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ACTIVITIES OF ANTIOXIDANT ENZYMES AFTER AMIFOSTINE APPLICATION DURING CYCLOPHOSPHAMIDE ANTICANCER THERAPY

ANNA STANKIEWICZ[1*], ELŻBIETA SKRZYDLEWSKA[1], MAREK MAKIEŁA[1], MARIOLA SULKOWSKA[2], WALDEMAR FAMULSKI[2], TOMASZ ZAWADZKI[2]

[1]Departments of Analytical Chemistry,
[2]Pathological Anatomy,
Medical University of Białystok, Białystok, Poland

Cyclophosphamide, an alkylating compound, used in chemotherapy is metabolised with free radical formation. The potent scavenger of oxygen free radicals acting selectively in normal tissues is amifostine. This paper reports data on the effect of amifostine on the rat liver and lung antioxidants enzymes activity after cyclophosphamide injection. Cyclophosphamide causes a decrease in the liver Cu,Zn-SOD, glutathione peroxidase (GSH-Px) and glutathione reductase (GSSG-R) activities and an increase in catalase (CAT) activity. At the same time this compound causes a decrease in lung Cu,Zn-SOD and CAT, but it does not change GSSG-R activity and causes an increase in GSSG-R activity. Administration of cyclophosphamide with amifostine causes smaller changes in activities of the examined enzymes. In conclusion, amifostine partially protects cells from cyclophosphamide and its metabolites.

INTRODUCTION

Amifostine (S-2-3(aminopropylamino)ethylphosphorothioate) is a phosphorylated aminothiol prodrug, which is an analogue of cystamine. It is a selective cytoprotective agent for normal tissues from the toxicities associated with chemotherapy and radiotherapy (Spencer & Goa, 1995). The active metabolite of amifostine is S-2-(3-aminopropylamino)ethanethiol (WR-1065) which is selectively produced by normal cells through dephosporylation of amifostine by membrane-bound alkaline phosphatase (Capizzi, 1996). This compound may provide cytoprotective action directly by bind to and thus detoxify the active species of alkylating and platinum agents (DeNeve, Everett, Suminski & Valeriote 1988; Treskes, Holwerda, Nijmans, Ficthinger-Schepman & Van der Vijgh, 1992). It also acts as a potent scavenger of drug- or radiation-induced oxygen free radicals (Ohnishi, Ohnishi, Glick & Schein, 1992).

Many anticancer drugs, like alkylating and platinum agents, are highly reactive electrophilic compounds and thus generate reactive oxygen species (Bachur, Gordon & Gee, 1978). One of such alkylating compounds used in chemotherapy is cyclophosphamide. Metabolic transformations of cyclophosphamide produce therapeutically active compounds which exert toxic effects, such as acrolein, phosphotamide mustard and nitrogen mustard hydroxylated by the cytochrom P-450 mixed function oxidase (MFO) system. Phosphoramide mustard and acrolein reduce glutathione concentration in the mouse liver rendering cells more sensitive to oxidant injury (Patel & Block, 1990; Venkatesan & Chandrakasan, 1995). However, increased glutathione concentration in the lung after cyclophosphamide administration has been found in other study (Patel & Block, 1985).

The activity of the respective enzymes involved in metabolic transformations of cyclophosphamide shows significant tissue differentiation affecting the selective toxicity of cyclophosphamide. The lack of detoxifying enzymes, aldehyde oxidase and aldehyde dehydrogenase, in the lungs contributes to selective cyclophosphamide toxicity against the lung tissue. MFO-mediated metabolism of cyclophosphamide and of other similar drugs is an important, but not the only pathway of their metabolic changes. Other metabolic pathways such as co-oxidation via arachidone acid metabolism through prostaglandin H synthetase (PHS) have

*Address for correspondence: Dr hab. Elżbieta Skrzydlewska, Department of Analytical Chemistry, Białystok Medical University, Ul. Mickiewicza 2, P.O. Box.14, 15–230 Białystok 8, e-mail: skrzydle@amb.ac.bialystok.pl

also been reported (Patel, 1990; Fraiser, Kanekal & Keherer, 1991).

