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STUDIES ON THE NATURE OF HEMOLYTIC EFFECT INDUCED BY ETHYLENE GLYCOL ALKYL ETHERS

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Abstract: The nature of hemolytic effect induced by ethylene glycol alkyl ethers was analyzed taking into account G-6-PDH activity, ATP, pyruvate and thiols levels in peripheral blood of rats treated with single doses of 2-ethoxyethanol and 2-butoxyethanol. In addition, the susceptibility to autoxidation of rat erythrocyte lipids was evaluated. A decrease of ATP level in a dose-dependent manner and an increase in protein- and nonprotein-bound sulfhydryl groups were observed. These results indicate that an acute hemolytic effect of ethylene glycol alkyl ethers is not associated with alterations in G-6-PDH activity and the susceptibility of erythrocyte lipids to autoxidation. Increases in protein- and nonprotein-bound sulfhydryl groups seem to indicate the selective hemolysis of the aged erythrocytes. The increase in pyruvate and thiol levels may protect erythrocytes against the appearance of oxidative stress.

Keywords: ethylene glycol alkyl ethers, oxidative hemolysis, glucose-6-phosphate dehydrogenase, ATP, lipid peroxidation, thiol groups

Ethylene glycol alkyl ethers (EGAE) are extensively produced chemicals and used in a variety of industrial and household products. They are found in a number of paints, varnishes, engine fuels, hydraulic fluids, floor polishes and glass, leather, and upholstery cleaners. Some chemicals with similar structure, e.g., 2-phenoxyethanol are used also in pharmacy (1).

Animal and human studies have shown that EGAE can induce adverse reproductive, developmental, and hematological effects through inhalation, dermal absorption, and ingestion (2-4).

The hematopoietic system is a major target of EGAE, especially 2-butoxyethanol (BE) acute toxicity (4, 5). After administration of single doses of this compound to rats dose- and time-dependent anemia was observed (4, 6). BE-induced hemolytic anemia was characterized by an early swelling of red blood cells (RBC) as evidenced by an increase in packet cell volume (PCV) and mean corpuscular volume (MCV) (7), resulting in hemolysis and decline in the number of RBC, hemoglobin (HGB) concentration and PCV values, and also in an increase of plasma HGB level and reticulocyte count (4). In addition, BE caused secondary hemoglobinuria positively associated with the dose of this compound (8) and elevated spleen wt./body wt. ratio (9). Spleen enlargement was attributed to sequestration of swelled and deformed RBC, what was directly related to the effect of BE on circulating red blood cells. Stomatocytes, cup-shaped cells, and spherocytes are the main morphological features of erythrocytes from rats treated with BE or *in vitro* exposed to butoxyacetic acid (BAA), the metabolite of BE (3). *In vivo* studies clearly indicated that metabolic activation of EGAE via the alcohol/aldehyde dehydrogenases is a prerequisite for the development of hematotoxicity by this compound (6, 10, 11). Other EGAE, 2-ethoxyethanol (EE), was significantly less hematotoxic than BE (4).

Investigations of the mechanism of BE-induced hemolytic anemia indicated that this compound causes an increase in osmotic fragility of erythrocytes which results in increased sensitivity to osmotic lysis (12). Further *in vitro* examination of the cellular mechanisms involved in the hemolytic effect revealed that BAA and butoxyacetic aldehyde (BAL) caused a time- and concentration-dependent ATP depletion, suggesting that the erythrocyte membrane is the most likely target (10). The exact biochemical mechanism(s) of BE- and other EGAEs-induced hemolytic anemia remains essentially unknown.

BE was found to generate reactive oxygen species (ROS) including hydroxyl radicals, that in turn may produce lipid peroxidation, DNA damage, and/or protein modifications in the liver (13, 14). The production of ROS may be caused through both

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Kupffer cells activation and through the Fenton and/or Haber-Weiss reactions, involving iron and also the production of cytokines, e.g. TNF-alpha. The hepatic oxidative stress induced by BE *in vivo* occurs secondary to induction of hemolysis and iron deposition in the liver rather than as a direct effect of this compound or its metabolite, BAA (14).

Chronic exposure of rats and mice to BE led to an increase in hepatocellular carcinomas and liver hemangiosarcomas only in B6C3F1 mice (15). BE has been shown to be negative in standard genotoxicity assays (15, 16). It was suggested that neoplastic changes in liver induced by this compound may be attributed to oxidative stress as a result of Kupffer cells activation due to phagocytosis of hemolyzed or damaged RBC and iron deposition (14).

