Thiodiacetic Acid – a Metabolite of Ethylene Oxide

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Urine samples of premature babies contain high amounts of thiodiacetic acid (TDA). Since these pre-term infants are exposed to an increased oxygen atmosphere in the incubator, we supposed that these high levels of thiodiacetic acid might be produced from ethylene, generated in the course of lipid peroxidation processes. Considering that conversion of ethylene to ethylene oxide (EO) is well known in biology we investigated whether ethylene oxide is metabolised to thiodiacetic acid or not. Therefore Sprague-Dawley rats and NMRI mice were exposed to ethylene oxide for six hours. Urine specimens were collected after exposure and the amount of thiodiacetic acid was determined by gas chromatography/mass spectrometry. The quantity of excreted TDA increased enormously compared to control samples. So thiodiacetic acid seems to be a metabolite of ethylene oxide *in vivo*.

Introduction

Any human urine specimen contain a basic level of thiodiacetic acid (Müller *et al.*, 1978). A dramatic increased excretion of TDA was observed in neonatal urine of premature babies, delivered in gestation week 25–30 (Pettit *et al.*, 1984). In both cases the precursor molecules of urinary TDA remained unknown until now.

Thiodiacetic acid is a well known metabolite of some medicines and various small reactive molecules. Many 1,2-disubstituted ethane derivatives, e.g. 1,2-dichloroethane (Payan *et al.*, 1993), 2-bromoethanol (Jones and Wells, 1981) and 2-chloroethanol (Grunow and Altman, 1982) are metabolised by conjugation with glutathione. In addition vinylchloride (Müller *et al.*, 1976), 1,1-dichloro ethylene (Reichert *et al.*, 1979), acrylonitrile (Kedderis *et al.*, 1993) and also drugs like fotemustine (Brakenhoff *et al.*, 1993), mitozolomide (Gachon *et al.*, 1993) or chlomethiazole (Grupe and Spiteller, 1982) are converted to glutathione conjugates. All these conjugates are further degraded to *S*-carboxymethyl cysteine (Müller and Norpoth, 1977; Müller and Norpoth, 1978) which finally leads to thiodiacetic acid (Yllner, 1971; Müller *et al.*, 1976; Müller and Norpoth, 1978).

S-carboxymethyl cysteine was also reported to be a metabolite of ethylene oxide (EO), another reactive C-2 compound (Tardif *et al.*, 1987). The metabolism of ethylene oxide was studied rather intensively (Segerbäck, 1983; Törnqvist *et al.*, 1986; Törnqvist *et al.*, 1989) since it was found to be a mutagen and a carcinogen (Gennart *et al.*, 1983).

Detoxification of ethylene oxide is connected with an decrease of glutathione levels in lung and liver of animals treated with EO (McKelvey and Zemaitis, 1986). Ethylene glycol (Martis *et al.*, 1982), *N*-acetyl-*S*-(2-hydroxyethyl) cysteine (Gerin and Tardif, 1986) and *S*-(2-hydroxyethyl) cysteine (Ehrenberg *et al.*, 1977) were identified, besides *S*-carboxymethyl cysteine (Tardif *et al.*, 1987), as intermediates of the metabolic pathway of EO. Later on 2-hydroxymercapturic acid, 2-methylthioethanol and 2-mercaptoethanol were found to be further metabolites of EO (Koga *et al.*, 1987). But TDA was obviously not considered to be a final product of ethylene oxide metabolism (see p. 75: note added in proof).

Endogenous sources of ethylene oxide exist too: It was shown that plants, e.g. *vicia faba* (Abeles and Dunn, 1985) as well as rat liver microsomes

Abbreviations: EO, ethylene oxide; FID, flame ionisation detector; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; M^+, molecular ion; TDA, thiodiacetic acid.

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(Schmiedel *et al.*, 1983) are able to produce ethylene oxide by epoxidation of ethylene. Therefore rats exposed to ethylene exhaled EO (Filser and Bolt, 1983).

