Inhibition of creatine kinase activity by ethylene oxide

M Matsuoka, H Igisu, N Inoue, H Hori, I Tanaka

Abstract

Exposure of rats to 500 ppm ethylene oxide for six hours a day, three times a week, for 12 weeks, lowered serum creatine kinase activity by more than 40%. The only other change was a slightly decreased triglyceride concentration. After four weeks of exposure, neither aspartate aminotransferase nor lactate dehydrogenase activity in brain, spinal cord, and muscle was affected but creatine kinase activity was clearly inhibited. In vitro, ethylene oxide inhibited creatine kinase activity in brain homogenate and in a purified muscle enzyme preparation. Dithiothreitol did not counteract the effect of ethylene oxide. Though the amount of sulphhydryl groups in purified creatine kinase was decreased considerably by exposure to ethylene oxide, the enzyme still showed moderate activity. Thus ethylene oxide inhibits creatine kinase activity in vivo and in vitro and the inhibition appears to be unrelated to the disruption of sulphhydryl groups in the enzyme.

Ethylene oxide (EO) is a highly reactive gas widely used in the chemical industry and for the sterilisation of steam and heat sensitive materials in hospitals. Recently, cases of encephalopathy or poly-neuropathy of employees engaged in sterilisation have been reported.¹⁻⁶ Electrophysiological and morphological studies in these cases indicated neuro-axonal degeneration.^{3,6} Previous experiments in our laboratories have shown that chronic exposure to EO causes central peripheral distal axonal degeneration in rats.⁷ Nevertheless, the mechanism(s) underlying the neurotoxicity have not been elucidated.

Creatine kinase (CK) (ATP: creatine phosphotransferase, EC 2.7.3.2) is widely distributed in various tissues, particularly in the nervous system and muscles.⁸ Moreover, acrylamide and methyl *n*-butyl ketone which are typical toxins causing distal

axonopathy have been reported to inhibit CK.⁹ Therefore, we studied the effects of EO on this enzyme in vivo and in vitro.

Materials and methods

MATERIALS

Purified (crystalline) creatine kinase (from rabbit muscle) was obtained from Boehringer-Mannheim-Yamanouchi (Tokyo, Japan). CO₂ gas (99.5%) and ethylene oxide gas (pure EO 20 Wt%, Co₂ 80 Wt%) were purchased from Showa Tansan Co, Ltd (Tokyo, Japan). All other chemicals were reagent grade.

ANIMALS AND TREATMENT

Male Wistar rats weighing 160-180 g were used. The exposure chamber was made of stainless steel with a total inner volume of 1.0 m³. Air and EO gas were constantly introduced into the chamber through a mass flow meter. The concentration of EO in the exposure chamber was kept at 500 ppm and monitored periodically by gas chromatographic analysis. Such an exposure caused peripheral neuropathy in rats after 13 weeks of exposure.⁷ One exposure lasted for six hours and the exposure was repeated three times in a week. Control animals were exposed to ambient air in the same manner. Water was given ad libitum except during exposure to EO. (Hence, the water and the food were not in contact with EO.) As exposure to EO suppresses food intake, to minimise the effect of nutritional difference, food intake was matched by pair feeding between exposure and control groups. Forty hours after the final exposure, animals were killed by decapitation. Blood was collected from the cervical veins and kept on ice. The brain, entire spinal cord, and a part of gastrocnemius muscles were immediately kept at -40°C.

PREPARATION OF TISSUE

Frozen tissues were thawed at 37°C for five minutes. Subsequent procedures were all carried out at 0-4°C. Each tissue was homogenised with 9 vols of cold 20 mM NaHCO₃ using a Potter-Elvehjem homogeniser; a Teflon pestle was used for brains (800 rpm, 7 strokes), and a glass pestle for the spinal cords and muscles (400 rpm, 7 strokes). The muscle homogenates were centrifuged at 6000 rpm for 30 minutes. Supernatants of muscle homogenate and the whole

Departments of Environmental Toxicology and Environmental Health Engineering, University of Occupational and Environmental Health, Kitakyushu 807, Japan

