formation of NADH were determined at 25° as described in the legend to Table 1. Initial PDHC activity (A_0) was 1.7 U/mg (100%). Slopes of the plots of $\ln(A_t/A_0)$ versus t were computed as described in the legend to Table 1. A rapid but partial (~30%) decrease in the PDHC activity was noted within the first few minutes after additions of TFEC or cytGTK (data not shown). Thus, inactivation rates were obtained from the plots at 4–30 min. Each value represents the mean ± SEM of at least three independent measurements.

LDH activity was not affected by the presence of TFEC, cytGTK, or TFEC/cytGTK. Some slight thermal denaturation of GDH and GAPDH was noted (~10%), but no effect on the remaining activity was observed when TFEC and/or cytGTK were present. Mitochondrial MDH was somewhat more unstable. Only ~40% of the original activity remained after incubation for 1 hr at 37°, but the presence of TFEC and/or cytGTK had no effect on the remaining activity (data not shown).

Of the series of highly purified enzymes investigated, only cytosolic AspAT was inactivated by TFEC. The percent activities of AspAT (relative to the time zero activities) after a 1-hr incubation in the presence of no addition (control; 98 ± 3%), cytGTK (97 ± 2%), TFEC (57 ± 4%), and TFEC plus cytGTK (71 ± 7%) were measured ($N = 6$). Note that inactivation of cytosolic AspAT by TFEC did not require the concomitant addition of a lyase. The susceptibility of cytosolic AspAT in PC12 cell homogenates to inactivation by TFEC also was investigated. An aliquot (20 μL) of the untreated PC12 homogenate was heated to destroy the mitochondrial AspAT (see Materials and Methods). An aliquot (15 μL) of this heat-treated sample was added to a 100-μL incubation mixture contain-

TABLE 3. Specific activities of selected enzymes of energy metabolism in PC12 cells after treatment with 1 mM TFEC

Enzyme*	Specific activity (mμ/mg protein)		% Relative to control†
	− TFEC (control)	+ TFEC	
KGDHC	9.26 ± 0.77	0.84 ± 0.12	9
PDHC			
Active	0.88 ± 0.52	2.94 ± 0.87	334
Total	30 ± 5	3.67 ± 0.92	12
Total AspAT (1 hr)	212 ± 22	218 ± 25	
Total AspAT	185 ± 10	93 ± 4	50
Cyt AspAT	98 ± 13	85 ± 4	
Mit AspAT	87 ± 13	8 ± 2	9
TFEC-lyase (1 hr)	0.55 ± 0.01	0.59 ± 0.15	
TFEC-lyase	0.56 ± 0.05	0.48 ± 0.05	
MDH	1160 ± 94	982 ± 204	
GDH	34 ± 6	31 ± 2	
LDH	268 ± 24	272 ± 30	
GAPDH	94 ± 5	95 ± 5	

Each value represents the mean ± SEM of 3–6 independent measurements.

*Except where noted, specific activities were measured in the cell homogenate after cells were exposed to TFEC for 24 hr. Enzyme activities are expressed as mU (= nmol/min)/mg of protein (30°).

†Only values that were significantly different ($P < 0.05$) from the control (− TFEC) values are listed.

ing 100 mM potassium phosphate buffer (pH 7.4) with or without 5 mM TFEC. Aliquots (10 μL) of this incubation mixture then were assayed in triplicate for AspAT activity. The percent activity of cytosolic AspAT remaining after 1 hr relative to that obtained before incubation was 98 ± 3 in the absence of TFEC and 60 ± 4 in the presence of TFEC (N = 6 separate cell homogenates).

To gain insights into the mechanism by which TFEC reacts with cytosolic AspAT, we investigated the nature of the cofactor bound to the inactivated enzyme, and compared the results with those obtained after inactivation of the enzyme with β-chloro-L-alanine. After a 2-hr incubation at 37°, reaction mixtures containing (i) no addition, (ii) 5 mM TFEC, or (iii) 5 mM β-chloro-L-alanine (see Materials and Methods) were analyzed for PLP content in the pH 11 filtrate. The amount of PLP recovered (nmol) was 3.5 ± 0.1, 0.5 ± 0.1, and <0.2, respectively (N = 4). After raising the pH to 13 and boiling for 4 hr, the recovery of PLP (nmol) was 3.2 ± 0.2, 3.2 ± 0.1, and 2.7 ± 0.2, respectively (N = 4).