Ethylene is generated from ω-3 polyunsaturated fatty acids in mammalians (Dumelin and Tappel, 1977) and also released during lipid peroxidation from peptides containing methionine (Scheick and Spiteller, 1996). Pre-term infants exposed to an increased oxygen atmosphere in the incubator often suffer from retrolental fibroplasia caused by oxidative stress (Bast, 1989). Therefore we presumed that the high TDA levels in urine specimens of premature babies might be due to the metabolism of ethylene to TDA, *via* ethylene oxide, in the course of increased lipid peroxidation. To confirm this assumption we exposed rats and mice to ethylene oxide and checked their urine specimens for thiodiacetic acid.

Materials and Methods

Chemicals

50 l of 1.01% ethylene oxide (CAS-no. 75-21-8) in nitrogen were obtained from Messer Griesheim (Düsseldorf, Germany). All other chemicals were purchased from Fluka (Neu Ulm, Germany). Solvents, obtained from Merck (Darmstadt, Germany), were distilled before use.

Animals

Five male (individuals 1.1-1.5) and five female (individuals 2.1-2.5), 6-7 weeks old Sprague-Dawley rats (Crl: CD®BR) and five female, 8 weeks old NMRI mice (individuals 3.1-3.5) (Crl: NMRI) were purchased from Fa. Charles River (Sulzfeld, Germany). The animals were housed in stainless steel wire mesh cages and acclimatised for 21/2 weeks to the environmental conditions (24 °C ± 2 °C temperature, 55% ± 15% relative humidity, 12 h light/dark cycle). Drinking water and pelleted food (rat/mice maintenance diet „1324 N, specially prepared" from Altromin, Lage, Germany) were offered *ad libitum*. One week prior to EO exposure the body weights were determined (male rats: 285-301 g, female rats: 219-234 g and female mice: 27-33 g) and during 24 h the urines of the individual animals were collected to obtain unexposed controls.

Inhalation experiment

Animals were whole body exposed to a target concentration of 233 ppm EO for 6 h. Exposure was conducted in a glass/stainless steel inhalation chamber (0.86 m^3) under dynamic flow conditions. Total flow of 10 m^3/h was maintained constant by a vacuum pump on the exhaust side. EO was introduced into the chamber with a flow of 2.7 l/min (1% EO in N_2). EO concentration was monitored by constant gas chromatography using a FID.

The 6 h EO whole body exposure was performed between 7.40 a.m. and 1.40 p.m., the mean concentration was 233 ppm ± 35 ppm EO. No clinical abnormalities were observed in the animals during and after the EO exposure. At that time the body weights ranged from 321 g to 339 g for male rats; from 234 g to 249 g for female rats and from 28 g to 32 g for the female mice.

