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PLASMA MEMBRANE AS A SITE OF REDOX ACTIVATION OF DAUNOMYCIN IN INTACT HUMAN ERYTHROCYTES

QUANTITATIVE EVALUATION OF THE HYDROGEN PEROXIDE PRODUCED BY THE MEMBRANE WITH RESPECT TO THE CYTOSOL

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Abstract—The relative importance in human red blood cells of the plasma membrane as a site of redox activation of anthracyclines as compared to hemoglobin was evaluated by assaying the H_2O_2 produced upon exposure to daunomycin. The method of H_2O_2-dependent irreversible inhibition of catalase (EC 1.11.1.6) activity by 3-amino-1,2,4-triazole was applied to intact erythrocytes, as well as to isolated membranes with added purified catalase. The results obtained indicate a secondary role in daunomycin activation for the plasma membrane from a quantitative point of view, although membrane pathways can be more harmful than cytosolic pathways, especially towards extracellular targets, when the high efficiency of the cytosolic antioxidative defences and the external location of the membrane activation site are considered.

Red blood cells (RBCs§) are potential sites of redox activation of antitumor anthracycline antibiotics. In fact, Adriamycin® and daunomycin have been shown to be reduced to their semiquinones by human erythrocytes [1] and to generate H_2O_2 in this reaction [2]. Moreover, formation of hydroxyl radicals as well as O_2^- has been monitored in intact RBCs exposed to anthracyclines by ESR spectroscopy [1, 3]. An involvement of oxyhemoglobin (HbO_2) in H_2O_2 production by anthracyclines in RBCs has been established [2]. On the other hand, the role of the outer side of the erythrocyte membrane in the generation of an ESR-detectable anthracycline radical in the presence of carbon monoxide, and of O_2^- in the presence of oxygen, has also been well documented [1].

RBCs are not normally considered a main target for the anthracyclines, although 30–50% of drug circulating in the blood has been reported to be associated with erythrocytes following intravenous administration [4]. In addition, the use of anthracycline-loaded autologous RBCs has been proposed recently as a new pharmacological strategy able to increase the therapeutic index of the drug [5]. In this context, a better characterization of the redox activation of anthracyclines by RBCs could be relevant for the clinical usefulness of these drugs. In particular, it is important to assess the relative contribution of cytosolic and membrane sites to drug activation and to the consequent production of reactive oxygen species.

In this paper we report results obtained by applying the method of H_2O_2-dependent inactivation of catalase (EC 1.11.1.6) by 3-amino-1,2,4-triazole (3-AT) to quantitative measurements of daunomycin-mediated H_2O_2 fluxes generated by intact erythrocytes, as well as by isolated RBC plasma membranes. These results indicate a larger contribution by the cytosolic sources of H_2O_2, mainly HbO_2, with respect to membrane pathways, although the cytosolic pathways show negligible damaging effects towards the cells.

MATERIALS AND METHODS

Materials

Bovine liver catalase in crystalline form, 3-AT, *N*-ethylmaleimide (NEM) 1-chloro-2,4-dinitrobenzene (CDNB) and 5,5′-dithiobis-(2-microbenzoic acid) (DTNB) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Benzylamine hydrochloride was purchased from ICN Pharmaceuticals Inc. (Costa Mesa, CA, U.S.A.). Purified bovine serum amine oxidase (EC 1.4.3.6) was a kind gift of Prov. B. Mondovi', University of Rome "La Sapienza". NADPH and NADH were purchased from Boehringer Mannheim (Mannheim, F.R.G.). Daunomycin was a kind gift of Farmitalia (Milan, Italy). Sephadex gels was purchased from Pharmacia

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§ Abbreviations: 3-AT, 3-amino-1,2,4-triazole; CDNB, 1-chloro-2,4-dinitrobenzene; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); GSH, reduced glutathione; GSSG, oxidized glutathione; (H)RBC, (human) red blood cell; HbO_2, oxyhemoglobin; HbO_2-HRBC, human red blood cell containing oxyhemoglobin; HbCO, carbon monoxyhemoglobin; HbCO-HRBC, human red blood cell containing HbCO; HEM, *N*-ethylmaleimide; PBS, phosphate-buffered saline.

LKB (Uppsala, Sweden). Sigma catalase preparations were purified from contaminating superoxide dismutase (EC 1.15.1.1) by gel filtration on Sephadex G-75 [6]. All other chemicals were of the best quality commercially available and were used without further purification.

Preparation of samples

Human blood was drawn from healthy donors at the Transfusion Center of University of Rome "La Sapienza", (courtesy of Prof. G. Isacchi) and was centrifuged to remove plasma and buffy coat. The RBCs were washed three times in phosphate-buffered saline (PBS) (140 mM NaCl, 20 mM phosphate buffer pH 7.4), resuspended at appropriate concentrations in the same saline buffer and used.

