Acta Poloniae Pharmaceutica – Drug Research, Vol. 57 No. 6 pp. 449–454, 2000

ISSN 0001-6837
Polish Pharmaceutical Society

EFFECT OF THIOL DRUGS ON THE OXIDATIVE HEMOLYSIS IN HUMAN ERYTHROCYTES

MAŁGORZATA ICIEK, MARTA POLAK and LIDIA WŁODEK

Institute of Medical Biochemistry, Jagiellonian University Collegium Medicum
7 M. Kopernika Str., 31–034 Cracow, Poland

Abstract. The effect of different thiol drugs and 2–methyl–thiazolidine–2,4–dicarboxylic acid on the oxidative stress, induced by hydrogen peroxide, was examined in human erythrocytes. The results indicated that captopril (CA), methimazole, N–acetylcysteine (NAC), penicillamine and precursor of L–cysteine 2–methyl–thiazolidine–2,4–dicarboxylic acid (CP) might protect the erythrocyte membrane against lipid peroxidation in the experimental conditions. Captopril, methimazole and penicillamine had the strongest antioxidative properties at the concentration level of 0.5 mM. The protective effects gradually decreased at higher and lower concentrations of these drugs. Contrary, the antioxidative properties of N–acetylcysteine increased with its levels growing in the reaction mixture, and only N–acetylpenicillamine did not protect erythrocytes against oxidative damages. The effect of 2–methyl–thiazolidine–2,4–dicarboxylic acid showed in these *in vitro* experimental conditions that it could act as an antioxidant at the concentration as high as 5 mM and higher.

Keywords: captopril / methimazole / N–acetylcysteine / penicillamine / N–acetylpenicillamine / 2–methyl–thiazolidine–2,4–dicarboxylic acid / oxidative hemolysis

Studies *in vitro* demonstrate that thiols can have both anti– and prooxidative effect depending on their concentration, the presence of transitory metal ions, the pK value of the sulphydryl group, and even on the polarity of the environment (1). The protective effect of thiols is associated with their ability to react with reactive oxygen species, while their prooxidative effect is related to their spontaneous oxidation to sulphides, as well as to the formation of thiyl radicals (2,3). Numerous drugs characterized by a different pharmacological profile contain sulphydryl groups the presence of which may trigger both a pro– and antioxidative effect. In other words, their side effect may be both, beneficial and disadvantageous for the cells. The investigations in this paper included drugs containing the –SH group and having diversified pharmacological properties, such as captopril, methimazole, N–acetylcysteine and penicillamine, as well as compounds which have no medical application, 2–methyl–thiazolidine–2,4–dicarboxylic acid and N–acetylpenicillamine. Captopril reduces blood pressure as a specific competitive inhibitor of angiotensine convertase. It is known to manifest antioxidative properties (4,5,6). Methimazole, another drug under our investigation, is employed in hyperthyroidism. To date, it is little known about its anti– or prooxidative effect but it was found to act as an antioxidant reducing the nephrotoxic activity of gentamycin (7). N–acetylcysteine (NAC) is a commonly used mucolytic and hepatoprotective agent which shows antioxidative properties (8). 2–Methyl–thiazolidine–2,4–dicarboxylic acid (CP) is a product of non–enzymatic concentration of pyruvate and cysteine and shows hepatoprotective properties (8,9,10).

The effect of the above mentioned thiols on oxidative processes was studied in human erythrocytes where oxidation was induced by hydrogen peroxide in the presence of natrium azide, a catalase inhibitor. The effect of the above thiols was studied within a given concentration ranges 0.1 mM – 2.0 mM for CA, methimazole, NAC, penicillamine, N–acetylpenicillamine, and 0.1 mM – 25 mM for CP. The levels of malonyldialdehyde (MDA), reactive oxygen species (ROS) and hemoglobin released were evaluated as the measure of red cell peroxidative hemolysis.

EXPERIMENTAL

Chemicals

2–Thiobarbituric acid, N–acetylcysteine, 2', 7'–dichlorofluorescein were obtained from Sigma Chemical Co (Germany) whereas methimazole and N–acetyl–DL–penicillamine from Sigma Chemical Co (St. Louis USA). Trichloroacetic acid was purchased from Ubichem plc Sigma Chemical Co, captopril from Fluka Chemie AGCH–9470 Buchs. D–L– penicillamine and 2',7'–dichlorofluorescein diacetate were purchased from Serva Feinbioche-

mica (Heidelberg) and Molecular Probes Eugene OR (USA), respectively. 2–Methyl–thiazolidine–2, 4–dicarboxylic acid was synthesized in the Laboratory of Chemical Synthesis of the Jagiellonian University.