M Matsuoka, H Igisu, N Inoue, H Hori, I Tanaka

Table 1 Serum chemistry (mean $\pm$ 02 SD) after 12 week exposure to ethylene oxide. (Number of samples in each group in parentheses)

	Control	Test		Control	Test
Total protein (g/dl)	7.1 $\pm$ 0.4 (8)	6.9 $\pm$ 0.5 (8)	Total lipid (mg/dl)	221.9 $\pm$ 57.6 (9)	232.4 $\pm$ 49.2 (9)
Albumin (g/dl)	2.5 $\pm$ 0.2 (8)	2.4 $\pm$ 0.2 (8)	Phospholipids (mg/dl)	114.3 $\pm$ 24.6 (9)	123.2 $\pm$ 10.0 (9)
Total bilirubin (mg/dl)	0.09 $\pm$ 0.04 (8)	0.15 $\pm$ 0.08 (8)	Total cholesterol (mg/dl)	76.6 $\pm$ 19.6 (8)	73.6 $\pm$ 8.1 (8)
Aspartate aminotransferase (Karmen unit)	160.8 $\pm$ 35.0 (8)	168.8 $\pm$ 48.6 (8)	High density lipoprotein cholesterol (mg/dl)	34.0 $\pm$ 10.9 (9)	41.1 $\pm$ 12.4 (9)
Alanin aminotransferase (Karmen unit)	27.8 $\pm$ 4.6 (8)	35.8 $\pm$ 10.3 (8)	Triglyceride (mg/dl)	47.4 $\pm$ 7.4 (8)	37.5 $\pm$ 7.1 (8)*
Alkaline phosphatase (King-Armstrong unit)	169.8 $\pm$ 26.1 (8)	193.5 $\pm$ 66.4 (8)	Free fatty acids (mEq/L)	0.71 $\pm$ 0.28 (9)	0.70 $\pm$ 0.10 (9)
Lactate dehydrogenase (Wróblewski unit)	357.2 $\pm$ 172.4 (8)	339.9 $\pm$ 129.3 (8)	Blood urea nitrogen (mg/dl)	13.9 $\pm$ 1.7 (8)	14.9 $\pm$ 3.5 (8)
Cholinesterase (IU)	68.4 $\pm$ 10.7 (8)	60.4 $\pm$ 14.3 (8)	Creatinine (mg/dl)	0.6 $\pm$ 0.1 (8)	0.5 $\pm$ 0.1 (8)
Creatine kinase (IU)	4501 $\pm$ 1234 (8)	2572 $\pm$ 659 (8)**	Uric acid (mg/dl)	1.5 $\pm$ 0.3 (8)	1.3 $\pm$ 0.3 (8)
Amylase (IU)	2423 $\pm$ 289 (8)	2307 $\pm$ 743 (8)	Tri-iodothyronine (ng/dl)	44.7 $\pm$ 8.1 (6)	35.7 $\pm$ 5.7 (6)
Glucose (mg/dl)	67.0 $\pm$ 12.2 (4)	63.2 $\pm$ 12.6 (5)	Thyroxine (μ g/dl)	4.57 $\pm$ 0.47 (6)	4.92 $\pm$ 1.26 (6)
			Thyroid stimulating hormone (μ U/ml)	2.22 $\pm$ 0.94 (6)	1.63 $\pm$ 0.29 (6)

Difference from control: *p < 0.05; **p < 0.005.

homogenates of brain and spinal cord were used for enzyme assay.

ANALYTICAL PROCEDURE

Activities of CK, aspartate aminotransferase (ASAT) (L-aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1), and lactate dehydrogenase (LDH) (L-lactate: NAD oxidoreductase, EC 1.1.1.27) in tissue were determined using the method of Oliver,¹⁰ Karmen *et al.*,¹¹ and Wróblewski and LaDue¹² respectively. Serum chemistry (total protein, albumin, total bilirubin, ASAT, alanin aminotransferase, alkaline phosphatase, LDH, cholinesterase, CK, amylase, glucose, total lipid, phospholipids, total cholesterol, high density lipoprotein cholesterol, triglyceride, free fatty acids, blood urea nitrogen, creatinine, uric acid) was determined by Hitachi H-736 autoanalyser. Tri-iodothyronine, thyroxine, and thyroid stimulating hormone in serum were determined by radioimmunoassay.

