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THIODIGLYCOLIC ACID IS EXCRETED BY HUMANS RECEIVING IFOSFAMIDE AND INHIBITS MITOCHONDRIAL FUNCTION IN RATS

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ABSTRACT:

Thiodiglycolic acid has been identified as a major metabolite of the anticancer drug ifosfamide in humans. Patients treated with 12–16 g ifosfamide/m^2·day excreted thiodiglycolic acid ranging from 0.10 ± 0.02 mmol on the first day of therapy, to a maximum of 3.27 ± 0.15 mmol on the fourth day of ifosfamide infusion. This amounted to 5.4 ± 0.2% of the administered dose of ifosfamide appearing as thiodiglycolic acid in urine during a 5 days' continuous ifosfamide infusion. Thiodiglycolic acid (50mg/kg) administered to rats inhibited the carnitine-dependent oxidation of [1-^{14}C]palmitic acid by 55%, but affected neither the oxidation of [1-^{14}C]octanoic acid, which is carnitine-independent, nor the oxidation of [1,4-^{14}C]succinic acid, a marker of Kreb's cycle activity. Additionally, thiodiglycolic acid (30μM) inhibited oxidation of palmitic acid but not palmitoyl-L-carnitine in isolated rat liver mitochondria, indicating that it either sequesters carnitine or inhibits carnitine palmitoyltransferase I. This study elucidates a specific mitochondrial dysfunction induced by thiodiglycolic acid which may contribute to the adverse effects associated with ifosfamide chemotherapy.

Ifosfamide (IFO)[1] is an oxazaphosphorine alkylating agent effective against a variety of cancers (Sladek, 1988; Sechant *et al.*, 1991). It is a prodrug which requires metabolic activation by cytochrome P450, specifically CYP3A4 (Walker *et al.*, 1994) to produce the active alkylating agent isophosphoramide mustard. In addition to the production of isophosphoramide mustard, *N*-dechloroethylation of the parent compound yields the inactive metabolites 2- and 3-dechloroethylifosfamide and chloroacetaldehyde (CAA). CAA has been associated with neurotoxicity (Goren *et al.*, 1986) and suggested to be responsible for inducing IFO related CNS toxicity (Kurowski *et al.*, 1991), although the underlying mechanism remains poorly understood. Recently, CAA was shown to inhibit mitochondrial function (Visarius *et al.*, 1997), but little attention has been given to the study of potential mitochondrial dysfunction induced by products of CAA biotransformation. CAA and its associated oxidation/reduction products, 2-chloroacetic acid and 2-chloroethanol, respectively, may react with endogenous cysteine or glutathione forming *S*-conjugates which may in turn be metabolized to *S*-carboxymethyl-L-cysteine (Hoffman *et al.*, 1991). Further biotransformation of CMC, by oxidative desamination and decarboxylation yields TDGA (Hoffman *et al.*, 1991), a 3-thia dicarboxylic acid and thus a potential inhibitor of mitochondrial function.

In addition to its identification as a major human biotransformation product of the mucolytic agent CMC, TDGA has also been found as a metabolite of the industrially important compounds vinyl chloride (Draminski and Trojanowska, 1981; Chen *et al.*, 1983; Sharma *et al.*, 1980; Jedrychowski *et al.*, 1984), ethylene dichloride (Payan *et al.*, 1993; Cheever *et al.*, 1990), vinylidine chloride (Reichert *et al.*, 1979), acrylonitrile (Fennell *et al.*, 1991; Kedderis *et al.*, 1993), of 2,2′-bis-(chloroethyl)-ether (Müller *et al.*, 1979; Lingg *et al.*, 1982), and 2-(chloroethyl) nitrosoureas (Godeneche *et al.*, 1993). The presence of TDGA in urine has been used as an index for monitoring occupational exposure to vinyl chloride (Heger *et al.*, 1982); however, adverse effects of exposure to TDGA have not as yet been reported. Since TDGA is structurally similar to other known inhibitors of mitochondrial function (Tonsgard and Getz, 1985; Passi *et al.*, 1984) and sulfur-substituted fatty acid analogues may inhibit β-oxidation (Skrede *et al.*, 1997), the purpose of this study was to investigate the effects of TDGA on mitochondrial function *in vivo* and *in vitro* and to quantify the importance of pathways leading to the production of TDGA in patients treated with IFO.

