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QUARTERLY

Liver and serum antioxidant status after methanol intoxication in rats*

Elżbieta Skrzydlewska and Ryszard Farbiszewski✉

Departments of Instrumental Analysis and Analytical Chemistry, Medical Academy, 5-230 Białystok 8, PO Box 14, Poland

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Activities of superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px) and glutathione reductase (GSSG-R) and concentration of ascorbate, α-tocopherol, non-protein and protein-bound sulfhydryl compounds and thiobarbituric acid-reactive substances (TBA-rs) were measured in liver and serum of rats 6, 12 and 24 h and 2, 5 and 7 days after intoxication with 1.5 g or 3.0 g methanol/kg b.w. Liver GSH-Px and GSSG-R activities and SH-groups and ascorbate content were significantly diminished at 6 and 24 h, while TBA-rs were increased. Serum SOD, GSH-Px and GSSG-R activities and SH-groups concentration were reduced while TBA-rs were elevated. The changes were more intensive after application of the higher dose of methanol. It is concluded that methanol impairs the liver and blood serum antioxidant mechanisms in rats.

Methanol toxicity, either acute or chronic, is characterized by a severe derangement of subcellular metabolism and structural alteration of liver cells. These changes may be associated with mitochondrial injury, especially with regard to the respiratory chain; partial inhibition of this chain may cause increased oxidation of redox carriers, resulting finally in enhanced production of oxygen radicals [1, 2]. However, the mechanism of their formation is very intricate. Lipid peroxidation is considered to be an efficient triggering mechanism of the disassembly of microsomal membranes and cytochrome P-450. An inverse correlation exists between the steady state concentration of lipid peroxidation products and cytochrome P-450 content in liver endoplasmic reticulum membranes [3]. Lipid peroxidation may induce activation of endogenous phospholipases and proteases resulting in disassembly of cytochrome P-450 [4].

In addition to its involvement in oxidative metabolism cytochrome P-450 also catalyzes reductive transformation of xenobiotics. This type of pathway often results in toxic effects due to formation of reactive free radicals.

The main pathway for methanol degradation involves alcohol dehydrogenase, the microsomal ethanol oxidizing system and catalase, which catalyze the conversion of methanol to aldehyde and then by aldehyde dehydrogenases to formate. These processes are accompanied by increase in NADH con-

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✉Corresponding author: Ryszard Farbiszewski, Department of Analytical Chemistry, Medical Academy, 15-230 Białystok 8, PO Box 14, phone 42 20 21 ext. 2226, fax 85 42 49 07

Abbreviations: SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; GSSG-R, glutathione reductase; TBA-rs, thiobarbituric acid-reactive substances.

centration what causes the stimulation of enzymes generating the superoxide anion that may be involved in lipid peroxidation [5].

Data on the antioxidant defence potential in the liver and blood serum after methanol intoxication in rats are lacking. In this report we draw particular attention to the antioxidant status expressed in enzyme activities and in the content of low molecular mass antioxidants in liver and serum of rats after acute methanol intoxication.

MATERIAL AND METHODS

Male Wistar rats (approx. 230 g body weight — b.w.) fed on a standard diet (containing 0.55% of cysteine and methionine) were used. All procedures were in strict accordance with regulations for the care and use of laboratory animals and were approved by the local Animal Care Committee. The minimal lethal dose of methanol in the rat is 9.5 g/kg b.w. The doses from 1 to 3 g of methanol/kg b.w. were used in the experimental methanol intoxication of rats [6]. In the present work 72 rats were given (by the intragastric route) 50% methanol in two doses (36 rats 1.5 g and 36 rats 3.0 g methanol/kg b.w.) in isotonic NaCl solution with a syringe through a plastic tube. Equivalent volume of saline was given to six control rats (three of them were killed after 6 h and the remaining three 7 days after NaCl administration). The intoxicated rats were divided into six groups (six rats in each group).

Six, 12, 24 hours and 2, 5 and 7 days after methanol administration the animals were killed under ether anaesthesia, then livers were removed quickly and placed in ice-cold 0.15 M NaCl, perfused with the same solution in order to remove completely the blood cells, then blotted on filter paper, weighed and homogenized in 9 ml of ice-cold 0.25 M sucrose or 0.15 M NaCl (both solutions contained 6 µl of 250 mM butylated hydroxytoluene in ethanol, to prevent formation of new peroxides during the assay). Homogenization procedure was performed under completely standardized conditions: 10% homogenates were centrifuged at 10000 × *g* for 15 min at 4°C and the supernatants were kept on ice until assayed. The supernatant obtained from homogenate prepared in sucrose was used to measure the activity of the enzymes (sucrose shows protective action): superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and glutathione reductase (GSSG-R). To evaluate catalase activity the homogenate was incubated with 0.1% Triton X-100 for 30 min at 37°C and then centrifuged at 10000 × *g* for 15 min at 4°C; the supernatant was used for enzyme assay. The supernatant obtained from homogenate prepared in 0.15 M NaCl was used to analyse the SH-groups, ascorbate and TBA-rs content.