Isolation of erythrocytes

Human blood was obtained from healthy volunteers and was sampled into natrium citrate containing tubes. The cells were separated by centrifugation at 2500 g for 10 min. The erythrocytes were washed three times with the same volume of physiological saline.

Reaction mixtures

Each reaction mixture in a final volume of 2 ml contained 0.2 ml of packed red cells. The final concentration of natrium azide was 2 mM, of hydrogen peroxide 10 mM, and of the thiol compounds 0.1, 0.5, 1.0, and 2.0 mM. Simultaneously, control samples without thiols were prepared. The samples were adjusted to a final volume with phosphate–buffered saline (0.9% NaCl, 5 mM sodium/potassium phosphate, pH 7.4) and then were incubated for 30 min at 37°C.

Lipid peroxidation assay

Lipid peroxidation was measured in reaction with thiobarbituric acid (TBA) in acid medium (11) and was expressed as micromoles of malonyldialdehyde per g of hemoglobin in reaction mixture. After incubation, 0.25 ml of reaction mixture was deproteinized with 2 ml of 28% trichloroacetic acid and then centrifuged at 2500 g for 10 min. Next 2 ml of the supernatant was mixed with 0.5 ml of 0.9% TBA and heated at 100°C for 10 min. The absorbance of the coloured product was measured at 535 nm.

Reactive oxygen species (ROS) assay

Reactive oxygen species were assayed fluorometrically according to the method of Bondy (12). 0.01 ml of erythrocytes suspension was incubated with 0.01 ml of 2',7'–dichlorofluorescein diacetate in a final volume of 1ml phosphate buffer pH 7.4 at 37°C for 30 min. Then samples were centrifuged at

Table 1. Effect of various captopril concentrations on lipid peroxidation (MDA), reactive oxygen species (ROS) and free hemoglobin levels.

	Control	Captopril			
		0,1 mM	0,5 mM	1 mM	2 mM
MDA µmol/g Hb	0,304 ± 0,022	0,211** ± 0,017	0,179** ± 0,006	0,183** ± 0,005	0,262* ± 0,025
ROS µmol/g Hb	4,236 ± 0,373	3,125* ± 0,141	2,789* ± 0,119	3,119* ± 0,155	3,535* ± 0,266
Hb g/cm³	0,120 ± 0,004	0,083* ± 0,012	0,071* ± 0,004	0,076* ± 0,003	0,101* ± 0,006

*p<0,05; **p<0,001

Table 2. Effect of various methimazole concentrations on lipid peroxidation (MDA), reactive oxygen species (ROS) and free hemoglobin levels.

	Control	Methimazole			
		0,1 mM	0,5 mM	1 mM	2 mM
MDA µmol/g Hb	0,295 ± 0,020	0,229* ± 0,012	0,201* ± 0,018	0,229* ± 0,020	0,262 ± 0,022
ROS µmol/g Hb	4,216 ± 0,199	3,515 * ± 0,129	3,150** ± 0,077	3,353** ± 0,065	3,403 ± 0,411
Hb g/cm³	0,120 ± 0,004	0,106* ± 0,006	0,084* ± 0,004	0,088* ± 0,005	0,107 ± 0,006

*p<0,05; **p<0,001

Table 3 .Effect of various penicillamine concentrations on lipid peroxidation (MDA), reactive oxygen species (ROS) and free hemoglobin levels.

	Control	Penicillamine			
		0,1 mM	0,5 mM	1 mM	2 mM
MDA µmol/g Hb	0,292 ± 0,008	0,234* ± 0,012	0,221* ± 0,011	0,233* ± 0,010	0,253* ± 0,010
ROS µmol/g Hb	4,048 ± 0,262	3,465** ± 0,221	3,262** ± 0,145	3,432* ± 0,115	3,635* ± 0,122
Hb g/cm^3	0,120 ± 0,004	0,107* ± 0,004	0,103* ± 0,003	0,108* ± 0,004	0,115* ± 0,006

*p<0,05; **p<0,001

Table 4. Effect of various D,L–acetylpenicillamine concentrations on lipid peroxidation (MDA), reactive oxygen species (ROS) and free hemoglobin levels.