The highest concentration of MFO was found in the liver, while the highest activity of PHS and lipooxygenase was revealed in the lungs and the bladder, the organs most severely affected by cyclophosphamide. Studies on the effect of MFO and PHS inhibitors on cyclophosphamide metabolism allow for the assumption that cyclophosphamide activation in the liver and lungs occurs, at least partly, in the presence of various enzymes. Thus, MFO inhibitors decrease the production of alkylating cyclophosphamide metabolites in the liver, but not in the lungs, while PHS inhibitors are effective in the lungs, but not in the liver. None of the inhibitors completely stops the production of alkylating compounds nor protects against damage and subsequent pulmonary fibrosis observed after cyclophosphamide administration (Marinello, Gurtoo, Struck & Paul, 1978; Patel, 1987; Kanekal & Kehrer, 1994).

Recent studies suggest that cyclophosphamide forms reactive metabolites, generates reactive oxygen species during its oxidative metabolism, and depresses the antioxidant defence mechanisms of the lung (Venkatesan & Chandrakasan, 1995; Sulkowska & Sulkowski, 1997). On the other hand, amifostine can scavenge reactive oxygen species (Marzatico, Porta, Moroni, Bertorelli, Borasio, Finotti, Pansarasa & Castagna, 2000; Ohnishi, Ohnishi, Glick & Schein, 1992). There are no reports on the combined effect of amifostine and cyclophosphamide on the lung and liver antioxidant system. The aim of this work has been to evaluate the activity of basic antioxidant enzymes in the lung and liver tissues after cyclophosphamide and amifostine treatment.

MATERIAL AND METHODS

Male Wistar rats (approximately 230 g b.w.) fed standard diet (containing 0.55% of cysteine and methionine but no antioxidant) were used for the experiment. The experimental protocol was approved by the Ethical Committee on Human and Animal Experimentation of the Medical University of Białystok.

The rats were divided into 4 groups:

A. Control group (n = 18).
B. Cyclophosphamide group. The rats were treated intraperitoneally with cyclophosphamide at a dose of 150 mg/kg b.w. (n = 18).
C. Amifostine group. The rats received intraperitoneally amifostine at a dose of 200 mg/kg b.w. (n = 18).
D. Cyclophosphamide and amifostine group. The rats were treated intraperitoneally with amifostine at a dose of 200 mg/kg b.w. and subsequently with cyclophosphamide at a dose of 150 mg/kg b.w. (n = 18)

1, 5, and 14 days after drugs treatment rats were sacrified following ether anaesthesia (six animals in each group). Livers and lungs were removed quickly and placed in iced 0.15 M NaCl solution, perfused with the same solution to remove blood cells, blotted on filter paper, weighed and homogenised in 9 ml of ice-cold 0.25 M sucrose with addition of 6 µl 250 mM BHT (butylated hydroxytoluene) in ethanol, to prevent formation of new peroxides during the assay. Homogenization procedure was performed under standardized conditions; 10% homogenates were centrifuged at 10 000 × *g* for 15 min at 4°C and the supernatant was kept on ice until assayed.

Superoxide dismutase Cu,Zn-SOD (E.C. 1.15.1.1) activity was determined by the method of Misra and Fridovich (1972) modified by Sykes, McCormac and O'Brein (1978). Superoxide dismutase activity was determined spectrophotometrically by measuring the inhibition of epinephrine oxidation to adenochrome. Mn-SOD of the liver mitochondria is known to be destroyed during this procedure. A standard curve for SOD activity was made using SOD from bovine erythrocytes (Sigma Biochemicals, St. Louis, MO). One unit of SOD was defined as the amount of the enzyme, which inhibits epinephrine oxidation to adrenochrome by 50%.