Cu,Zn-SOD (EC 1.15.1.1) activity in the liver tissue and in the blood serum was determined by the method of Misra & Fridovich [7] as modified by Sykes *et al.* [8], which measures the activity of cytosolic SOD. Mn-SOD of liver mitochondria is known to be removed during the separation procedure. A standard curve for SOD activity was constructed using SOD from bovine erythrocytes (Sigma Biochemicals, St. Louis MO, U.S.A.). One unit of SOD is defined as the amount of the enzyme which inhibits epinephrine oxidation to adrenochrome by 50% [8]. The enzyme specific activity was expressed in units per mg protein.

Glutathione peroxidase (EC 1.11.1.6) activity was measured spectrophotometrically using a technique based on the procedure of Paglia & Valentine [9] whereas GSH formation was monitored by measurement of oxidation of NADPH to NADP. One unit of the enzyme activity is defined as the amount of enzyme catalyzing the oxidation of 1 mmol of NADPH/min per mg protein.

Glutathione reductase (EC 1.6.4.2) activity was measured by the method of Mize & Langdon [10] by monitoring oxidation of NADPH at 340 nm.

Catalase (EC 1.11.1.9) activity was measured by the decrease in absorbance of hydrogen peroxide at 240 nm [11]. The reaction rates were determined at 25°C using 10 mM hydrogen peroxide and the activity was expressed as micromoles of H_2O_2 decomposed per min and per mg protein.

Sulfhydryl groups were estimated according to Ellman [12] using 5,5′-dithiobis(ni-

trobenzoic acid) in liver homogenates and blood serum untreated or deproteinized (with perchloric acid).

Ascorbic acid in the supernatant of liver homogenates and serum was measured according to Kyaw [13].

Serum α-tocopherol was analysed by HPLC [14].

Lipid peroxidation products in the supernatant of liver homogenates and blood serum were assayed as described by Buege & Aust [15].

The results are expressed as mean values ±SD. Statistical analysis was performed using Student's t test for unpaired data, and values of $P<0.05$ were considered to be significant.

RESULTS

After administration of 1.5 g methanol/kg b.w. activity of the liver Cu,Zn-SOD was not changed during 7 days of observation, while the dose of 3.0 g of methanol caused an increase in the activity of this enzyme during the first day of intoxication (Tables 1 and 2). The activity of catalase was significantly increased during the first 24 h after either dose of methanol and then returned to normal values. GSH-Px and GSSG-R activities were significantly decreased 6 to 24 h after the smaller dose of methanol; this decrease remained whole time of the experiment after application of 3.0 g of methanol. The non-protein and protein-bound SH groups were significantly reduced after 12 h to 5 days after ingestion of either dose of methanol. The concentration of ascorbate was also diminished after 12 h and subsequently it returned to normal values, while at the same time the level of TBA-rs was considerably increased.

Antioxidant status of blood serum is presented in Tables 3 and 4. SOD activity in intoxicated rats was elevated in the first 24 h, then it returned to normal values. GSH-

Table 1. Antioxidant parameters in the liver from control rats and from rats treated with 1.5 g methanol/kg b.w.

Parameters	Time after intoxication						
	Control	6 h	12 h	24 h	2 days	5 days	7 days
Cu, Zn-SOD (U/mg protein)	11.6±0.5	12.0±0.7	11.0±0.8	11.6±0.7	11.6±0.7	11.4±0.7	11.2±0.6
Catalase (U/mg protein)	221±15	262 ±14*	248±22*	220±21	222 ±18	219±17	215±16
GSH-Px (μmol NADPH/min per mg protein)	144±11	144±13	133±13	112±11*	124±13*	133±12	142±7
GSSG-R (nmol NADPH/min per mg protein)	40.9±2.91	36.0±3.8*	34.6±3.6*	31.9 ±3.6*	38.2±3.6	40.0±3.4	40.7±3.3
Non-protein SH-groups (μmol/g tissue)	4.68±0.32	4.41±0.40	3.87±0.38*	3.60 ±0.35*	4.14±0.36*	4.32±0.44	4.41±0.30
Protein SH-groups (μmol/g tissue)	16.1±1.2	14.7±1.4	12.1±1.3*	7.8±1.3*	10.7±1.2*	14.1±1.3*	15.5±1.2
Ascorbate (μg/g tissue)	83.9±4.9	81.9±7.1	74.2±7.7*	79.1±6.3	81.9±6.4	82.6±5.6	81.4±6.3
TBA-rs (nmol/g tissue)	85.2±6.2	92.0±7.9	99.7±8.4*	100.0±7.7*	91.6±6.9*	87.5±8.2	85.5±7.1

* Significantly different from control ($P < 0.05$)

Px, GSSG-R activities, non-protein and protein bound SH-groups, ascorbate and tocopherol content gradually diminished, while TBA-rs were consistently elevated after methanol ingestion. The extent of the changes observed depended on the methanol dose.