	Control	D,L–acetylpenicillamine			
		0,1 mM	0,5 mM	1 mM	2 mM
MDA µmol/g Hb	0,290 ± 0,016	0,268 ± 0,012	0,272 ± 0,014	0,272 ± 0,019	0,276 ± 0,010
ROS µmol/g Hb	4,145 ± 0,150	4,156 ± 0,112	4,162 ± 0,095	4,151 ± 0,162	3,940 ± 0,156
Hb g/cm^3	0,120 ± 0,004	0,121 ± 0,005	0,121 ± 0,020	0,120 ± 0,004	0,120 ± 0,004

Table 5 . Effect of various N–acetylcysteine concentrations on lipid peroxidation (MDA), reactive oxygen species (ROS) and free hemoglobin levels.

	Control	N–acetylcysteine			
		0,1 mM	0,5 mM	1 mM	2 mM
MDA µmol/g Hb	0,280 ± 0,011	0,243* ± 0,010	0,225* ± 0,015	0,214* ± 0,017	0,203* ± 0,012
ROS µmol/g Hb	4,145 ± 0,220	3,594* ± 0,150	3,430* ± 0,165	3,289* ± 0,167	3,084* ± 0,226
Hb g/cm^3	0,120 ± 0,006	0,107* ± 0,005	0,101* ± 0,009	0,095* ± 0,010	0,086* ± 0,008

*p<0.05

12000 g for 8 min at 4^{0}C. The excitation wavelength was 488 nm and the emission wavelength 525 nm. The level of ROS was expressed as micromoles of 2',7'–dichlorofluoresceine per g of hemoglobin in reaction mixture.

Hemoglobin released assay

The amount of hemoglobin released was determined according to the method of Drabkin (13). The reaction mixture after incubation was centrifuged at 2500 g for 10 min, then the amount of hemoglobin in the supernatant was determined. To 5 ml of Drabkin reagent 0.2 ml of supernatant was added and after 20 min extinction was measured at 540 nm.

Statistical methods

Student's t–test was used for statistical analysis and a p value of <0.05 was considered

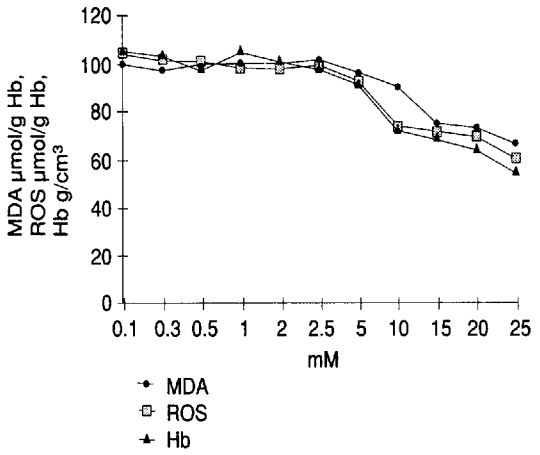

Figure 1. Effect of various 2–metylthiazolidine–2,4–dicarboxilic acid concentrations on lipid peroxidation (MDA), reactive oxygen species (ROS) and free hemoglobin levels.

significant. Values are expressed as means ± SEM.

RESULTS

The control samples containing none of the studied compounds were characterized by a high level of MDA, ROS and hemoglobin released.

All the investigated concentration values of captopril were found to significantly decrease oxidative hemolysis (Table 1). The greatest drop in the level of MDA, ROS and released hemoglobin was noted at 0.5 mM, when the level of MDA decreased to 58%, ROS diminished to 65% and hemoglobin to 59% of the control samples. At higher concentration values, the antioxidative effect of captopril was weaker.

Similarly as in the case of captopril, all the studied methimazole concentration values resulted in a significant decrease of the MDA, ROS and hemoglobin levels (Table 2). All these parameters reached their lowest values at the methimazole concentration of 0.5 mM. At this level the value of MDA dropped to 68%, ROS to 74% and free hemoglobin to 70% of the control values. These levels gradually increased at higher and lower methimazole concentrations.

Penicillamine was proved to exert a beneficial effect on a drop of all the investigated parameters (Table 3). Also in this case the lowest level of MDA, ROS and free hemoglobin was noted at 0.5 mM. The level of MDA reached 75% of the controls while for ROS, the value was 80%, for free hemoglobin 90%. These values also increased at higher and lower penicillamine levels.