Cysteine S-Conjugate β-Lyase Activity in PC12 Cells Treated with TFEC

PC12 cells contain cysteine *S*-conjugate β-lyase activity with TFEC as substrate (Table 3). Treatment with 1 mM TFEC did not alter the total lyase activity in the PC12 cells. The presence of cysteine *S*-conjugate β-lyase activity suggested that PC12 cells in the presence of TFEC would be a good cellular system for studying the effects of toxic cysteine *S*-conjugates on mitochondrial energy metabolism.

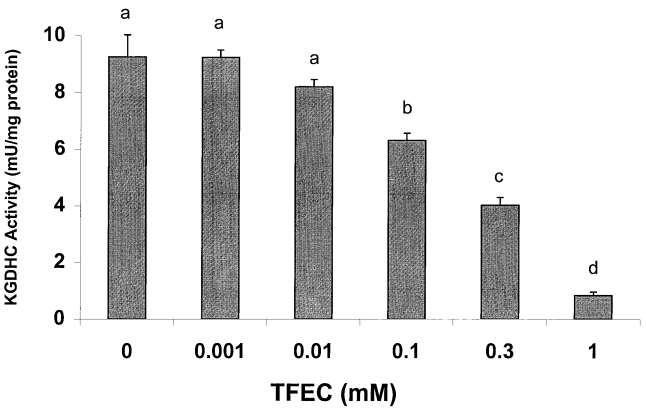

FIG. 1. Concentration-dependent inactivation of KGDHC by TFEC in PC12 cells. Cells were treated with the indicated concentrations of TFEC for 24 hr. KGDHC activity was measured in the cells as indicated in Materials and Methods. Statistical significance was tested by one-way ANOVA with post-hoc Student–Newman–Keuls comparison. Bars with different letters differ from each other ($P < 0.05$). Each data point represents the mean ± SEM of six independent measurements.

Effect of TFEC on KGDHC and PDHC Activities in PC12 Cells

Incubation of the cells with TFEC produced a concentration-dependent loss of KGDHC activity (Fig. 1). Although 1 mM TFEC was chosen for most of the ensuing studies, a significant inhibition was observed at 100 μM TFEC. The loss of activity was also time-dependent (Fig. 2). By 24 hr, only ~10% activity remained in the cells treated with 1 mM TFEC relative to control cells (Figs. 1 and 2; Table 3). The inactivation of KGDHC in the TFEC-treated cells appeared to be irreversible, because NADH production could not be re-established even upon prolonged incubation of the TFEC-treated PC12 homogenates (10 μL) with assay mixture (200 μL) (data not shown). Treatment of the PC12 cells with various concentrations of TFEC (up to 1 mM) for 24 hr had no effect on cell viability as judged by LDH release to the medium. In the presence of 0.1, 0.3, or 1.0 mM TFEC, the activity of LDH in the medium was ~3–5% relative to that in the cellular compartment. This value was not significantly different from control (~2–3%) (data not shown). The lack of change in LDH release demonstrated that the change in KGDHC activity in the TFEC-treated PC12 cells was not secondary to cell death.

The findings with PDHC were more complex. In control PC12 cells, the baseline activity of PDHC was very low compared with that of KGDHC (Table 3). This finding suggested that PDHC in the control PC12 cells was largely inactivated by phosphorylation. In support of this suggestion, intact PC12 cells treated with DCA/Mg^{2+} exhibited a >30-fold increase in activity ("total" activity, Table 3). Interestingly, the amount of active PDHC was 3 times greater in the PC12 cells treated with 1 mM TFEC for 24 hr than in control, untreated cells. However, stimulation of PDHC activity by DCA/Mg^2 was blocked completely in the TFEC-treated cells (Table 3).