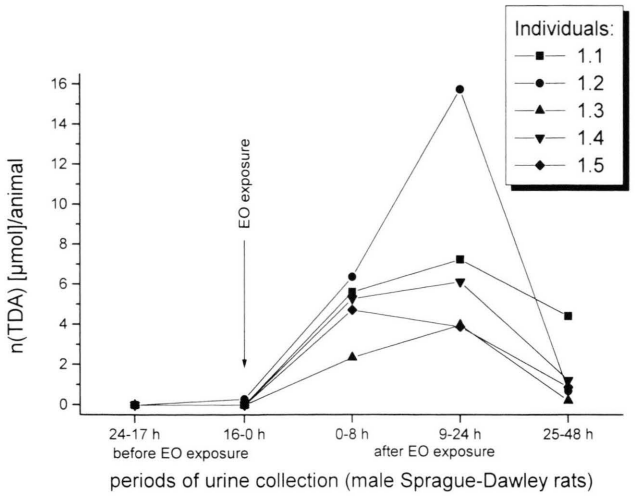
Collection of animal urine specimens

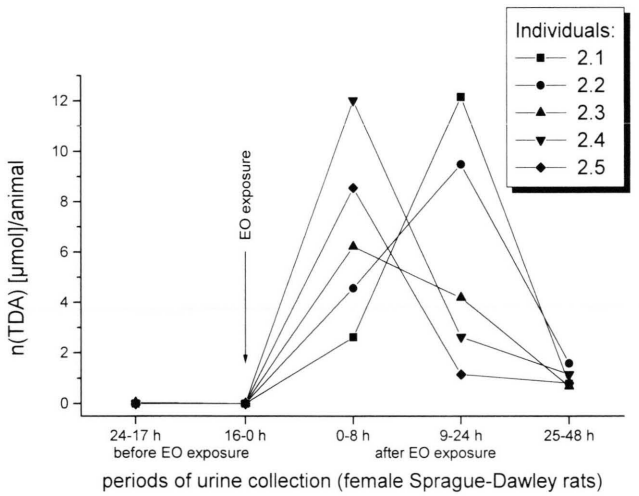
First urine samples were obtained during the acclimatisation period one week prior to the EO exposure. Individual urine specimens (amount between 0.4 g and 12.3 g) were collected to determine control values for the periods 24-17 h and 16-0 h prior to EO exposure. A second sampling period was started directly after the 6 h EO exposure. Individual urine fractions (weight: 0.5 g - 24.5 g) were taken for the periods 0-8 h, 9-24 h and 25-48 h after the EO exposure.

For urine collection the animals were individually transferred to metabolism cages „STT 150/200" (material: polycarbonate Makrolon®/stainless steel; EBECO, Castrop-Rauxel, Germany). During the first 8 h of urine sampling the animals had only free access to drinking water. Afterwards they were allowed to eat too. The first samples (24-17 h before exposure and 0-8 h after exposure) were collected in flasks surrounded with solid carbon dioxide. All other periods of urine collection were performed at room temperature. Each urine sample was weighed and stored in a brown glass flasks under argon at -18 °C until analysis.

Quantification of TDA in animal urines

The urine specimens were thawed and an aliquot of glutaric acid (internal standard) was





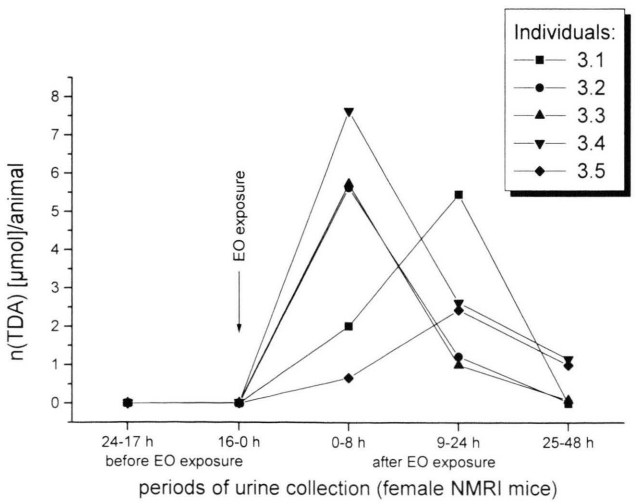


Fig. 1. Amounts of urinary thiodiacetic acid excreted by each animal during different periods before and after 6 h of ethylene oxide-exposure (beginning of ethylene oxide addition is marked by an arrow).
a: male Sprague-Dawley rats;
b: female Sprague-Dawley rats;
c: female NMRI mice.
Urine specimens were collected over five periods:
– 24–17 h before beginning of ethylene oxide exposure;
– 16–0 h before beginning of ethylene oxide exposure;
– 0–8 h after ethylene oxide exposure;
– 9–24 h after ethylene oxide exposure;
– 24–48 h after ethylene oxide exposure.

added. Afterwards they were freeze-dried, the residue dissolved in methanol, and etheral diazomethane was added. After 15 min the solvent as well as the excess of CH_2N_2 were removed with nitrogen. Each sample was dissolved in ethylacetate and an aliquot was subjected to GC and GC/MS. Peaks were identified by comparison of their mass spectra with those of authentic material. Quantification of thiodiacetic acid was done by comparing intensities of the ion trace of $m/z = 128$ for dimethylglutarate and $m/z = 146$ for dimethylthiodiacetate. Due to the small amounts of urine, obtained especially from mice (minimum value 0.4 g), quantification could be performed only one time for each urine sample.