Studies on N–acetylpenicillamine demonstrated that the compound exerted no protective effect against oxidative damage of the cells. None of the investigated D,L–acetylpenicillamine concentration levels resulted in the decrease of MDA, ROS and free hemoglobin values, or these values were diminished only negligibly (Table 4).

Investigations carried out on NAC (Table 5) confirmed its antioxidative properties. It is showed that the level of MDA, ROS, hemolysis and free hemoglobin decreased with the increase in NAC concentration. For the most beneficial and at the same time the highest NAC concentration level (2.0 mM), the value of MDA dropped to 72%, ROS to 74% and free hemoglobin to 71% of the control levels.

Figure 1 illustrates the effect of various CP concentration values on the level of MDA, ROS and free hemoglobin. The association was studied for the concentration range of 0.01–25.0 mM. The results indicated that in the experimental conditions *in vitro*, the protective effect of CP could be observed only at 5.0–25.0 mM. At the highest concentration (25.0 mM), the level of MDA decreased to 68%, ROS to 61%, and free hemoglobin to 59% of the control values.

DISCUSSION

The present investigations demonstrated that when human erythrocytes were exposed to hydrogen peroxide in the presence of natrium azide, the levels of MDA, ROS and hemolysis rose rapidly in comparison to samples containing hydrogen peroxide itself or natrium azide only. This supports our hypothesis that hemolysis results from peroxidative damages of biological membranes and other cellular structures inflicted by hydrogen peroxide. The studies showed that erythrocytes were characterized by high resistance to hydrogen peroxide and, thus, in our experiment it was necessary to employ high concentrations of the compound (10 mM) in the presence of natrium azide, a catalase inhibitor. Only in such conditions the erythrocyte antioxidative systems break down, which ultimately leads to hemolysis.

The investigations included the group of thiols which, as it is well known, due to their sulphydryl group could manifest changeable and contrary effects in pro– and antioxidative processes (4, 5, 14,15). The results demonstrate that in the experimental conditions at 0.5 mM concentration,

captopril, methimazole and penicillamine exert the strongest protective effect on erythrocytes. In this group, the most pronounced antioxidative activity is characteristic of captopril.

N–acetylcysteine behaves differently, because its antioxidative properties increase with increasing NAC concentration in reaction mixture. It seems to confirm its generally accepted low toxicity. Most likely, in order to visualize a drop in its protective effect, investigations should be carried out on the compound with higher concentration values. Contrary to penicillamine (Table 3), N–acetylpenicillamine (Table 4) has no effect on oxidative processes in erythrocytes and it shows that acetylation by nitrogen results in a total loss of protective properties of this compound.

In this work, 2–methyl–thiazolidine–2,4–dicarboxylic acid (CP), a product of non–enzymatic condensation of cysteine and pyruvate released in a reverse reaction simultaneously two antioxidants: cysteine necessary for the GSH biosynthesis and pyruvate. The latter ketoacid proved to exert the antioxidative properties (8, 16). In the previous studies *in vivo* and *in vitro,* CP was proved to have an effective and long–lasting (up to 12 hours) hepatoprotective effect in ethyl alcohol (17) and paracetamol intoxication (18). This is why the 30–minute incubation of 2–methyl–thiazolidine–2,4–dicarboxylic acid with erythrocyte suspension was most likely too short to allow any protective effect of lower CP concentrations to be noted.

The prooxidative activity of thiols demonstrated with use of free cysteine as an example, showing that both, its deficit and its excess can induce hemolysis (8). A similar concentration–dependent effect on hemolysis is exerted by penicillamine (19). In the present investigations, the concentration of penicillamine at which its maximum protective effect against hydrogen peroxide is noted is identical with the value observed by Lovstad (19) at which he also noted the lowest level of hemolysis. A decrease in protective properties observed at higher concentration levels of the studied thiols is most likely related to the oxidation of their sulphydryl groups to corresponding disulphides and the possible formation of disulphide and thiyl radicals. No such concentration of NAC was identified in the present studies at which its protective effect on erythrocytes would be decreased, as it could be observed in the case of the other thiol drugs. In the *in vivo* experiment conduced by Sprong et al. (20), lower doses of NAC were found to decrease the blood hydrogen peroxide level and to diminish the mortality rate in rats while high doses of NAC resulted in the decrease of pulmonary GSH and increase of mortality .