For in vitro studies, homogenates of the brain from male Wistar rats were suspended in 20 mM NaHCO₃ (0.33 mg/ml) with or without 10 mM dithiothreitol (DTT) and were bubbled with N₂ (100%), CO₂ (99.5%), and EO (EO 20 Wt%, CO₂ 80 Wt%) gas for two minutes. After being placed on ice for 30 minutes, the activity of CK was determined. For the determination of ASAT and LDH, homogenates of rat brain were made thus: 1.0 mg/ml for ASAT and 3.3 mg/ml for LDH.

Crystalline CK from rabbit muscle (1 mg/ml) was dissolved in 20 mM NaHCO₃ and bubbled with EO gas for three, 10, 30, and 60 seconds. After standing on ice for 10 minutes, the amount of sulphhydryl groups and CK activity were determined. The sulphhydryl group was measured by the method of Ellman using cysteine as the standard.¹³

The protein was measured by the method of Lowry *et al.*¹⁴ Statistical differences were examined by Student's *t* test.

Results

IN VIVO STUDIES

Body weight did not differ between exposed and control groups. The exposed rats began to show ataxic gait at the sixth week of the experiment.

The CK activity in the serum was lowered by more than 40% after 12 weeks of exposure (table 1). Serum triglyceride levels decreased by 20%, but no other biochemical alterations were found (table 1).

The CK activity was also inhibited in brain, spinal cord, and muscle after four weeks exposure but no inhibition was found in ASAT and LDH from these tissues (table 2). After 12 weeks exposure, CK activity in the brain was more suppressed than after four weeks.

After a single exposure (six hours), CK in the brain was suppressed by approximately 10%. The enzyme activity recovered gradually with time (data not shown).

Table 2 Effects of exposure to ethylene oxide on enzyme activities in tissues (mean $\pm$ SD). (Number of samples in each group in parentheses)

	CK	ASAT	LDH
Brain:			
Control (4)	315.9 $\pm$ 10.8	72.1 $\pm$ 1.6	136.5 $\pm$ 6.7
Test (6)	232.0 $\pm$ 4.5*	71.7 $\pm$ 2.5	136.5 $\pm$ 4.6
Control (6)†	298.1 $\pm$ 31.3		
Test (6)†	203.2 $\pm$ 21.0*		
Spinal cord:			
Control (4)	195.1 $\pm$ 9.5	40.8 $\pm$ 2.3	63.4 $\pm$ 1.4
Test (6)	150.8 $\pm$ 3.3*	40.0 $\pm$ 1.5	63.6 $\pm$ 2.8
Muscle:			
Control (4)	1854.8 $\pm$ 175.5	61.8 $\pm$ 5.7	202.6 $\pm$ 22.3
Test (6)	1101.6 $\pm$ 247.3*	59.4 $\pm$ 8.2	219.5 $\pm$ 32.9

The activities of CK (creatine kinase), ASAT (aspartate aminotransferase), and LDH (lactate dehydrogenase) are expressed in terms of international unit/g tissue, Karmen unit/mg tissue, and Wróblewski unit/mg tissue, respectively.

*Significantly different from the control (p < 0.001).

†The organs were obtained after 12 weeks exposure. All other organs were obtained after four weeks exposure.

Table 3 Effects of N_2 , CO_2 , and ethylene oxide on creatine kinase activity in brain homogenate

	Activity (IU/mg prot)	Per cent inhibition
Control	2.56 ± 0.02	—
+ 10mM DTT	2.63 ± 0.04	-2.7
+ N_2	2.61 ± 0.02	-2.0
+ CO_2	2.38 ± 0.03	7.0
+ EO	0.34 ± 0.04	86.7
+ EO + 10mM DTT	0.41 ± 0.02	84.0

Homogenates of the rat brain (0.33 mg/ml) with or without dithiothreitol (DTT) were bubbled with N_2 , CO_2 , or EO gas for two minutes and CK activity was determined. Each value is the mean ± SD of three experiments.

