Biochemical Pharmacology, Vol. 31, No. 4, pp. 521–525, 1982.
Printed in Great Britain.

0006-2952/82/040521-05 $03.00/0

H_2O_2 PRODUCTION, MODIFICATION OF THE GLUTATHIONE STATUS AND METHEMOGLOBIN FORMATION IN RED BLOOD CELLS EXPOSED TO DIETHYLDITHIOCARBAMATE *IN VITRO*

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(*Received* 5 *June* 1981; *accepted* 14 *August* 1981)

Abstract—Human red blood cells treated with the CuZn superoxide dismutase inhibitor diethyldithiocarbamate (DDC) undergo metabolic modifications in addition to the superoxide dismutase inhibition: oxidation of the reduced glutathione (GSH) to oxidized glutathione (GSSG), methemoglobin formation, and increased hexose monophosphate shunt activity were observed. The magnitudes of these changes are dependent on the DDC concentration. Under nitrogen, only superoxide dismutase inhibition occurs. After removal of the GSH with *N*-ethylmaleimide, production of H_2O_2 can be detected by measuring the red cell catalase inhibition in the presence of 3-amino-1,2,4-triazole. H_2O_2 production is not altered by conversion of oxyhemoglobin to methemoglobin by sodium nitrite prior to incubation. GSH oxidation and methemoglobin formation are stopped when DDC is eliminated from the incubation medium after completion of the superoxide dismutase inhibition. These data indicate that methemoglobin formation and modification of the GSH status in red cells treated by DDC are not a direct consequence of the CuZn superoxide dismutase inhibition but are due rather to a DDC-dependent production of H_2O_2.

Diethyldithiocarbamate (DDC), a copper chelator, has been found to bind [1] and inhibit [2–5] purified copper zinc superoxide dismutase (CuZn SOD). *In vivo* inhibition of this enzyme by DDC was first reported by Heikkila *et al.* [2] and proposed as a tool to explore the role of the superoxide radical (O_2^-) and of CuZn SOD in biological processes. Thus, DDC administration to living organisms or to cells *in vitro* has been used for this purpose in studies on the indoleamine 2,3-dioxygenase [6], the effects of ionizing radiation [7–11], the action of hemolytic drugs [12], the toxicity of ozone [13], paraquat [11, 13], and hyperoxia [15, 16].

Goldstein *et al.* [13], in their study on ozone and paraquat toxicity in mice, reported that injection of DDC to mice not only inhibited superoxide dismutase but also decreased non-protein sulfhydryl groups as well as glutathione peroxidase (GSHPx) in lungs and liver. This complicates the interpretation of their results in relation to the role of O_2^- and CuZn SOD in ozone and paraquat toxicity, especially because sulfhydryls such as glutathione (GSH) are implicated in the defence against oxygen toxicity. As this change in non-protein sulfhydryl groups occurred later than the superoxide dismutase inhibition, it could be either the consequence of the CuZn SOD inhibition or the results of another biological action of DDC.

We now report studies on human red blood cells and show that DDC induces H_2O_2 formation, modifications of the glutathione (GSH) status and formation of methemoglobin; the latter actions do not seem to be the consequences of SOD inhibition.

MATERIALS AND METHODS

Fresh blood from healthy volunteers was drawn in heparin. Serum and buffy coat were removed by centrifugation, and the erythrocytes were washed twice at 4°C with isotonic NaCl (ten volumes for one volume of blood). Unless specified, 0.75 ml of red cells were suspended in 10 ml of buffered saline (144 mM NaCl in 10 mM potassium phosphate buffer pH 7.4) with or without DDC (Sigma), in 25 ml flasks and incubated at 37° in a water bath rotating shaker (100 rpm). In some experiments, removal of DDC from the cell environment was carried out after 30 min of incubation with DDC; the red cell suspension was cooled in ice for 5 min and centrifuged; erythrocytes were then washed twice with buffered saline, resuspended in buffered saline without DDC and re-incubated at 37°.

Measurement of GSH was performed with 5,5-dithiobis-(2-nitrobenzoic acid) (Sigma) after deproteinization with metaphosphoric acid [17]. GSSG inside and outside the red cells [18] was measured according to [19]. After incubation, the red cell suspension was centrifuged and the supernatant removed for GSSG assay; the erythrocyte pellet was resuspended in buffered saline containing *N*-ethylmaleimide (NEM) (Sigma); after 10 min at 4°, the deproteinization was carried out with trichloroacetic acid (TCA); aliquots were taken for the estimation of GSSG using glutathione reductase (Sigma) and the oxidation of NADPH at 340 nm followed in a Gilford 240 spectrophotometer.

Methemoglobin formation was measured by spectrophotometry according to [20].