The antioxidative effect of captopril was first demonstrated by Andreoli (4). The observation that *in vitro,* in an acellular system captopril is a stronger antioxidant than cysteine appears very interesting. Fernandes (21) found that captopril was a more effective antioxidant than enalapril or lisinopril – other angiotensine convertase inhibitors which do not contain the thiol group. It suggests that the protective properties of CA are associated with the free –SH group. The investigations of Golik (5) and Altuntas (6) demonstrated that blood concentration levels of MDA in patients subjected to long–term captopril therapy significantly decreased which confirms the antioxidative properties of the drug. Other studies by Lappena proved that in the presence of iron or copper ions captopril exhibited prooxidative effects and, thus, it could not be treated as a simple antioxidative agent. A specific binding of copper by captopril induces its prooxidative activity. Simultaneously, penicillamine has an antioxidative effect in the presence of copper, and a prooxidative effect in the presence of iron (22). The prooxidative ability of captopril and penicillamine which is regulated by the presence of transitory metals must, then, be taken into consideration in experimental studies and clinical trails. These reports are confirmed by Bartosz who states that deoxyribose degradation induced by iron or copper ions is intensified under the influence of captopril (15).

Summing up, the investigations, of methimazole, captopril, NAC and penicillamine irrespectively of their main pharmacological usage can be said to exhibit *in vitro* concentration–dependent antioxidative activities which in this case can be regarded as a beneficial side effect. One should bear in mind that thiol drugs, as well as all the compounds containing the –SH groups, may also demonstrate adverse prooxidative properties within at certain concentration ranges and in the presence of transitory metals.

REFERENCES

1. Tocher J., Edwards D.: Biochem. Pharmacol. 9, 1367 (1995).
2. Yim M.B., Chae H.Z., Rhee S.G., Chok P.B., Stadtman E.R.: J. Biol. Chem. 269, 1621(1994).
3. Wardman P.: in Biothiols in Health and Disease, Packer L., Cadenas E. Eds., p.1, Dekker, New York 1995.
4. Andreoli S.P.: Am. J. Physiol. 264, in: Renal Fluid Electrolyte Physiol. 33, 120 (1993).

5. Golik A., Weissgarten J., Evans S., Cohen N., Zaidenstein R., Modai D., Cotariu D.: Clin. Chim. Acta. 240, 89 (1995).
6. Altuntas Y., Guven M., Ince E., Acbay O., Caner M., Kanigur–Sultuybek G.: J. Bas. Clin. Physiol. Pharmacol. 6, 281 (1995).
7. el Daly E.S.: Journal de Pharmacie de Belgique. 52, 149 (1997).
8. Meister A.: in Biothiols in Health and Disease, Packer L., Cadenas E. Eds., p.165, Dekker, New York 1995.
9. Włodek L.B. Wróbel M.: Neoplasma. 39, 223 (1992).
10. Cier M.P.: Sem. Hop. Paris. 39, 2173 (1980).
11. Stock S.J., Dormandy T.L.: Br. J. Hematol. 20, 95 (1971).
12. Bondy S.C., Guo S.X.: Eur. J. Pharmacol. 270, 349 (1994).
13. Drabkin D.L., Austin J.H.: J. Biol. Chem. 112, 105 (1936).
14. Vibert–Li J–L., Okada S.: Acta Med. Okayama. 50, 125 (1996).
15. Bartosz M., Kedziora J., Bartosz G.: Free Radic. Biol. Med. 23, 729 (1997).
16. Włodek L., Rommelspacher H.: Acta Biochim. Polon. 44, 759 (1997).
17. Włodek L., Rommelspacher H.: Alcohol and Alcoholism. 29, 649 (1994).
18. Włodek L., Rommelspacher H.: Fundam. Clin. Pharmacol. 11, 454 (1997).
19. Lovstad R.A.: Int. J. Biochem. 16, 1283 (1984).
20. Sprong R.C., Winkelhuyzen–Janssen A.M., Aarsman C.J., van Oirschot J.F., van der Bruggen T., van Asbeck B.S.: Am. J. Resp. Critic. Care Med. 157, 1283 (1998).
21. Fernandes A.C., Filipe P.M., Freitas J.P., Manso C.F.: Free Radic. Biol. Med. 20, 507 (1996).
22. Lappena D., De Gioia S., Mezzetti A., Ciofani G., Di Ilio C., Cuccurullo F.: Biochem. Pharmacol. 50, 27 (1995).

Received: 22.03.2000