DISCUSSION

Toxic effects of methanol on the liver and other tissues are well documented and confirmed by basic biochemical investigations, morphological studies and by clinical examinations in most cases of accidental intoxication in humans [1].

The data presented in this work indicate that subacute administration of methanol to rats led to an impairment of the antioxidant defence system both in liver and serum. Deficiency of some antioxidant enzymes (GSH-Px, GSSG-R) and of endogenous low molecular mass substances, e.g. α-tocopherol, ascorbate, non-protein SH compounds, was detected. A major role in the cellular defence against exogenous oxidants is played by reduced glutathione which is essential for the optimal functioning of numerous SH-dependent enzymes [16]. The content of non-protein SH compounds in our studies correlates positively with GSSG-R activity, and the decrease of the latter could be due to exacerbation of the damage to the liver which could become unable to remove effectively the reactive oxygen metabolites produced after methanol ingestion in rats.

Increased catalase expression in the liver 6 and 12 h after methanol ingestion could suggest that this enzyme and GSH-Px and GSSG-R are independently regulated when liver is damaged by methanol ingestion. Thus, it can be assumed that diminished activities of some enzymes and other compounds cause an impairment of the antioxidant defence potential of the liver. The lipid

Table 2. Antioxidant parameters in the liver from control rats and from rats treated with 3.0 g methanol/kg b.w.

Parameters	Time after intoxication						
	Control	6 h	12 h	24 h	2 days	5 days	7 days
Cu, Zn-SOD (U/mg protein)	12.1 ± 0.5	12.7 ± 0.7	13.3 ± 0.7*	13.1 ± 0.6*	12.4 ± 0.7	11.9 ± 0.5	12.0 ± 0.6
Catalase (U/mg protein)	230±14	294±15*	308±20*	287±19*	254±18*	232±19	228±15
GSH-Px (μmol NADPH/min per mg protein)	134.2±11.1	129.4±13.3	115.7±12.4*	92.7 ±8.6*	94.3±10.3*	112.7±12.1*	129.1±9.4
GSSG-R (nmol NADPH/min per mg protein)	39.1±2.7	32.9±3.8*	27.4±2.9*	21.3±2.5*	22.9±3.1*	29.4±3.4*	37.9±3.3
Non-protein SH-groups (μmol/g tissue)	4.50±0.29	4.31±0.36	3.89±0.34*	3.24±0.39*	3.79±0.38*	4.06±0.41	4.32±0.31
Protein SH-groups (μmol/g tissue)	16.0±1.2	14.1±1.3*	11.8±1.3*	9.3±1.4*	10.7±1.2*	11.1±1.1*	14.9±1.2
Ascorbate (μg/g tissue)	82.1±4.5	81.9±6.1	76.1±7.1*	73.2±6.5*	73.4±6.1*	75.3±6.7	79.7±6.1
TBA-rs (nmol/g tissue)	85.5±7.0	92.9±7.9	102.1±8.9*	109.3±7.1*	102.1±7.8*	93.8±8.2	86.7±7.1

* Significantly different from control ($P < 0.05$)

Table 3. Antioxidant parameters in blood serum from control rats and from rats treated with 1.5 g methanol/kg b.w.