Catalase (E.C. 1.11.1.9) activity was measured spectrophotometrically by determination of H_2O_2 decomposition (Aebi, 1977). After 30 min preincubation of the postmitochondrial fraction of the liver homogenate with 1% Triton X-100 a decrease in absorbance at 240 nm was determined.

Glutathione peroxidase (GSH-Px; E.C. 1.11.1.6) activity was measured spectrophotometrically by a method based on that of Paglia and Valentine (1967), whereas GSH formation was assayed by measuring conversion of NADPH to NADP.

Glutathione reductase (GSSG-R; E.C. 1.6.4.2) activity was measured by monitoring the oxidation of NADPH at 340 nm (Mize & Langdon, 1962).

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

RESULTS

Administration of cyclophosphamide caused a decrease in the activity of superoxide dismutase throughout the whole time of experiment while after amifostine injection the activity of this enzyme was significantly increased during 5 days and insignificantly increased after 14 days of treatment (Table 1). However, after administration of cyclophosphamide and amifostine the activity of Cu,Zn-SOD was similar to the control. After cyclophosphamide administration, the activities of the two GSH-related enzymes, glutathione peroxidase and glutathione reductase, were significantly decreased, while amifostine injection did not cause significant changes in the activities of these enzymes. Administration of cyclophosphamide and amifostine caused a decrease in the activity of examined enzymes but lower than after cyclophosphamide alone. Cyclophosphamide injection caused a progressive increase in the catalase activity, while amifostine administration caused a non-significant increase in the activity of this enzyme. Injection of cyclophosphamide and amifostine brought about a progressive increase in CAT activity, which was smaller than after cyclophosphamide administration.

Administration of cyclophosphamide to rats caused a decrease in the lung superoxide dismutase activity while amifostine caused an increase in the activity of this enzyme throughout the experiment (Table 2). After cyclophosphamide and amifostine injection, activity of this enzyme was similar to values of the control group but was significantly increased in comparison cyclophosphamide group. Activity of GSH-Px was signifi-

Table 1. The activity of antioxidant enzymes in the liver of rats treated with cyclophosphamide, amifostine and cyclophosphamide with amifostine and in control rats.; data points represent mean ± SD; n = 6; (*P < 0.05 in comparison with the control group, **P < 0.05 in comparison with the cyclophosphamide group)

Analyzed enzyme	Time after drug administration	Control group	Cyclophosphamide group	Amifostine group	Cyclophosphamide and amifostine group
SOD U/mg protein	1 day	14,6 ± 1,0	13.2 ± 1.1*	20.5 ± 1.6*	14.2 ± 1.1
	5 days	13,4 ± 1,0	12.6 ± 1.1	19.4 ± 1.4*	13.1 ± 1.2
	14 days	14.2 ± 1.1	13.1 ± 1.0	15.0 ± 1.2	13.5 ± 1.1
GSH-Px U/mg protein	1 day	154 ± 11	95 ± 6*	139 ± 10*	124 ± 9*,**
	5 days	145 ± 10	73 ± 5*	144 ± 12	115 ± 10*,**
	14 days	151 ± 11	131 ± 12*	152 ± 12	134 ± 11*
GSSG-R U/mg protein	1 day	32.5 ± 2.5	15.2 ± 1.2*	35.7 ± 2.8	29.5 ± 2.5**
	5 days	30.4 ± 2.3	22.8 ± 1.8*	34.6 ± 2.7*	27.6 ± 2.4**
	14 days	31.7 ± 2.3	29.7 ± 2.3	33.2 ± 2.3	30.5 ± 2.5
CAT U/mg protein	1 day	27.2 ± 2.0	29.3 ± 2.5	29.3 ± 2.4	28.9 ± 2.5
	5 days	25.9 ± 2.0	33.5 ± 2.8*	30.3 ± 2.5*	32.9 ± 2.8*
	14 days	28.5 ± 1.9	40.1 ± 3.4*	27.8 ± 2.3	33.7 ± 2.7*,**