Materials and Methods

Chemicals. [1-^{14}C]PA was purchased from Amersham (Buckinghamshire, UK), [1-^{14}C]OA, and [1,4-^{14}C]SA from DuPont NEN (Boston, MA). IFO was obtained from ASTA Medica (Wangen, Switzerland), TDGA, PA, palmitoyl-L-carnitine (PLC), and SA were from Sigma (St. Louis, MO).

Patients. Thirteen patients (five female, eight male) with advanced soft-tissue sarcoma were studied during one to three chemotherapy cycles admin-

[1] Abbreviations used are: IFO, ifosfamide; CAA, chloroacetaldehyde; CMC, *S*-carboxymethyl-L-cysteine; TDGA, thiodiglycolic acid (dicarboxymethylsulfide); PA, palmitic acid; OA, octanoic acid; SA, succinic acid; PLC, palmitoyl-L-carnitine; mesna, 2-mercaptoethane sulfonate; TDPA, 3,3′-thiodipropionic acid; DNP, 2,4-dinitrophenol.

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istered 3 months apart. In each cycle of 5 days' treatment, the patients received 12–16 g IFO/m^2·day by continuous infusion together with adequate hydration (2 liters 5% glucose/day) and the uroprotective agent mesna which was administered intravenously at a dose of 1.9–2.8 g/m^2·day. Urine was collected prior to the administration of IFO and during the infusion of IFO. Aliquots were stored frozen until analysis. TDGA was measured in the urines obtained during 25 chemotherapy cycles of 5 days' duration. All patients gave informed consent to participate in the study, which had been approved by the local ethics committee.

Analysis of TDGA. Patient urine (0.5ml) together with 75μl thiodipropionic acid (TDPA) (0.22mM), which was used as an internal standard, were lyophilized, and TDGA- and TDPA-dimethyl esters were then formed by reaction with borontrifluoride/methanol (BF_3 ~10% [~1.3M] in MeOH), for 20 min at 60°C. Dimethyl esters were extracted into ethylacetate and subsequently detected by gas chromatography. Quantitative analyses were performed on a Perkin Elmer 3920 gas chromatograph equipped with a sulfur-specific flame photometric detector on an OV-17, 3% packed glass column. Injector and detector temperatures of 200°C and isothermal oven temperature of 150°C allowed for the elution of TDGA and TDPA derivatives at 2.1 and 5.6 min, respectively. Calibration curves for the derivatized standards were linear over the range of concentrations studied (1.25μM - 2.5mM) after transformation to account for exponential response of the sulfur specific detector. Peak areas were calculated with a Spectra Physics 4290 integrator.

Animals. Male Wistar rats (200–250 g) were bred and housed in the University of Berne vivarium, allowed access to food and water *ad libitum*, and kept on a 12-hr light/dark cycle. Rats anesthetized with pentobarbital (50mg/kg, ip), whose body temperatures were regularly monitored and maintained at 38°C, were used for all experiments.

***In Vivo* Oxidation of Labeled Substrates.** To collect breath of anesthetized rats, a cylindrical vessel attached to a vacuum pump was placed over the rodent's head. TDGA (50mg/kg, ip), dissolved in sterile saline (0.9%), was applied 5 min preceding the ip administration of [1-^{14}C]PA (3μCi/kg, 55.0mCi/mmol), [1-^{14}C]OA (0.3μCi/kg, 55.0mCi/mmol), or [1,4-^{14}C]SA (0.3μCi/kg, 59mCi/mmol). [1-^{14}C]PA and [1-^{14}C]OA were prepared in commercially available thistle oil, and [1,4-^{14}C]SA in sterile saline (0.9%). Control animals received an equivalent volume of saline without TDGA (ip) 5 min before receiving either [1-^{14}C]PA, [1-^{14}C]OA, or [1,4-^{14}C]SA. $^{14}CO_2$ produced from the oxidation of labeled substrates was collected from a constant air stream while an applied vacuum (200 ml/min) pulled exhaled breath through successive solutions of ethanol, to dry exhaled breath, and ethanolamine (4M, in ethanol), to trap exhaled $^{14}CO_2$. The $^{14}CO_2$ was then quantified by scintillation spectroscopy.