The current study was undertaken to investigate the effects of EE and BE on: (a) glucose-6-phosphate dehydrogenase (G-6-PDH) activity in RBC, (b) ATP and pyruvate concentrations in peripheral blood; (c) thiols levels in red cells; and (d) the susceptibility of erythrocyte lipids to oxidation. The examinations were performed at 24 h after EE, and BE administration to rats in single doses, i.e. when the majority of hematological effects were dramatic.

EXPERIMENTAL

Chemicals

EE, and BE were purchased from Sigma-Aldrich Ltd, Poland. Other chemicals were obtained from POCh (Poland). EE, and BE were dissolved in saline, immediately before dosing, and administered by subcutaneous injections in a fixed volume of 2 mL/kg body weight, regardless of dose.

Animals and treatment

Experiments were performed on 12-week-old male Wistar rats (Krf: (WI)WUBR), weighing about 300 g and obtained from Jagiellonian University, Faculty of Pharmacy, Breeding Laboratory (Kraków, Poland). They were kept under constant temperature (21 ± 2^{o}C) with a 12 h : 12 h (light : dark) cycle and had free access to rat chow (Murigran, Poland) and tap water throughout the experimental period.

The rats were arbitrarily divided into groups of five animals each. The rats were treated with EE at single doses of 2.5, 5.0, and 7.5 mM/kg. Another groups of rats were given in the same manner BE at doses of 0.625, 1.25, 2.5, and 5.0 mM/kg. Control rats obtained the equivalent volume of saline. Following a 24-h period after EE, and BE administration, the rats were anesthetized by thiopental *i.p.* injection (65 mg/kg), and bled by puncture of the heart. Blood samples were used for hematological and biochemical analyses.

The experiments were conducted in compliance with the Polish regulations for animals experimentation and the agreement of the Animal Welfare Committee of the Jagiellonian University.

Hematological and biochemical analyses

In heparin-added whole blood samples, immediately after collection, RBC, and HGB were analyzed by means of a COBAS MICROS (Roche, Palo Alto, CA, USA) analyzer. G-6-PDH activity and both ATP and pyruvate concentrations in rat blood were determined by means of Sigma Assay Kits No. 345-UV, 366-UV, and 726-UV (Sigma Diagnostics), respectively. The levels of total sulfhydryl groups (TSH) and nonprotein sulfhydryl groups (NPSH) in isolated erythrocytes were analyzed according to Sedlak & Lindsay (17) and Maron et al. (18), respectively. The protein sulfhydryl groups (PSH) level was calculated from TSH and NPSH concentrations. For the analysis of erythrocyte lipids oxidation the method of Stocks et al. (19) was employed.

Statistical analyses

The results are expressed as the mean ± S.D. Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett test. The analysis was performed with the SPSS 12.0 statistical packet (SPSS Inc., Chicago, IL, USA). Probabilities less than 0.05 were considered significant.

RESULTS

Administration of a single doses of BE (1.25 – 5.0 mM/kg) to rats resulted in significant hemolysis of erythrocytes as evidenced by a decreased RBC and HGB (Table 1). This effect became more pronounced with increasing doses. No hemolysis was observed in rats treated with EE. After administration of this compound at the highest dose of 7.5 mM/kg a decrease in HGB was seen. The G-6-PDH activities in both experimental groups were unchanged. The ATP concentrations declined in a dose-dependent manner only in rats treated with BE. The pyruvate concentrations in rats given EE and BE were slightly elevated, but these changes were not dose-dependent.

The level of erythrocyte thiols, especially NPSH and PSH, were elevated in rats treated mainly with BE at each dose. These increases were not

Table 1. Effects of various doses of EE, and BE on G-6-PDH activity, and ATP, pyruvate, RBC, and hemoglobin levels in peripheral blood of rats *in vivo*.