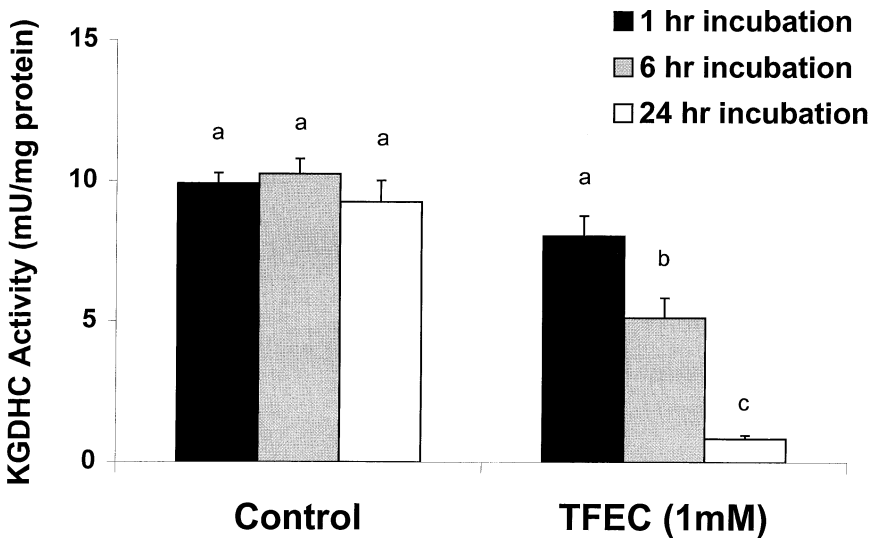

FIG. 2. Time-dependent inactivation by TFEC of KGDHC in PC12 cells. The cells were treated with 1 mM TFEC for 1, 6, or 24 hr. KGDHC activity was measured as indicated in Materials and Methods. Statistical significance was tested as described in the legend to Fig. 1. Bars with different letters differ from each other ($P < 0.05$). Each data point represents the mean ± SEM of six independent measurements.

Effect of TFEC on Selected Enzymes of Energy Metabolism in PC12 Cells

To determine whether the effects of TFEC on KGDHC or PDHC were selective, the actions of TFEC on other mitochondrial and cytosolic enzymes were determined in intact PC12 cells. No significant loss of specific activity was noted for GAPDH, MDH, GDH, or LDH in PC12 cells exposed to 1 mM TFEC for 24 hr (Table 3). The specific activity of total (i.e. cytosolic plus mitochondrial) AspAT was decreased ~50% by this treatment after 24 hr, but not after 1 hr (Table 3). To determine which isoform was more susceptible to TFEC treatment, the cells were heated at 65° (as described in Materials and Methods). In the untreated PC12 cells, destruction of mitochondrial AspAT by heating reduced the total AspAT activity from 185 ± 10 to 98 ± 13 mU/mg protein, indicating that 47 ± 7% of total AspAT activity in these cells was due to the mitochondrial isozyme (N = 6 independent cell harvests). The specific activity of total AspAT activity in the cells treated with TFEC for 24 hr (93 ± 4 mU/mg protein) was similar to that of the thermostable AspAT activity of the control cells (98 ± 13 mU/mg protein). The minor decline in specific activity upon heating (from 93 ± 4 to 85 ± 4 mU/mg protein) indicated that only 9% of the mitochondrial isozyme remained active in the TFEC-treated cells. These data show that the TFEC treatment did not affect the cytosolic AspAT significantly (compare 98 ± 13 with 85 ± 4 mU/mg protein), but almost exclusively inactivated the mitochondrial isozyme, the activity of which declined by ~90% (Table 3). Thus, although cytosolic AspAT is susceptible to inactivation by TFEC in cell homogenates, it was unaffected in the intact PC12 cells exposed to TFEC, where only the mitochondrial isozyme was inhibited.

DISCUSSION

Selective Inhibition of Purified Enzymes by TFEC

The current studies have shown that TFEC or a sulfur-containing fragment of TFEC selectively inhibits purified enzymes. Only two of the seven highly purified enzymes of energy metabolism studied in the present work were inactivated by TFEC or by the fragment released from TFEC. The sulfur-containing fragment released from TFEC by the action of cysteine *S*-conjugate β-lyases is a potent thioacylating agent of protein lysine residues (see Materials and Methods). Thus, either lysine residues in the resistant enzymes were "buried" or surface lysine residues were not necessary for catalytic activity. The activity of the mitochondrial enzyme KGDHC was not particularly sensitive to TFEC *per se*, but was sensitive to the TFEC fragment generated by the action of cysteine *S*-conjugate β-lyases, presumably through thioacylation of protein lysine residues.