Isolation and Incubation of Mitochondria. Mitochondria from the livers of male Wistar rats (200–250 g) were isolated as previously described (Johnson and Lardy, 1967). Briefly, livers were quickly removed from decapitated rats and immediately transferred to ice-cold mannitol (0.25 M)-sucrose (0.07 M) buffer (MSB). Livers were finely minced and EDTA (2 mmol/L) was added prior to homogenization. Mitochondria were isolated by means of differential centrifugation, suspended in MSB at a concentration of 1g original liver weight per ml, and stored on ice. Protein concentrations in mitochondrial preparations were determined with the biuret method using bovine serum albumin as standard (Cornall *et al.*, 1949).

Oxygen consumption by intact mitochondria was measured at 37°C in a chamber equipped with a Clark-type oxygen electrode. Buffer for all mitochondrial incubations contained potassium phosphate (0.1 M), triethanolamine (0.1 M), and magnesium sulfate (0.15 M) in addition to MSB, pH 7.4. The respiratory control ratio (RCR = state 3/state 4) in mitochondria energized with succinate was used to control the quality of each preparation and was greater than 5 in each preparation used.

Uncoupling of mitochondria was achieved by the addition of 2,4-dinitrophenol (DNP) (125 μM). Addition of PA or PLC (10 μM) to uncoupled mitochondria allowed for the assessment of palmitic acid oxidation, PA being dependent on the endogenous mitochondrial carnitine pool for its activation and PLC independent. The effect of TDGA on mitochondrial function was assessed by incubating mitochondria together with DNP and TDGA (30 μM) for 2 min prior to substrate addition. Mitochondria incubated with DNP alone for 2 min prior to addition of substrate served as control.

Statistical Analysis. Results are presented as mean ± SEM. The urinary excretion of TDGA was evaluated by repeat measure one-way analysis of variance using Bonferroni correction for multiple comparisons. The results of the animal and *in vitro* experiments were assessed by Student-*t* test. Two-tailed probabilities of less than 0.05 were considered to be significant.

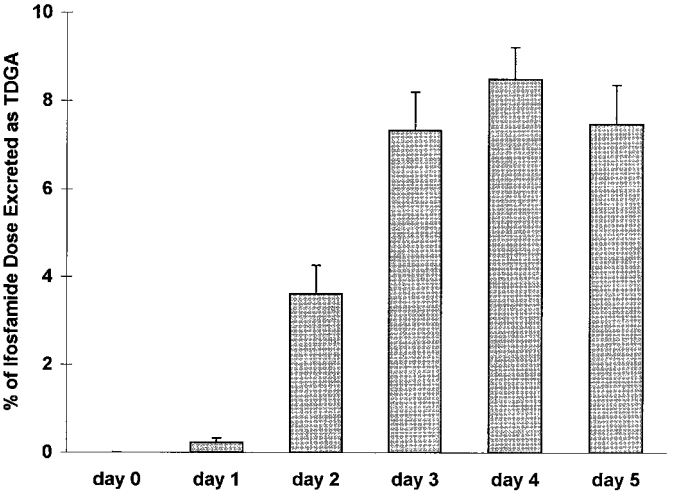

FIG. 1. *Excretion of TDGA in urine by patients receiving IFO.*

Data represent 25 IFO cycles in 13 patients who received 12–16g IFO/m^2·day (mean ± SEM).

Results

Prior to the infusion of IFO, TDGA was found in the urine samples of two patients who excreted 22 and 4 μmol TDGA/24 hr. During the infusion of IFO the excretion of TDGA gradually increased to 0.10 ± 0.02 mmol on day 1, 1.44 ± 0.15 mmol on day 2, 2.87 ± 0.21 mmol on day 3, 3.27 ± 0.15 mmol on day 4, and 2.84 ± 0.14 on the last day of IFO infusion. Amount of TDGA excreted on days 2–5 was significantly different ($p < 0.01$) from the excretion of TDGA prior to IFO infusion. The fraction of the administered dose of IFO recovered as TDGA in urine ranged from 0.23 ± 0.05% on the first day of IFO infusion to 8.49 ± 0.36% on the day of maximal TDGA excretion (fig. 1). This amounted to 5.4 ± 0.2% of the administered dose of IFO appearing as TDGA during the 5 days' continuous IFO infusion.