Group/ Compound (mM/kg)	G-6-PDH (U/g HGB)	ATP (µM/dL)	Pyruvate (mM/L)	RBC (x 10^6/µL)	HGB (g/dL)
Control	11.1 ± 0.95	52.0 ± 8.7	0.230 ± 0.06	9.1 ± 1.1	17.4 ± 1.1
EE					
2.5	13.0 ± 1.5	41.3 ± 1.5	0.231 ± 0.03	8.2 ± 0.5	16.3 ± 0.7
5.0	11.9 ± 1.9	45.6 ± 6.9	0.305 ± 0.08***	8.7 ± 0.2	15.6 ± 1.0
7.5	12.2 ± 1.7	42.7 ± 5.7	0.247 ± 0.09*	7.7 ± 0.7	14.1 ± 2.1*
BE					
1.25	14.5 ± 3.5	30.3 ± 5.5**	0.267 ± 0.03***	5.5 ± 1.5**	6.8 ± 0.8***
2.5	9.8 ± 3.1	24.8 ± 4.0**	0.239 ± 0.05	3.4 ± 1.0***	6.1 ± 0.6***
5.0	9.5 ± 2.5	21.2 ± 2.5***	0.232 ± 0.06	2.2 ± 0.6***	5.1 ± 0.4***

The mean ± S.D. values are given.
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, statistically significant difference from the control group.

Table 2. Influence of EE and BE on level of thiols in erythrocytes of peripheral blood in rats.

Group/ Compound (mM/kg)	TSH (µM/g HGB)	NPSH (µM/g HGB)	PSH (µM/g HGB)
Control	285.6 ± 10.5	7.8 ± 0.5	277.8 ± 10.5
EE			
2.5	266.0 ± 33.5	7.5 ± 0.9	258.5 ± 33.5
5.0	318.3 ± 18.2*	6.9 ± 0.9	311.4 ± 18.1*
7.5	215.2 ± 13.8***	9.7 ± 1.1*	205.5 ± 13.8***
BE			
1.25	339.4 ± 39.0*	16.6 ± 5.5***	322.8 ± 38.2
2.5	318.0 ± 18.2*	13.0 ± 2.2**	305.0 ± 17.6*
5.0	304.3 ± 42.0	10.9 ± 2.3*	293.4 ± 41.7

The mean ± S.D. values are given.
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, statistically significant difference from the control group.

dose-dependent. Administration of EE in a dose of 5.0 mM/kg increased a level of PSH, whereas the maximum dose of this compound (7.5 mM/kg) increased NPSH concentration and decreased PSH level (Table 2).

MDA levels in erythrocytes induced by hydrogen peroxide *in vitro* were about 32 – 46 times higher than in not-induced ones. No effects of EE and BE on not-induced and induced by hydrogen peroxide oxidation of erythrocyte lipids were observed. The obtained results were not statistically different in relation to control group (Table 3).

DISCUSSION

As expected on the basis of previous studies (4, 6), administration of EE at single doses in the range of 1.25–5.0 mM/kg did not induce hemolytic anemia. On the contrary, BE at the same doses led to dramatic intravascular hemolysis evidenced by the decrease in RBC, and HGB values in peripheral blood. The various changes observed in peripheral blood in rats treated with BE are typical of hemolytic anemia with an associated reticulocytosis and hyperplasia of both bone marrow and spleen (extramedullary hemopoiesis) (5).

The erythrocyte metabolism is designed for simply maintaining the cell structure, a specific composition of organic compounds, the cation flux between red cell and the blood plasma, and to keep the hemoglobin function. Therefore, for this cell only few metabolic pathways are necessary, such as glycolysis, the hexose monophosphate shunt (also called pentose phosphate pathway), nucleotide

metabolism and glutathione synthesis. The metabolic energy is supplied anaerobically with glucose as the exclusive nutrient. The key product for a proper function of the red cell is ATP, which is produced during the glycolysis. The G-6-PDH deficiency is the most frequent enzyme defect responsible for inherited hemolytic anemia. In erythrocytes with deficiency of this enzyme exposured to an oxidative stress, the level of reduced glutathione (GSH) decreases at first in the older red blood cells, entailing Heinz body formation, which indicates a damage to hemoglobin, and hemolysis due to cell membrane injury. The average lifetime of the erythrocytes is shortened and the production of new cells, i.e. reticulocytes is increased. The latter may help compensate for the impending anemia (20).

The survival of erythrocytes in the microcirculation system depends primary on their deformability. A decrease in cell deformability may result from a direct or indirect oxidative damage of the cell membrane due to lipid peroxidation or cross-linking via disulfide bridges in the membrane proteins. If the antioxidative defense and repair systems are deficient, hemolysis and Heinz bodies are already produced by potentially hemolytic agents. In case of G-6-PDH deficiency, nearly all the chemicals able to induce methemoglobinemia also produce hemolysis with or without Heinz bodies (20). Some chemicals, e.g. EGAE, are able to induction of hemolysis rather than methemoglobinemia or Heinz bodies.

