ACCELERATED COMMUNICATION

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

THERESA M. VISARIUS, HEINZ BÄHLER, ADRIAN KÜPFER, THOMAS CERNY, AND BERNHARD H. LAUTERBURG

Clinical Pharmacology (T.M.V., A.K., B.H.L.), University of Berne; and Medical Oncology (H.B., T.C.), University Hospital

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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/m² 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 (50 mg/kg) administered to rats inhibited the carnitine-dependent oxidation of [1-14C]palmitic acid by 55%, but affected neither the oxidation of [1-14C]octanoic acid, which is carnitine-independent, nor the oxidation of [1,4-14C]succinic acid, a marker of Kreb’s cycle activity. Additionally, thiodiglycolic acid (30 μM) 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.
istered 3 months apart. In each cycle of 5 days’ treatment, the patients received 12–16 g IFO/m²/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/m²-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 (0.5 ml) together with 75 μl thiodipropionic acid (TDPA) (0.22 mM), which was used as an internal standard, were lyophilized, and TDGA- and TDPA-dimethyl esters were then formed by reaction with boron trifluoride/methanol (BF₃/MeOH) for 20 min at 60°C. Dimethyl esters were extracted into ethyl acetate 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 were 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 (1.25 μM - 2.5 mM) after transformation to account for exponential response of the sulfur specific 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 (50 mg/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 (50 mg/kg, ip), dissolved in sterile saline (0.9%), was applied 5 min preceding the ip administration of [1-14C]PA (3 μCi/kg, 55 mCi/mmol), [1-14C]OA (0.3 μCi/kg, 55 mCi/mmol), or [1,4-14C]SA (0.3 μCi/kg, 55 mCi/mmol), [1-14C]PA and [1-14C]OA were prepared in commercially available thistle oil, and [1,4-14C]SA in sterile saline (0.9%). Control animals received an equivalent volume of saline without TDGA (ip) 5 min preceding the ip administration of [1-14C]PA (3 μCi/kg, 55 mCi/mmol), or [1,4-14C]SA. O2 production 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 (Johnston 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 mM) was added prior to homogenization. Mitochondria were isolated by means of differential centrifugation, suspended in MSB at a concentration of 1 g 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.

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 at 5% 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 appreciable fractions 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;
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 \text{ mM} (Cerny and Küpper, 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 \textit{N}-dechloroethylation and subsequently reflected in

![Figure 2](image1.png)

**FIG. 2.** Effect of TDGA on the exhalation of \( ^{14} \text{CO}_2 \) formed from labeled palmitic, octanoic and succinic acid, respectively.

Filled symbols mark the exhalation of \( ^{14} \text{CO}_2 \) from the oxidations of: (a) \([1-^{14} \text{C}]\)palmitic acid, (b) \([1-^{14} \text{C}]\)octanoic acid, and (c) \([1,4-^{14} \text{C}]\)succinic acid, after the administration of TDGA (50 mg/kg). Open symbols represent \( ^{14} \text{CO}_2 \) produced by control animals which received the respective labeled substrates (mean \( \pm \) SEM, \( N = 3 \)).

![Figure 3](image2.png)

**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.

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tr>
<td><strong>Effect of TDGA on metabolism in vivo and in vitro (( N = 3 ) for each substrate)</strong></td>
</tr>
<tr>
<td><strong>Control</strong></td>
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<tr>
<td>In vivo</td>
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<tr>
<td>([1-^{14} \text{C}])palmitic acid</td>
</tr>
<tr>
<td>([1-^{14} \text{C}])octanoic acid</td>
</tr>
<tr>
<td>([1,4-^{14} \text{C}])succinic acid</td>
</tr>
<tr>
<td>In vitro</td>
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<tr>
<td>Palmitic acid</td>
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<td>Palmitoyl-L-carnitine</td>
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* In vivo data represent the percent of substrate exhaled as \( ^{14} \text{CO}_2 \) in 70 min. In vitro data are reported as nmol \( \text{O}_2 \) min/\( \text{mg} \) mitochondrial protein.

* Significant difference in comparison to controls (\( p < 0.01 \)).

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 (Küpper et al., 1996). Because TDGA, in analogy to other dicarboxylic acids and 3-thia fatty acids, has the potential to impair 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 affects either the activity of carnitine palmitoyltransferase 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 cytotoxic 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.

References