Of the other purified enzymes studied, only cytosolic AspAT was susceptible to the action of TFEC. However, the mechanism of inactivation of cytosolic AspAT is different from that of inactivation of KGDHC. Purified cytosolic pig heart AspAT has weak lyase activity with DCVC [38]. The enzyme is inactivated by DCVC in a time-dependent fashion. Moreover, DCVC inactivates AspAT in mitochondrial and cytosolic fractions of rat kidney and brain [38]. TFEC also inactivates AspAT in these preparations, but the process requires the presence of αKG [38]. The fact that the addition of cytGTK (which has cysteine *S*-conjugate β-lyase activity) did not accelerate the TFEC-induced inactivation of cytosolic AspAT (present work) suggests that this isoenzyme is inactivated largely by aminoacrylate addition to the PLP cofactor and not by thioacylation of a lysine residue. A precedent exists for inactivation of cytosolic AspAT by enzyme-generated aminoacrylate.

Previous work has shown that inactivation of pig heart cytosolic AspAT by various amino acids with good leaving groups in the β position (e.g. L-serine *O*-sulfate and β-chloro-L-alanine) proceeds via attack of the aminoacrylate intermediate on the PLP cofactor bound as a Schiff base to the ε amino group of an active site lysine [35, 39]. The resulting PLP–lysine–aminoacrylate complex eliminates lysine slowly and is hydrolyzed to the tightly bound

aldol product of pyruvate with PLP. This aldol product can be released from the active site by denaturing the enzyme at pH ≥ 11. The aldol product can be converted back to free PLP by boiling in 0.1 M NaOH for 4 hr [35, 39]. We carried out a similar experiment with commercial cytosolic AspAT inactivated by TFEC. For comparison, another experiment was carried out in which cytosolic AspAT was inactivated by β-chloro-L-alanine, a compound now firmly established as inactivating the enzyme through an aminoacrylate intermediate [39]. The data showed that inactivation of cytosolic AspAT by TFEC proceeds through chemical modification of the PLP cofactor similar to that obtained with β-chloro-L-alanine. Thus, the findings provide additional, strong evidence that inactivation of cytosolic AspAT by TFEC proceeds in large part through attack of an aminoacrylate intermediate on the PLP cofactor.

Mechanism of Inhibition of Enzymes by a Fragment Derived from Toxic Cysteine S*-Conjugates*

The current results support the suggestion that a product derived from the interaction of TFEC with a cysteine *S*-conjugate β-lyase inactivates susceptible enzymes. Available evidence suggests that the toxic metabolite generated from cysteine *S*-conjugates is produced by PLP-dependent enzymes. Several PLP-dependent mammalian cysteine *S*-conjugate β-lyases, including cytGTK, have been characterized that catalyze the conversion of toxic cysteine *S*-conjugates to a reactive sulfur-containing fragment [1–4]. In agreement with the importance of PLP-containing enzymes in promoting the toxicity of certain halogenated cysteine *S*-conjugates was the finding that toxicity of DCVC is attenuated by inhibitors of PLP-enzymes. Moreover, the α-methyl analogue of DCVC, which cannot undergo PLP-enzyme-catalyzed β elimination, is not toxic (reviewed in Refs. 1–4).

Mitochondrial Enzymes as Prime Targets of the TFEC Fragment

Results with purified enzymes (Table 1), isolated mitochondria [15], cultured cells (Figs. 1 and 2), and intact animals [18] all indicate that KGDHC is a prime target of reactive sulfur-containing fragments generated by cysteine *S*-conjugate β-lyase(s). The ability of DCVC to depress αKG-supported respiration in liver mitochondria [15] or of TFEC to inhibit KGDHC within cells (current results) requires an incubation period. Interestingly, Bruschi *et al.* [18] showed that after administration of TFEC to rats a pronounced time-dependent loss of KGDHC activity occurs in kidney mitochondria and that this inactivation is accompanied by labeling of the E2k and E3 subunits by the TFEC fragment. TFEC had no effect on two other mitochondrial enzymes (i.e. mitochondrial MDH and GDH) either in purified form or in intact PC12 cells (present results). Our findings suggest that KGDHC may be particularly vulnerable to the breakdown products of toxic cysteine *S*-conjugates.