As shown in fig. 2, TDGA resulted a 55% inhibition of the oxidation of PA. To localize the inhibitory effect to the carnitine-dependent transport of long chain fatty acids into the mitochondria, β-oxidation, or the Kreb's cycle, the effects of TDGA on the oxidation of OA and SA were also studied. Neither the oxidation of OA, a substrate for β-oxidation which does not require carnitine for its import into the mitochondrial matrix, nor the oxidation of SA, a substrate for the Kreb's cycle, were inhibited by TDGA (fig. 2 and table 1).

Experiments with isolated mitochondria allowed for the comparison between PA and PLC oxidation and, thus, mechanistic evaluation of long chain fatty acid oxidation. In uncoupled mitochondria, TDGA markedly inhibited PA oxidation but did not affect PLC metabolism (table 1).

Discussion

The results depicted indicate that large quantities of TDGA are excreted by patients treated with IFO. TDGA may be produced from the pathways indicated in fig. 3. The key metabolite of IFO resulting in the formation of TDGA is most likely CAA. It is possible that an appreciable fraction of the IFO dose is converted into TDGA as each IFO molecule possesses two chloroethyl side chains which may be metabolized to CAA through *N*-dealkylation (Sechant *et al.*, 1991;

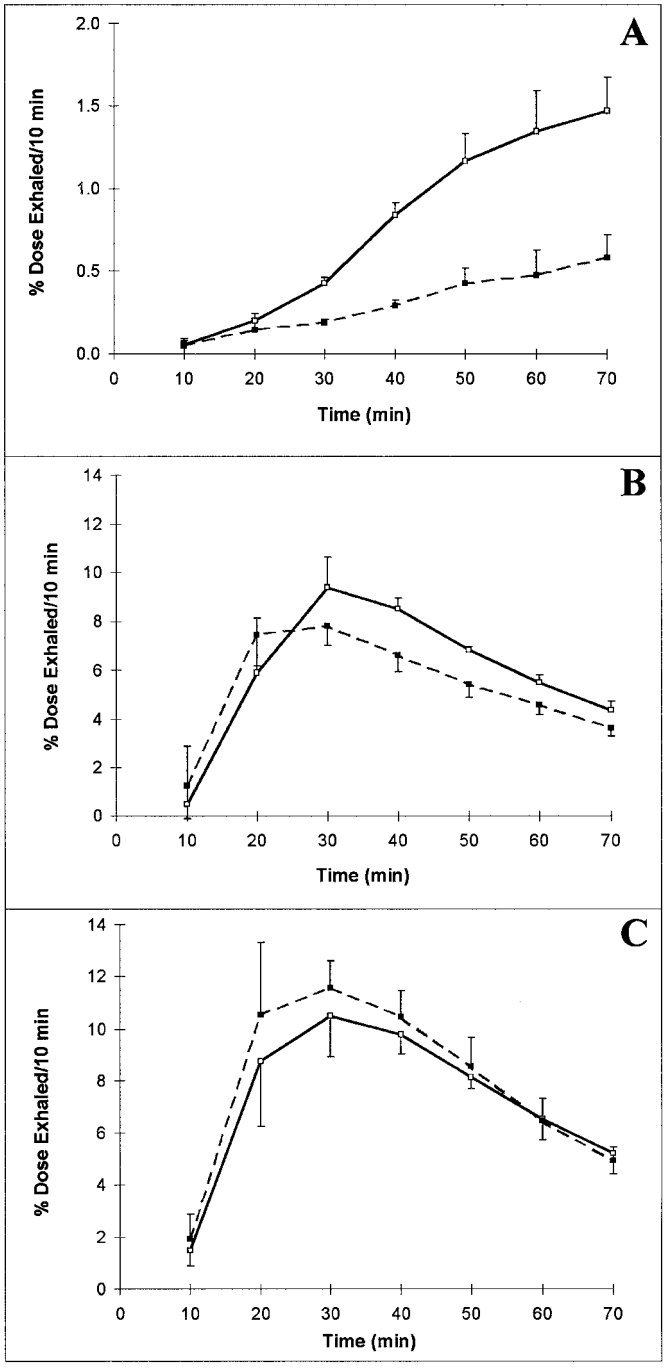