Hexose monophosphate shunt activity was carried out as in [21] measuring the $^{14}CO_2$ evolution from [1-^{14}C]glucose. Red blood cells were suspended (7.5 vol. p. 100) in a solution kept at 4° containing 0.1 M HEPES/NaOH pH 7.4, 4 mM KCl, 5 mM $MgCl_2$, 55 mM NaCl, 12 mM Na_2HPO_4, 12 mM D-glucose and 0.4 μCi per ml of [^{14}C]-D-glucose (Amersham). 9.5 ml of this suspension was placed into flasks containing 0.5 ml of DDC solution in buffered saline. The flasks were immediately capped (zero time), put at 37° and shaken as described above. The $^{14}CO_2$ produced was trapped by 0.2 ml of 1 N NaOH placed into a plastic cupule hung on the stopper. The reaction was stopped by injecting through the rubber stopper 0.2 ml of perchloric acid. The incubation was pursued for 30 min to allow a complete CO_2 release from the aqueous phase. The radioactivity contained in the NaOH was measured using Tricarb liquid scintillation counting equipment. For measurement of the glycolysis, glucose was assayed in the perchloric acid extracts by using a commercial kit (glucose Auto Test, Boehringer–Mannheim). The total radioactivity of the buffered cell suspension was also quantitated for determination of the specific activity of [1-^{14}C]-D-glucose.

Anaerobic experiments were carried out by blowing continuously nitrogen into flasks for 30 min before addition of DDC to the erythrocyte suspension and afterward during the time of incubation.

Experiments with 3-amino-1,2,4-triazole (AT) (Sigma, recrystallized) were carried out as follows: red cell GSH was first depleted with NEM as in [19]; cells were washed twice and incubated 15 min with AT; then DDC was added (zero time) and aliquots removed 30 min later for the catalase assay. No catalase inhibition was observed in control flasks either without DDC or with DDC and ethanol (0.034 M). Catalase was determined by measuring the disappearance of added H_2O_2 in the presence of the red cell extract at 0° [22, 23]. In some experiments, red cell oxyhemoglobin was converted to methemoglobin prior to incubation with AT and DDC, i.e. by adding 25 mg of sodium nitrite per ml of erythrocytes to the medium containing NEM [28] followed by the usual washing.

Measurement of the SOD inactivation was carried out as follows: at different times, an aliquot (0.15 ml) of the red cell suspension was removed and centrifuged at 15,000 g for 2 min. The supernatant was removed and water (0.1 ml) was added to the red cell pellet. SOD activity was assayed immediately after, by using the technique of Heikkila and Cabbat [25] based on the inhibition by SOD of the autooxidation of 6-hydroxydopamine. The assay was performed at 25° instead of 37° as in [25]. DDC, at the concentrations used in our experiments, did not affect the spontaneous auto-oxidation of 6-hydroxydopamine (Sigma). One unit of SOD activity is arbitrarily defined as the amount of enzyme which inhibits by 50 per cent the spontaneous auto-oxidation of 6-hydroxydopamine.

Glutathione peroxidase (GSHPx) activity was measured on hemolysates prepared as described above for SOD assay. GSHPx activity was measured as described in [26] by a coupled enzyme procedure with glutathione reductase and NADPH using t-butyl-hydroperoxide as substrate [27]. One unit of GSHPx activity is defined as the amount of enzyme inducing the oxidation of one μmole of NADPH in our assay conditions.

RESULTS

Figure 1 shows the time-course of the CuZn SOD inactivation in red cells incubated in the presence of DDC (1 g/l). The remaining SOD activity at 15 min was 34 ± 9 per cent of the initial value of 844 ± 88 units per ml of packed red cells (mean ± S.D. from 7 experiments). Figure 1 shows also the time-course of the GSH disappearance within red cells incubated with DDC. It was found that this drop in GSH corresponds exclusively to an oxidation of GSH to oxidized glutathione (GSSG), since the molar ratio between GSH decrease and GSSG formation is equal to 2:1 (Table 1). Furthermore, DDC induced the formation of methemoglobin (Table 1). We also observed, as had been reported previously by Goldstein *et al.* [13], a progressive inactivation of GSHPx. Results from four experiments showed that after 1 hr with DDC at 1 g/l the GSHPx activity was decreased from 202 ± 0.015 to 0.125 ± 0.014 units per ml of packed red cells which represents an average of 38 per cent inactivation of GSHPx. Moreover, the hexose monophosphate shunt (HMPS) activity measured in the presence of glucose increased significantly, indicating that the GSSG produced was reduced back to GSH via the glutathione reductase at the expense of NADPH supplied by the HMPS (Fig. 2).