Synthesis of standards

Dimethylthiodiacetate and dimethylglutarate were obtained by reaction of thiodiacetic acid respectively glutaric acid with an etheral solution of diazomethane in methanol for 15 min. The solutions were brought to dryness with a stream of nitrogen.

Gas chromatography

GC analysis was performed with a Carlo Erba HRGC chromatograph using a fused silica DB-1 capillary column (length: 30 m; inner diameter: 0.32 mm; carrier gas: H_2 2 ml/min; temperature program: 80–280 °C at 3 °C/min; detector: FID; injector temperature: 190 °C; detector temperature: 210 °C; split ratio: 1:30). Peak area integration: Shimadzu C-R3A Chromatopac. Retention indices were calculated according to Kováts (Kováts, 1958) by co-injection of *n*-alkanes.

GC/MS

Measurements were performed on a double focusing mass spectrometer Finnigan MAT 95 running under EI conditions at 70 eV. A HP 5980 series II gas chromatograph with a 30 m x 0.3 mm (i.d.) DB-1 fused-silica column was used for sample separation. The carrier gas was hydrogen and the temperature programme was the same as used for GC.

Results

Quantification of thiodiacetic acid was achieved by comparison of ion trace intensities. The ion $m/z = 128$ derived from dimethylglutarate added as internal standard was compared with $m/z = 146$ obtained from dimethylthiodiacetate. Both fragments correspond to the loss of methanol from the M^+. The molecular ions are not very intensive and therefore less suited for quantification than the M-32 fragments which are of high intensity.

In the animal urine specimens collected before EO exposure only traces of TDA were detected by GC/MS (Fig. 1a–1c). Immediately after addition of ethylene oxide to the air (marked by an arrow in Fig. 1a–c) the content of TDA in the animal urines rose and reached a maximum value of 15.7 μmol in male rat 1.2 (Fig. 1a).