Effects of ethylene oxide on sulphydryl group (○) and enzyme activity (●) of purified CK. Crystalline creatine kinase from rabbit muscle dissolved in 20 mM $NaHCO_3$ (1 mg/ml) was bubbled with EO gas for three, 10, 30, and 60 seconds. Control experiments (0 sec) were done in same manner without bubbling EO. Sulphydryl (SH) group and CK activity were determined in duplicate and triplicate, respectively.

IN VITRO STUDIES

CK activity in the brain homogenate was not affected by bubbling with N_2 . Exposure to CO_2 caused a slight suppression of CK activity but EO inhibited CK activity considerably (table 3). No inhibition was found in ASAT activity ($98.2 \pm 2.9\%$ of control, $n=3$) and LDH activity ($97.6 \pm 1.1\%$ of control, $n=3$) when the brain homogenate was exposed to EO.

The inhibition of CK activity by EO was not counteracted by 10 mM DTT (table 3). The sulphydryl groups in purified CK were considerably decreased by brief exposure to EO. Nevertheless, the decrease of CK activity was much less than that of the sulphydryl groups (figure).

Discussion

Because of its high explosiveness, EO is usually mixed with CO_2 in sterilising processes,¹⁵ and it was in the present study. Our in vitro experiments, however, showed that the inhibition of CK was not influenced by CO_2 .

In cases of EO polyneuropathy routine laboratory studies are reported to be normal.¹⁻⁵ In a cross

sectional study of workers with potential occupational exposure to EO, haematological and biochemical studies (total bilirubin, alkaline phosphatase, LDH, ASAT, alanine aminotransferase, γ -GTP, BUN, and serum creatinine) showed no significant difference between the potentially exposed and unexposed groups.¹⁶ In these studies little attention was paid to CK activity. We found that exposure to EO causes suppression of serum CK activity, whereas none of the other serum enzymes we examined was inhibited. It is well known that serum CK activity is affected by thyroid status; serum CK activity decreases in hyperthyroidism and increases in hypothyroidism. After 12 weeks exposure, however, there were no alterations in serum triiodothyronine, thyroxine, and thyroid stimulating hormone, showing that thyroid function was not impaired by exposure to EO. Furthermore, our in vitro study indicated that EO can attack CK directly.

The level of ATP seems to be important to maintain normal function of the nervous system. For instance, fast axonal transport, which delivers essential materials to different parts of the nerve cells, is an energy (ATP) requiring process¹⁷ and the ATP levels in tissues are partially conserved by CK with phosphocreatine as the phosphate donor.¹⁸ Therefore, if CK activity is inhibited, it may cause dysfunction of the nervous system. We cannot conclude, however, that the inhibition of CK activity is the sole cause of encephalopathy or neuropathy because there is a possibility that EO impairs other important enzyme(s) or structure(s) in the nervous system.

CK has cysteine residues in its active site.⁸ In our in vitro study EO induced inhibition of CK was not counteracted by DTT, a protective agent for sulphydryl groups. This is in contrast with the suppression of the effects of acrylamide or methyl *n*-butyl ketone by DTT.⁹ There is, however, a possibility that EO acted on residues other than cysteine. Though EO decreased the amount of sulphydryl groups considerably, CK still retained its activity. Thus it appears that the sulphydryl groups that were attacked by EO may not be concerned directly in the catalytic action of CK.

To monitor human exposure to EO, measurement of the haemoglobin-histidine alkylation product, N -(2-hydroxyethyl)histidine has been proposed,^{19,20} although the background level of this product apparently limits the sensitivity.²¹ Among the enzyme activities examined, CK activity was the most sensitive to EO. Since the concentration of EO used in our experiment was high, however, further studies are necessary to determine whether or not CK activity may be used clinically as an indicator of EO intoxication.

We thank T Sakimura, M Muramoto, and S Ishimatsu for technical help.