The results of the present study indicate that EE and BE administered in single doses to rats not led to the changes in the erythrocytic G-6-PDH activity. Similar result was obtained *in vitro* by other author (10). Therefore, the G-6-PDH activity in erythrocytes plays no significant role in hemolytic action of EGAE.

The decrease in ATP concentration in blood of rats treated with BE, but not with EE, in dose-dependent manner was observed in the present study. Also, similar effect was found in rat blood incubated with BAA *in vitro* and in rat *in vivo* (10, 21, 22). It was suggested that ATP depletion is due to inhibition of its synthesis (21). It seems that the decrease in ATP level, in time- and dose-dependent fashion, may be related to impairment of glycolysis. It is believed that a decrease in pyruvate kinase (PK) and glucose phosphate isomerase (GPI) activities, enzymes involved in glycolytic pathway and energy production in red blood cells, may cause a reduction of ATP level which in turn leads to an increased mechanical rigidity and to reduced deformability of erythrocytes. The life span of erythrocytes becomes shortened and cells are subjected to an earlier sequestration in the spleen and hemolysis.

Table 3. Susceptibility of erythrocytes to oxidation in rats treated with single doses of EE and BE.

Group/Compound (mM/kg)	MDA (nM/g HGB)	
	Not induced oxidation	Induced oxidation
Control	4.0 ± 1.5	141 ± 8.8
EE		
2.5	3.4 ± 1.1	135 ± 6.8
5.0	3.5 ± 0.8	138 ± 5.5
7.5	3.9 ± 0.5	134 ± 7.1
BE		
0.625	4.3 ± 0.8	139 ± 5.9
1.25	3.0 ± 0.6	137 ± 7.6
2.50	3.2 ±0.7	136 ± 6.2

The mean ± S.D. values are given.
The results are not statistically significantly different from the control group.

The PK is magnesium-dependent enzyme; it plays an important role in controlling the flux of glycolysis from fructose-1.6-diphosphate to pyruvate. GPI is an essential enzyme in all cells, catalyzing the interconversion of fructose-6-phosphate and glucose-6-phosphate; both substrates play an important role in glycolysis, gluconeogenesis and in the pentose phosphate cycle (23).

The small, not a dose-dependent increase in pyruvate concentration in rat blood observed in present experiment, may be associated with a diminished utilization of this metabolite in tissues. Pyruvate demonstrates the antioxidative activity in relation to hydrogen peroxide in the reaction of its non-enzymatic oxidative decarboxylation (24). Pyruvate and other α-keto acids can exert a protective effect against the oxidative hemolysis and other damages triggered in the red blood cells by hydrogen peroxide. Pyruvate appears to be non-toxic metabolite which can freely diffuse between the extra- and intracellular spaces (25, 26).

The increase in protein- and nonprotein-bound sulfhydryl groups in erythrocytes from rats treated with EE and BE may suggest the selective hemolysis of older erythrocytes. It is well evidenced that aging of erythrocytes results in several biochemical changes, e.g., in GSH, ATP, and 2,3-diphosphoglycerate depletion, in a decrease of antioxidant enzymes activity, and in an increase of lipid peroxides concentration (27, 28). In addition, older erythrocytes are less deformable and more osmotically fragile (29), whereas during exposure to BAA at

sub-hemolytic concentrations *in vitro* human and rat erythrocyte deformability decreases, while MCV and osmotic fragility increase (12).

In conclusion, the current *in vivo* findings support earlier data which indicated that EE and BE differ in their hemolytic activity. BE induced hemolysis was distinctly associated with ATP depletion and pyruvate increase, but not with G-6-PDH activity changes. Nonprotein- and protein sulfhydryl groups in erythrocytes from rats treated with both EE and BE demonstrate the opposite dependence upon doses. These data suggest that older erythrocytes are more sensitive to the hemolytic effect of EE and BE. The lack of changes in susceptibility of the erythrocytes derived from rats treated with single doses of EE and BE to oxidation induced by hydrogen peroxide, expressed by MDA levels seems to exclude the oxidative mechanism of hemolysis.

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