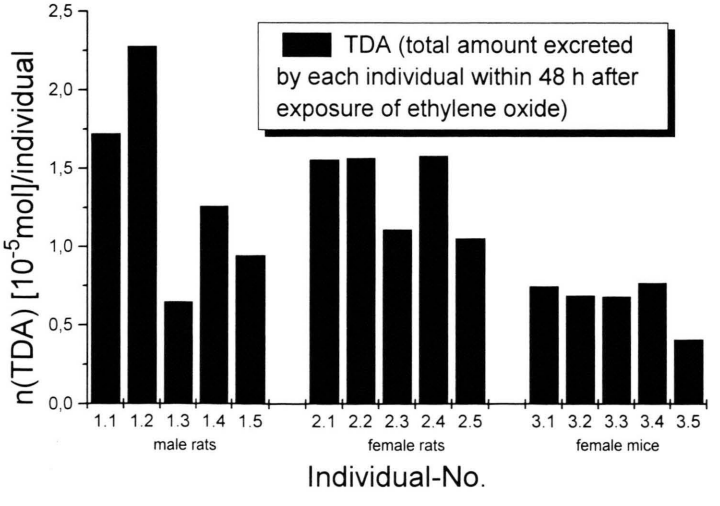


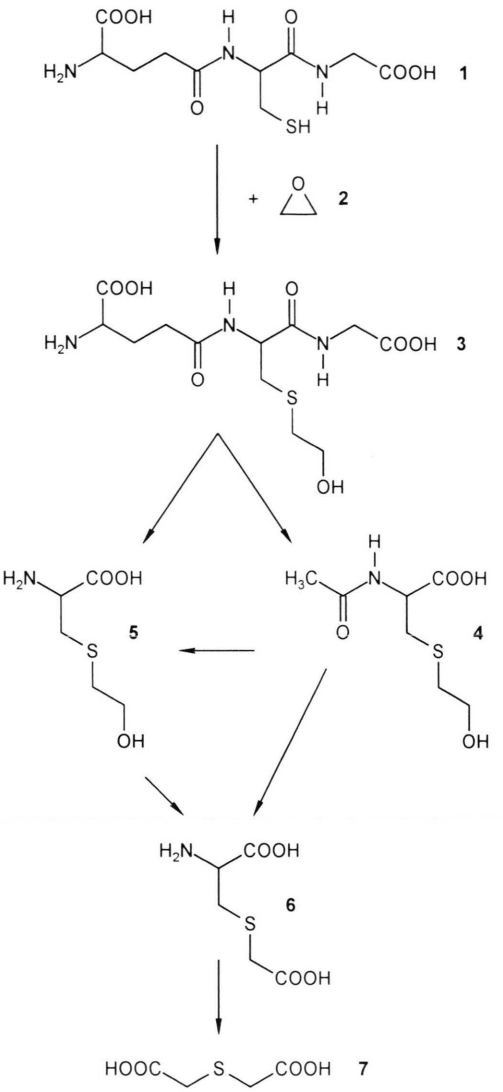
Fig. 2. Total amount of urinary thiodiacetic acid excreted by rat and mice during 48 h after 6 h of ethylene oxide-exposure.

Maximal excretion was observed in seven animals within the period of 0–8 h after EO exposure. Eight individuals reached the climax of excretion in the 9–24 h period. Afterwards (period 25–48 h after exposure) the amount of excreted TDA in the urines decreased. Fig. 2 shows the total amounts of TDA excreted by the animals during 48 h after ethylene oxide exposure. The maximum value was measured for male rat 1.2 ($2.28\ 10^{-5}$ mol).

Discussion

The results presented in Fig. 1a–1c indicate unambiguously that EO inhalation causes an increase of TDA in the urine of all animals. Therefore TDA is identified as a metabolite of ethylene oxide.

Since it was proven earlier that ethylene oxide is metabolised to *S*-carboxymethyl cysteine (Tardif *et al.*, 1987) which was shown to be a precursor of thiodiacetic acid (Yllner, 1971; Müller *et al.*, 1976; Müller and Norpoth, 1978) the following pathway for the formation of TDA from EO could be deduced (Yllner, 1971; Müller and Norpoth, 1978) (Scheme 1). Ethylene oxide (**2**) reacts with glutathione (**1**) forming the intermediate product **3**. Compound **3** suffers further degradation leading to *S*-(2-hydroxyethyl) cysteine (**5**), perhaps *via N*-acetyl-*S*-(2-hydroxyethyl) cysteine (**4**). Both compounds (**4, 5**) are precursors for *S*-carboxymethyl cysteine (**6**). Finally TDA (**7**) is obtained (Scheme 1).



Scheme 1. Proposed mechanism for the formation of urinary thiodiacetic acid by reaction of ethylene oxide with glutathione (according to Yllner, 1971; Müller and Norpoth, 1978).

It was already shown that ethylene is converted to ethylene oxide in liver microsomes of rats (Schmiedel *et al.*, 1983) and in the course of oxidative stress by activated cytochrome P-450 linked epoxidases (Capdevila *et al.*, 1990). In addition the metabolism of substituted vinyl compounds must be assumed to proceed *via* enzymatic epoxidation. Because of that there seems to be no doubt that TDA is also a metabolite of ethylene. Ethylene is generated during lipid peroxidation (Törnqvist *et al.*, 1989b) and based on our investigation it should be transformed to TDA, *via* intermediate formation of EO.

Therefore we conclude that the high TDA levels in urine specimens of premature babies were a result of lipid peroxidation processes induced by an increased oxygen content of the atmosphere. The investigation in our laboratory of urine samples of patients suffering from various inflammatory diseases in which increased lipid peroxidation was reported (Halliwell and Grootveld, 1987) showed not a major increase. Because of that the unnormal high TDA excretion of premature infants is obviously caused by a very high oxidative stress.

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Note added in proof: We were just informed that TDA is considered as metabolite of EO in Leon Golberg's monograph "Hazard Assessment of Ethylene Oxide"; CRC Press, Boca Raton, 1986, p. 12.

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