Parameters	Time after intoxication						
	Control	6 h	12 h	24 h	2 days	5 days	7 days
Cu, Zn-SOD (U/mg protein)	0.80±0.06	1.00±0.09*	0.98±0.08*	0.88±0.06*	0.83±0.06	0.78±0.05	0.82±0.06
GSH-Px (μmol NADPH/min per mg protein)	15.0±0.9	14.5±1.3	12.9±1.0*	12.6±0.9*	12.2±0.9*	13.8±1.0	15.1±1.0
GSSG-R (nmol NADPH/min per mg protein)	0.82±0.06	0.71±0.08*	0.62±0.07*	0.66±0.06*	0.69±0.06*	0.72±0.06*	0.79±0.06
Non-protein SH-groups (μmol/ml)	0.30±0.02	0.23±0.03	0.19±0.02*	0.15±0.01*	0.19±0.01*	0.27±0.01*	0.32±0.02
Protein SH-groups (μmol/ml)	2.15 ±0.11	1.92±0.14*	1.78±0.13*	1.63±0.13*	1.49±0.12*	1.66±0.13*	1.94 ±0.13*
Ascorbate (μg/100 ml)	2.66±0.21	2.24±0.25*	2.09±0.24*	2.15±0.21*	2.25±0.21*	2.38±0.21	2.55±0.20
α-Tocopherol (mg/100 ml)	1.71 ±0.14	1.77±0.17	1.65±0.17	1.54±0.13*	1.38±0.13*	1.16±0.11*	1.33±0.11*
TBA-rs (nmol/ml)	2.08±0.15	2.39±0.17*	2.57±0.20*	2.66±0.18*	2.60±0.18*	2.40±0.15	2.20±0.13

Table 4. Antioxidant parameters in blood serum from control rats and from rats treated with 3.0 g methanol/kg b.w.

Parameters	Time after intoxication						
	Control	6 h	12 h	24 h	2 days	5 days	7 days
Cu, Zn-SOD (U/mg protein)	0.80±0.06	0.89±0.08*	1.07±0.09*	1.04±0.08*	0.95±0.06*	0.88±0.06*	0.78±0.06
GSH-Px (μmol NADPH/min per mg protein)	14.9±0.7	14.1±1.2	11.5±1.1*	10.2±0.9*	10.3±0.8*	11.3±1.0*	13.1±0.9*
GSSG-R (nmol NADPH/min per mg protein)	0.86±0.06	0.77±0.06*	0.61±0.08*	0.53±0.05*	0.60±0.06*	0.68±0.07*	0.75±0.06*
Non-protein SH-groups (μmol/ml)	0.31±0.02	0.24±0.04*	0.15±0.03*	0.13±0.02*	0.14±0.02*	0.19±0.02*	0.27±0.02
Protein SH-groups (μmol/ml)	2.18±0.13	1.95±0.15*	1.74±0.15*	1.42±0.14*	1.47±0.12*	1.49±0.13*	1.82±0.15*
Ascorbate (μg/100 ml)	2.71±0.19	2.44±0.23	2.14±0.24*	1.99±0.20*	2.04±0.18*	2.18±0.21*	2.47±0.18*
α-Tocopherol (mg/100 ml)	1.65±0.12	1.67±0.15	1.57±0.17	1.39±0.15*	1.18±0.13*	1.17±0.11*	1.37±0.12*
TBA-rs (nmol/ml)	2.09±0.14	2.35±0.17*	2.57±0.20*	2.79±0.20*	2.91±0.19*	2.61±0.17*	2.34±0.15*

peroxidation process expressed by an increase in TBA-rs concentration may intensify the impairment of this organ.

Ascorbate is an effective water-soluble antioxidant which, in blood plasma, is the first to become depleted on exposure to peroxyl radicals and other types of oxidative stress [17, 18]. When plasma is exposed to a free radical-generating source, lipid peroxidation does not occur until all ascorbate present has been oxidized [18]. In addition, ascorbate can recycle tocopherol when the latter is oxidized [19]. That is why, the decrease of ascorbate in liver and serum of methanol intoxicated rats may be of considerable significance. The normal ascorbate level is of therapeutic benefit as a result of its ability to reduce oxidative stress; low ratio of ascorbate radical/ascorbate monoanion couple [20] allows ascorbate to serve as a terminal reductant in oxidizing free radical chain reactions [21]. Ascorbate reacts rapidly with superoxide and hydroxyl radicals, as well as with alkyl, peroxyl and alkoxyl radicals, thereby it can neutralize these radicals and stop the initiation and propagation of the reaction chain. An interaction between tocopherol and ascorbate in inhibiting oxidation has been proposed [22]. The function of ascorbate is not confined to being an electron donor in redox processes.

α-Tocopherol is a major lipid-phase antioxidant which reduces peroxyl radicals from polyunsaturated fatty acids in the cell membrane phospolipids or lipoproteins [23]. In our studies α-tocopherol content in serum was significantly diminished after methanol poisoning.

The decrease of ascorbate and α-tocopherol concentration in the liver and serum might slow down the healing process of the damaged liver. This slowing down could be due to overconsumption of these compounds caused by abnormal production of reactive oxygen metabolites.

It is concluded that protection mechanisms against oxidative disturbances are less efficient after methanol ingestion than under physiological conditions. The present study is the first attempt at measuring the antioxidant potential in rat liver and blood serum after methanol ingestion.

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