FIG. 2. *Effect of TDGA on the exhalation of $^{14}CO_2$ formed from labeled palmitic, octanoic and succinic acid, respectively.*

Filled symbols mark the exhalation of $^{14}CO_2$ from the oxidations of: (a) [1-^{14}C]palmitic acid, (b) [1-^{14}C]octanoic acid, and (c) [1,4-^{14}C]succinic acid, after the administration of of TDGA (50mg/kg). Open symbols represent $^{14}CO_2$ produced by control animals which received the respective labeled substrates (mean ± SEM, $N = 3$).

TABLE 1

Effect of TDGA on metabolism in vivo and *in vitro* ($N = 3$ for each substrate)

	Control	TDGA
In vivo		
[1-^{14}C]palmitic acid	6.2 ± 0.7	2.8 ± 0.7*
[1-^{14}C]octanoic acid	40.9 ± 3.1	36.7 ± 1.9
[1,4-^{14}C]succinic acid	52.1 ± 3.4	55.9 ± 2.6
In vitro		
Palmitic acid	293 ± 24	78 ± 6*
Palmitoyl-L-carnitine	5172 ± 191	5066 ± 107

In vivo data represent the percent of substrate exhaled as $^{14}CO_2$ in 70 min.
In vitro data are reported as nmol O_2/min · mg mitochondrial protein.
* Significant difference in comparison to controls ($p < 0.01$).

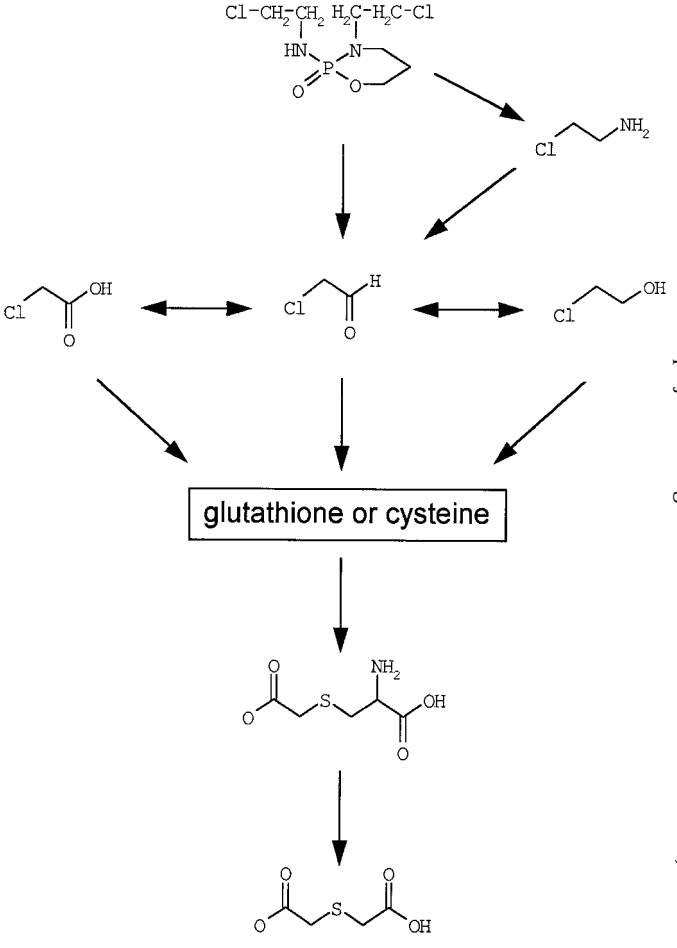

FIG. 3. *Proposed scheme of TDGA generation from IFO.*

For clarity, pathways indicated here represent only those discussed in this work. For more about IFO metabolism see: Sechant *et al.* (1991) *Drugs* **42**:428–467.