All these events were dependent upon the concentration of DDC added to the incubation medium (Table 1 and Fig. 2). When the incubation of the red cells was carried out under anaerobic conditions (i.e. under nitrogen) no oxidation of GSH was observed but CuZn SOD inhibition was comparable to that in air (Table 1). This result shows that oxygen is required for GSH oxidation induced by DDC but not for SOD inactivation. In order to test if the GSH

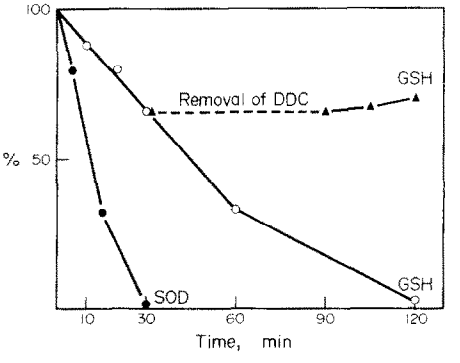

Fig. 1. Effect of DDC upon GSH and CuZn SOD activity in red blood cells. The red blood cell suspension was 7.5 ml per 100 ml; the DDC final concentration was 1 g/l. Abscissa: incubation time (min) after DDC addition; ordinate: remaining amount (%) of CuZn SOD activity. (●——●); GSH (○——○); GSH after removal of DDC (▲——▲) (see Methods). Results of a representative experiment are presented.

Table 1. Metabolic changes in red blood cells (RBC) in the presence of DDC

	SOD Activity remaining after 15 min		GSH oxidation after 30 min				Methomoglobin formation after 1 hr
		%	GSH RBC[a]	GSSG RBC[a]	GSSG medium[b]	Total GSH equivalents[c]	%
1. Under Air							
DDC (g/L)	0	100	2500	16	0	2532	0
	0.1	92	2409	32	14	2501	0
	0.5	45	2120	149	26	2458	1.2
	1.0	26	1609	396	33	2467	5.5
	2.0	3.5	560	920	57	2523	7.2
2. Under Nitrogen							
DDC (g/L)	0	100	2329	-	-	-	0
	0.1	0[d]	2320	-	-	-	0[d]

The RBC concentration was 7.5 ml/100 ml and the final volume was 10 ml.

[a] nmoles/ml RBC.

[b] nmoles of GSSG in the incubation medium containing 1 ml of RBC.

[c] nmoles of GSH equivalents for 1 ml of RBC = [GSH] in RBC + 2 ([GSSG] in RBC + [GSSG] in medium).

[d] The incubation time after DDC addition was 30 min in the experiments under nitrogen. Results of a typical experiment are presented.

oxidation was the consequence of the absence of SOD activity in red cells, we removed the DDC from the incubation medium after 30 min by washing the cells twice and re-incubated them without DDC (Fig. 1). We observed that the CuZn SOD remained fully inactivated while GSH oxidation stopped.

One route for glutathione oxidation is the reduction of hydrogen peroxide (H_2O_2) by glutathione peroxidase (GSHPx). We tested for the production of H_2O_2 within the erythrocytes by a method first described by Cohen and Hochstein [28], based on the inhibition of endogenous catalase by 3-amino-1,2,4-triazole. This reaction requires the presence of H_2O_2 and, therefore, catalase inhibition can serve indirectly to detect endogenous H_2O_2; furthermore, the H_2O_2-dependent inhibition of catalase is prevented by ethanol. In our system, no H_2O_2 formation was detectable in the first hour of incubation unless the red cells had been previously depleted of GSH by treating them with *N*-ethylmaleimide [29] (Table 2). Moreover, the conversion of red cell oxyhemoglobin to methemoglobin prior to the incubation with DDC did not change the rate of H_2O_2 production (Table 2) indicating that oxyhemoglobin was not required for the DDC-induced production of H_2O_2.

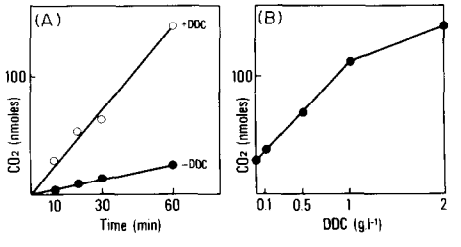

Fig. 2. Effect of DDC upon the HMP shunt in red blood cells. The red blood cell suspension was 7.5 ml per 100 ml and the glucose final concentration was 10 mM. The results show the total CO_2 production by 9.5 ml (final volume) of this suspension. (A) CO_2 production in the absence (●——●) and in the presence of 2 g/l DDC (○——○). (B) CO_2 production as a function of DDC concentration. The time of incubation was 60 min. The glucose consumption was not significantly affected by DDC and equal to 2053 ± 240 nmoles/hr ($n = 5$; mean ± S.E.M.). Results of a typical experiment are presented.