Despite the fact that TFEC/cysteine *S*-conjugate β-lyase had no effect on the activity of purified PDHC (current studies) or on PDHC in intact kidney cells [18], chronic treatment with TFEC did alter the activity of PDHC in intact PC12 cells. In PC12 cells, exposure to TFEC increased basal activity and abolished the enormous activation (>30-fold) of PDHC by treatment with DCA/Mg^{2+}. Possibly, TFEC-mediated metabolic derangements lead to loss of protein components of PDHC. Alternatively, the PDHC subunits may remain intact, but the phosphorylation/dephosphorylation machinery may be compromised. The 3-fold increase of residual PDHC activity in TFEC-treated cells compared with controls may represent a feedback mechanism to recruit what is left of the PDHC activating system to maintain metabolic integrity. Bruschi *et al.* [18] showed that both E2k and E3 subunits of rat kidney KGDHC are labeled with the TFEC-derived fragment *in vivo*, whereas PDHC is unaffected, despite the fact that PDHC also contains E3 subunits. To explain this remarkable finding, Bruschi *et al.* [18] suggested that KGDHC is in closer proximity to the mitochondrial cysteine *S*-conjugate β-lyase(s) than is PDHC. However, our finding that purified PDHC was not inhibited by a fragment derived from TFEC (Table 2, text) suggests an alternative mechanism. E3 binds to E2p more strongly in PDHC than does E3 to E2k in PDHC [40, 41]. Therefore, it is possible that E3 binding to E2k in KGDHC exposes critical lysines to the thioacylating agent, but that binding of E3 to E2p in PDHC does not lead to such exposure. The present findings suggest that the PDHC system is affected indirectly by a toxic cysteine *S*-conjugate, but the exact mechanism needs to be investigated further.

The actions of TFEC or its reactive sulfur-containing fragment on AspAT appear more complicated than their effects on KGDHC. Despite the fact that highly purified cytosolic AspAT and cytosolic AspAT in PC12 homogenates were susceptible to inactivation by TFEC *in vitro*, only the mitochondrial isozyme was inactivated in intact TFEC-treated PC12 cells (Table 3). As with the cytosolic isoform *in vitro*, it is probable that inactivation of mitochondrial AspAT in the intact PC12 cells takes place by attack of aminoacrylate on the PLP cofactor. However, it is also probable that some inactivation is due to the addition of a reactive sulfur-containing fragment to a catalytically important lysine residue. In this regard, it is important to note that after administration of TFEC to rats, six mitochondrial proteins were shown to be thioacylated, one of which was mitochondrial AspAT [17]. Whatever the mechanism, the data support the idea that in PC12 cells exposed to TFEC the cytosol is bypassed and TFEC accumulates in the mitochondria, where it is a substrate of local cysteine *S*-conjugate β-lyases. As noted above, KGDHC and mitochondrial AspAT are important components of the TCA cycle and malate–aspartate shuttle, respectively. Inhibitors of the mitochondrial AspAT block the malate–aspartate shuttle [42]. Therefore, inhibition of KGDHC and mito-

chondrial AspAT is expected to compromise aerobic energy metabolism in PC12 cells treated with TFEC.

Implications

The marked inhibition of KGDHC activity, the inability of TFEC-treated cells to attain full PDHC activity, and the reduced malate–aspartate shuttle activity all suggest that toxic cysteine *S*-conjugates impair mitochondrial oxidative/TCA cycle metabolism. Interestingly, the PC12 cells exposed to TFEC do not die, suggesting that other pathways of energy metabolism may be adequate for survival.

KGDHC is essential for mitochondrial function and plays a critical role in regulating oxidative metabolism and NADH production. Because of the central role of KGDHC in energy metabolism, inhibition of this enzyme may have profound effects on the function of cells and organs. KGDHC is especially vulnerable to oxidative insults [43, 44]. Its activity is severely reduced in affected brain regions in many neurodegenerative diseases, including Alzheimer's disease [24, 25, 27, 45] and Wernicke-Korsakoff syndrome [46]. Immunochemical staining shows that protein components of KGDHC are diminished in the brains of patients with Parkinson's disease [26]. Our previous studies suggest that the KGDHC deficit in the brains of patients with Alzheimer's disease may be primary or may be part of a critical cascade of events that leads to neurodegeneration [25]. The findings that a TFEC metabolite targets KGDHC ([18]; present work) and indirectly affects PDHC activity (present work) suggest that TFEC may be a useful tool to determine (i) the importance of these dehydrogenase complexes in brain energy metabolism, and (ii) the consequences of inhibition of α-keto acid dehydrogenase complexes in neurological function.

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