Table 2. The activity of antioxidant enzymes in the lung of rats treated with cyclophosphamide, amifostine and cyclophosphamide with amifostine and in control rats.; data points represent mean ± SD; n = 6; (*P < 0.05 in comparison with the control group, **P < 0.05 in comparison with the cyclophosphamide group)

Analyzed enzyme	Time after drug administration	Control group	Cyclophosphamide group	Amifostine group	Cyclophosphamide and amifostine group
SOD U/mg protein	1 day	4,61 ± 0.27	4.02 ± 0.34*	4.98 ± 0.32	4.76 ± 0.36**
	5 days	4.38 ± 0.25	3.91 ± 0.35*	4.64 ± 0.34	4.38 ± 0.36**
	14 days	4.25 ± 0.25	3.58 ± 0.34*	4.35 ± 0.34	4.35 ± 0.35**
GSH-Px U/mg protein	1day	65.2 ± 4.4	70.3 ± 5.6	68.4 ± 5.1	75.2 ± 5.9*
	5 days	63.7 ± 4.1	81.2 ± 6.5*	73.4 ± 5.7*	77.1 ± 6.1*
	14 days	65.9 ± 4.1	62.3 ± 5.8	81.5 ± 6.2*	65.5 ± 5.5
GSSG-R U/mg protein	1 day	24.5 ± 1.7	26.4 ± 1.9	29.1 ± 2.1*	28.7 ± 2.5*
	5 days	26.1 ± 1.9	22.7 ± 1.8*	26.6 ± 2.1**	24.6 ± 2.0
	14 days	25.0 ± 1.9	24.1 ± 2.0	22.2 ± 1.7*	26.1 ± 2.1
CAT U/mg protein	1 day	47.5 ± 3.1	49.5 ± 4.0	59.9 ± 4.7*	54.3 ± 4.4*
	5 days	45.3 ± 3.4	44.3 ± 3.8	53.3 ± 4.5*	55.3 ± 4.4*,**
	14 days	48.9 ± 3.4	38.2 ± 3.3*	52.4 ± 4.2	48.5 ± 3.4**

cantly increased during 5 days after cyclophosphamide administration and during two weeks after amifostine injection. After injection of both drugs, the GSH-Px activity was increased during 5 days. Activity of glutathione reductase was not significantly changed after administration of the drugs. However after cyclophosphamide administration a decrease in CAT activity was observed during two weeks, while amifostine caused an increase in the activity of this enzyme. During 5 days after cyclophosphamide and amifostine administration, the CAT activity was higher than in control group while after 5 and 14 days it was significantly increased in comparison with cyclophosphamide group.

DISCUSSION

The liver and the lung are the organs subjected to the action of cyclophosphamide and its metabolites (mainly acrolein and free radicals) to a low degree. The present study has revealed that cyclophosphamide administration caused changes in activities of antioxidant enzymes. After cyclophosphamide administration, the liver Cu,Zn-SOD, GSH-Px and GSSG-R activities were diminished and the lung Cu,Zn-SOD, GSSG-R and CAT activities decreased, too. The reduction of activities of these enzymes is probably connected with the damage of their structure. Reduction of Cu,Zn-SOD may also be due to an inhibited biosynthesis of enzyme molecules by cyclophosphamide or by its metabolites and/or to the effect of superoxide radicals. The damage to the Cu,Zn-SOD molecules might be related to the hydroxyl radical, the product of reaction of superoxide and hydrogen peroxide. SOD selectively scavenges superoxide anion radicals and the resulting hydrogen peroxide is removed by the GSH-Px system (Fridovich & Freeman,1986) and this suggests the importance of the NADPH-GSH-Px system in scavenging toxic species of oxygen. However the significant decrease in the liver GSH-Px activity might distrib this pathway. Moreover, the cyclophosphamide metabolite acrolein can react with sulphydryl compounds, mainly GSH, causing decreases in their concentrations. The combination of the decrease in the GSH-Px activity and in the GSH content with an increase in free radical formation during cyclophosphamide metabolism may be the reason of an increase in hydrogen peroxide concentration. The removal of hydrogen peroxide is mainly performed by CAT. The liver of rats after cyclophosphamide injection shows increased CAT activity. The mechanism of this is understood since it is known that oxidants such as hydrogen peroxide activate gene expression through the antioxidant responsive element (Rushmore, Morton & Pickett, 1991).