DISCUSSION

During the course of experiments with DDC on red cells from normal subjects and trisomy 21 patients who have a 50 per cent increase in CuZn SOD [30] we have been led to consider carefully the effects of DDC on red cell metabolism other than that of SOD inhibition. We have found a drastic change in the erythrocyte GSH status that is an oxidation of GSH to GSSG and an increased hexose monophosphate shunt activity secondary to GSH oxidation. Goldstein *et al.* [13] have also reported a decrease in non-protein sulfhydryl groups, presumably GSH, during *in vivo* experiments with DDC.

These results could be interpreted as a consequence of the CuZn SOD inhibition: the superoxide anion O_2^- could either oxidize GSH directly or participate in the formation of a powerful oxidant, the hydroxyl radical ($OH^\cdot$), by a Haber–Weiss type mechanism [31], $OH^\cdot$ promoting GSH oxidation.

We describe experiments indicating that, in red cells, GSH oxidation is not directly related to CuZn SOD inhibition by DDC. First, the rate of GSH disappearance is not proportional to the extent of the CuZn inhibition. As seen in Fig. 1, after 30 min, CuZn SOD is totally inhibited; nonetheless the rate of GSH oxidation remains the same as at the beginning of the incubation with DDC. Above all, we have created an experimental situation in which CuZn SOD is fully inactivated while GSH does not undergo oxidation; that is, after removing DDC from the incubation medium after 30 min of incubation as shown in Fig. 1. It could be concluded from these experiments that GSH oxidation results from another cause than SOD inhibition.

Table 2. Inhibition of catalase by aminotriazole in red blood cells treated with DDC

	Catalase Activity	Remaining Activity
	units.ml^{-1}	%
Zero Time	556 ± 34 (n = 13)	100
No NEM pretreatment		
- DDC	518	93
+ DDC	506	91
+ DDC + EtOH	573	101
After NEM pretreatment		
- DDC	537	97
+ DDC	252	45
+ DDC + EtOH	548	98
After $NaNO_2$ and NEM treatment		
- DDC	571	103
+ DDC	215	39
+ DDC + EtOH	529	95

The DDC concentration used was 1 g/l; the RBC concentration was 7.5 ml/100 ml; and the incubation time was 30 min.

The catalase activity is expressed as the number of units/ml of RBC suspension. Catalase units were calculated as in [23] (see Materials and Methods).

Results of a representative experiment are presented.

It was found that the presence of oxygen was required for GSH oxidation. That was a conclusion drawn from our experiments under anaerobic conditions, in which we found, as reported by others [5, 13], that CuZn SOD inhibition by DDC does occur to the same extent as under aerobic conditions. In other experiments, DDC was found to induce H_2O_2 production within the erythrocytes. H_2O_2 production, i.e. inhibition of catalase by 3-amino-1,2,4-triazole was not detectable unless GSH had been depleted with NEM [29]. This result confirms the concept first proposed by Cohen and Hochstein [32] that GSHPx is the primary agent for the elimination of H_2O_2 in erythrocytes. As a matter of fact, the absence of catalase inhibition without treatment by NEM, even after the disappearance of more than 65 per cent of the GSH at one hour of incubation, indicates that the H_2O_2 produced does not reach catalase but is metabolized by GSHPx.

Catalase inhibition in the presence of aminotriazole has been used in various *in vitro* [28, 29] and *in vivo* experiments [23, 34, 35] to detect the production of H_2O_2. In one of these studies [28] it has been reported that H_2O_2 production by hemolytic drugs which are not readily auto-oxidizable, such as primaquine and pamaquine, requires the presence of oxyhemoglobin. We tested this possibility for DDC by incubating red cells after a preliminary conversion of oxyhemoglobin [28] (Table 2). H_2O_2 production was not significantly changed by this treatment, indicating that the DDC-dependent production of H_2O_2 is not catalyzed by either oxyhemoglobin or methemoglobin.

In conclusion, H_2O_2 production induced by DDC and its consequences on the GSH status should be taken into account in the experiments with erythrocytes and possibly other cells *in vitro*. Removal of DDC from the incubation medium after CuZn SOD inhibition and before the complete disappearance of GSH(Fig. 1) may be helpful to explore more specifically the role of CuZn SOD and O_2^- in biological processes. Moreover, other thiocarbamic acid derivatives, which have been reported also to inhibit CuZn SOD [36], might not have the same 'side effects' as DDC.

Acknowledgements—This work was supported by the CNRS–ERA 47 and the University Rene Descartes. We thank Gerald Cohen and Richard Heikkila for their helpful comments.

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