Walker *et al.*, 1994) or by enzymatic conversion of IFO-produced chloroethylamine (Aeschlimann *et al.*, 1996). CAA is present in plasma of patients treated with IFO in concentrations up to 210 μM (Cerny and Küpfer, 1989). It may directly react with glutathione to produce equal amounts of *S*-(carboxymethyl)glutathione and *S*-(2-hydroxyethyl)glutathione (Jean and Reed, 1992). Alternatively, it can be oxidized or reduced to 2-chloroacetic acid or 2-chloroethanol, respectively, which will react with cysteine and glutathione, although markedly slower than CAA itself (Johnson, 1967), to form CMC. CMC in turn may undergo deamination and decarboxylation to form TDGA. When CMC, which is used as a mucolytic agent, is administered to patients, approximately 20% of the administered dose is excreted as TDGA (Hoffman *et al.*, 1991). Assuming a similar rate of conversion of CMC to TDGA in patients treated with IFO, the formation of CMC from IFO can be estimated at approximately 30%. The gradual increase in the excretion of TDGA during a continuous infusion of IFO suggests time-dependent changes in the metabolism of IFO or TDGA. One possible explanation for this phenomenon is an auto-induction of cytochrome P450 during the IFO infusion resulting in a higher rate of *N*-dechloroethylation and subsequently reflected in

an increase in CAA production. A time-dependent increase in CAA generation could explain observed decreases in total cysteine and glutathione reported in plasma during IFO therapy (Lauterburg *et al.*, 1994) and result in increasing amounts of TDGA excreted in urine.

Side effects of IFO therapy have been attributed to mitochondrial dysfunction (Küpfer *et al.*, 1996). Because TDGA, in analogy to other dicarboxylic acids and 3-thia fatty acids, has the potential to impair mitochondrial function, we studied its effect on mitochondrial oxidation of the long chain fatty acid PA, the medium chain fatty acid OA, and SA. The oxidation of PA, but not of OA or SA, was markedly inhibited *in vivo*. Contrary to previously studied dicarboxylic acids of chain length C_8 to C_{13} (Passi *et al.*, 1984), our results suggest that β-oxidation, tricarboxylic acid cycle function, and the mitochondrial respiratory chain are not affected by TDGA. Specifically, normal oxidation of SA in the presence of TDGA indicates intact Kreb's cycle function and an undisturbed mitochondrial respiratory chain, while normal oxidation of OA allows for the conclusion that depletion or sequestration of CoA cannot account for the inhibitory effect of TDGA observed with PA. Rather, TDGA affects either the formation of palmitoyl-carnitine ester or carnitine translocase. Since β-oxidation of PA and OA is almost exclusively initiated in mitochondria (Jacobs *et al.*, 1994), it is unlikely that peroxisomal metabolism of fatty acids influenced the present results.

To support our *in vivo* findings and further investigate the mechanism of TDGA induced inhibition of long chain fatty acid oxidation, the effect of TDGA on isolated mitochondria was studied. *In vitro* decreases in oxidative metabolism of PA but not PLC in the presence of TDGA indicate that TDGA effects either the activity of carnitine palmitoyl-transferase I or sequesters carnitine. Normal oxidation of PLC in the presence of TDGA demonstrates intact function of carnitine translocase, carnitine palmitoyltransferase II, β-oxidation, the Kreb's cycle, and the respiratory chain.

Taken together, this study has identified and quantified TDGA as a metabolite of the cytostatic drug IFO in humans and demonstrated its inhibitory effects on mitochondrial long chain fatty acid oxidation *in vitro* and *in vivo*. The appearance of TDGA in urine, whether it is IFO derived or otherwise, may thus indicate compromised mitochondrial function and signal potentially elevated concentrations of circulating long chain fatty acids. In view of these findings, further investigations on the severity of mitochondrial dysfunction in humans receiving IFO chemotherapy or those chronically exposed to known TDGA producing compounds seems to be important.

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