- 1 Gross JA, Haas ML, Swift TR. Ethylene oxide neurotoxicity: report of four cases and review of the literature. *Neurology* 1979;29:978-83.
- 2 Salinas E, Sasich L, Hall DH, Kennedy RM, Morris H. Acute ethylene oxide intoxication. *Drug Intell Clin Pharm* 1981;15:384-6.
- 3 Kuzuhara S, Kanazawa I, Nakanishi T, Egashira T. Ethylene oxide polyneuropathy. *Neurology* 1983;33:377-80.
- 4 Finelli PF, Morgan TF, Yaar I, Granger CV. Ethylene oxide-induced polyneuropathy. A clinical and electrophysiologic study. *Arch Neurol* 1983;40:419-21.
- 5 Zampollo A, Zacchetti O, Pisati G. On ethylene oxide neurotoxicity: report of two cases of peripheral neuropathy. *Ital J Neurol Sci* 1984;V:59-62.
- 6 Schröder JM, Hoheneck M, Weis J, Deist H. Ethylene oxide polyneuropathy: clinical follow-up study with morphometric and electron microscopic findings in a sural nerve biopsy. *J Neurol* 1985;232:83-90.
- 7 Ohnishi A, Inoue N, Yamamoto T, *et al.* Ethylene oxide induces central-peripheral distal axonal degeneration of the lumbar primary neurones in rats. *Br J Ind Med* 1985;42:373-9.
- 8 Bais R, Edwards JB. Creatine kinase. *CRC Crit Rev Clin Lab Sci* 1982;16:291-335.
- 9 Lapin EF, Weissbarth S, Maker HS, Lehrer GM. The sensitivities of creatine and adenylate kinases to the neurotoxins acrylamide and methyl *n*-butyl ketone. *Environ Res* 1982;28:21-31.
- 10 Oliver IT. A spectrophotometric method for the determination of creatine phosphokinase and myokinase. *Biochem J* 1955;61:116-22.
- 11 Karmen A, Wróblewski F, LaDue JS. Transaminase activity in human blood. *J Clin Invest* 1955;34:126-33.
- 12 Wróblewski F, LaDue JS. Lactic dehydrogenase activity in blood. *Proc Soc Exp Biol Med* 1955;90:210-3.
- 13 Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* 1959;82:70-7.
- 14 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951;193:265-75.
- 15 Boucher RMG. Advances in sterilization techniques. State of the art and recent breakthroughs. *Am J Hosp Pharm* 1972;29:661-72.
- 16 Currier MF, Carlo GL, Poston PL, Ledford WE. A cross sectional study of employees with potential occupational exposure to ethylene oxide. *Br J Ind Med* 1984;41:492-8.
- 17 Ochs S, Smith CB. Fast axoplasmic transport in mammalian nerve in vitro after block of glycolysis with iodoacetic acid. *J Neurochem* 1971;18:833-43.
- 18 Bradford HF. Brain glucose and energy metabolism: the linkage to function. In: Bradford HF, ed. *Chemical neurobiology. An introduction to neurochemistry*. New York: W H Freeman and Company, 1986:118-54.
- 19 Calleman CJ, Ehrenberg L, Jansson B, *et al.* Monitoring and risk assessment by means of alkyl groups in hemoglobin in persons occupationally exposed to ethylene oxide. *J Environ Pathol Toxicol* 1978;2:427-42.
- 20 Farmer PB, Bailey E, Gorf SM, *et al.* Monitoring human exposure to ethylene oxide by the determination of haemoglobin adducts using gas chromatography-mass spectrometry. *Carcinogenesis* 1986;7:637-40.
- 21 Van Sittert NJ, De Jong G, Clare MG, *et al.* Cytogenetic, immunological, and haematological effects in workers in an ethylene oxide manufacturing plant. *Br J Ind Med* 1985;42:19-26.

Accepted 10 March 1989

Correspondence and editorials

The *British Journal of Industrial Medicine* welcomes correspondence relating to any of the material appearing in the journal. Results from preliminary or small scale studies may also be published in the correspondence column if this seems appropriate. Letters should be not more than 500 words in length and contain a minimum of references. Tables and figures should be kept to an absolute minimum. Letters are accepted on

the understanding that they may be subject to editorial revision and shortening.

The journal now also publishes editorials which are normally specially commissioned. The Editor welcomes suggestions regarding suitable topics; those wishing to submit an editorial, however, should do so only after discussion with the Editor.