There appears to be a relationship between reduction of the liver GSH level, described elsewhere in literature, observed during cyclophosphamide treatment and the reduction of GSH-Px activity. This supposition is in accordance with the concept showing the reduction of GSH-Px activity, which is preceded by the consumption of GSH in fibroblast cell lines (Kinter & Roberts, 1996). The findings on decreased activities of GSH-related enzymes suggest that oxidative damage occurs due to certain oxidants, including superoxide radicals, phospholipid hydroperoxides and other organic hydroperoxides. The oxidation of a cysteine SH-group adjacent to a selenol group in the active site of GSH-Px is a likely cause of irreversible inactivation of this enzyme (Blum & Fridovich, 1985). The selenoprotein is synthesised in the liver and excreted into the blood (Yang, Morrison-Plunner & Burk, 1987). GSH-Px has a broad range of acceptor substrates, reacting with hydroperoxyl group of phospholipids and cholesterol integrated into biomembranes.

Cellular proteins, particularly sulphydryl proteins, are known to be susceptible to free radicals formed during cyclophosphamide administration. The selective oxidative modification of amino acids in proteins can have a significant impact on cellular function (Gebicki & Gebicki, 1993). Another important redox enzyme is glutathione reductase, which catalyses the conversion of GSSG to GSH. These enzymes contain one or more sulphydryl residues which are essential for its catalytic activity (Mize & Langdon, 1962). The modification of SH-groups in an enzyme molecule by oxygen radicals may be the cause of significant inactivation of this enzyme. In addition, the acrolein may also modify the sulphydryl groups of glutathione peroxidase and glutathione reductase molecules, so the significant decrease in these enzymes activity in the liver which is the main place of acrolein generation, may be due to this compound.

The combined deficiency of antioxidant enzymes may potentiate cyclophosphamide toxicity by simultaneously increased oxidative stress, while decreasing oxidative defences. In such a situation the hydroxyl, superoxide and peroxyl radicals generated during cyclophosphamide metabolism may react with cell components damaging their structure and functions. Therefore the use of pro-

tective compounds is very important for normal tissues.

An active metabolite of amifostine (WR-1065) has free sulphydryl group and can interact with electrophillic compounds preventing the reaction of cyclophosphamide metabolites with important cell components such as GSH, proteins, lipids and DNA. Moreover an *in vitro* study demonstrated that the active metabolite of amifostine, W-1065, was able to scavenge superoxide anions and lipoperoxyl radicals and especially the hydroxyl radicals (Ohnishi, Ohnishi, Glick & Schein, 1992; Marzatico, Porta, Moroni, Bertorelli, Borasio, Finotti, Pansarasa & Castagna, 2000). It is very important because hydroxyl radical is an aggressive species, reacting with extremely high rate constants with nearly every type of cell molecules and probably with disulphide compounds such as amifostine. The known preferential activity of amifostine as a selective scavenger of OH appears to be the key element in the efficacy of the drug, since OH radicals are surely the most dangerous ROS from a biological viewpoint. Indeed, they are extremely aggressive against cell structures and may both initiate and then self-propagate cell damage while $O_2^{\cdot}$ seems to play a secondary role (Halliwell & Gutteridge, 1989).

The biological activity of amifostine seems to be particularly important from a clinical point of view since it may be exerted in chemotherapy, e.g. during cyclophosphamide therapy.

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