HbO_2 was transformed into carbon monoxy-hemoglobin (HbCO) by flushing 80% (v/v) cell suspensions with 100% CO [7]. The erythrocytes were then diluted to 50% (v/v) final concentration with aerated buffer and exposed to daunomycin and 3-AT in air. During the treatment, the stability of HbCO was monitored by the absorption spectra of aliquots after 20-fold dilution in water [8].

Depletion of intracellular glutathione was performed by incubating in the presence of 1 mM CDNB [9] at 37° for 15 min 30% (v/v) human RBC (HRBC) suspensions in PBS. At the end of the incubation the cells were washed three times with 1000 vol. of cold PBS, resuspended in the buffer at 50% (v/v) concentration and then used for the experiments or assays reported below.

For preparation of RBC plasma membranes, erythrocytes from a known volume of 50% (v/v) suspension (in some experiments, after exposure to daunomycin) were washed with 0.172 mM Tris–HCl buffer pH 7.6 and lysed in hypotonic buffer according to Dodge *et al.* [10]. The membrane pellet obtained by centrifugation, after repeated washings with hypotonic saline to remove the hemoglobin, was then suspended in PBS to the original volume and used in the experiments or assays as described below.

Membrane sulphydryl groups were derivatized with NEM as reported previously [11]. Suspensions of ghosts in PBS, at a concentration corresponding to 50% (v/v) HRBCs, were treated with 20 mM NEM at 4° for 2 hr. The membranes were washed four to five times with 1000 vol. of PBS, resuspended to their original volumes and used.

Treatment with daunomycin of H_2O_2

Fifty per cent (v/v) HRBCs containing 50,000 U/mL of endogenous catalase, suspensions of isolated plasma membranes added with 10,000 U/mL of bovine liver catalase or solutions of different concentrations of bovine liver catalase were treated in PBS with varied daunomycin concentrations or known fluxes of H_2O_2 for 1 hr at 37° in the presence of 50 mM 3-AT. Glucose (5 mM) or NAD(P)H was used as exogenous substrate for cells or isolated plasma membranes, respectively. Control samples were also incubated in the absence of 3-AT and/or daunomycin (or H_2O_2). H_2O_2 was generated at controlled rates by the oxidative deamination of 10 mM benzylamine to benzaldehyde in the presence of appropriate amounts of bovine serum amine oxidase.

Assays

H_2O_2. The formation of H_2O_2 in RBCs or isolated plasma membranes exposed to daunomycin was estimated by evaluating the 3-AT-dependent irreversible inhibition of the catalase activity present in the system [12–15]. For quantitative assays the rate of catalase inhibition measured in the presence of daunomycin was referred to calibration plots, obtained by measuring in the same experimental systems the rate of catalase inhibition by 3-AT in the presence of known fluxes of H_2O_2.

At time intervals, aliquots of the incubation mixtures above described were diluted in 100 vol. of 0.45 M ethanol and incubated at 25° for 20 min. This treatment terminates the reaction of catalase with 3-AT [16]. Each aliquot was then assayed for catalase and the enzyme activity was plotted as a function of time. The rates of catalase inhibition were determined from the slope of the time-course plots of the catalase activity decrease measured after 15, 30, 45, 60 min of treatment with either varied concentrations of daunomycin or known fluxes of H_2O_2. In both cases the time-course plots were linear. The rates were calculated as $[100 - A_t/t]$ and were expressed as per cent per min: A_t was the per cent of residual enzyme activity at time t with respect to that at 0 time (100%). The slowest appreciable time-course of the decrease in enzyme activity, ranging between approximately 12 and 20% inhibition during 1 hr of incubation, was considered suitable for determining the minimal detectable value of catalase inhibition rate, i.e. 0.27 ± 0.07 %/min.

Enzymes. Bovine serume amine oxidase activity was assayed in the presence of benzylamine as substrate, either spectrophotometrically or by polarographic determination of oxygen uptake using an YSI oxygraph model 53 equipped with a Clark electrode. One unit of enzyme catalysed the formation of 1 μmol benzaldehyde/min [17]. Control experiments demonstrated that 50 mM 3-AT did not affect the enzyme: the specific activity was 0.28 U/mg protein in either the absence or the presence of 3-AT.

The activity of catalase and glutathione peroxidase (EC 1.11.1.9) was assayed as reported previously [18, 19]. One unit of catalase was defined as the amount dismuting 1 μmol H_2O_2/min. Glutathione peroxidase activity was expressed as micromoles of NADPH oxidized per minute per gram of hemoglobin by the glutathione reductase coupled in the assay.

Hemoglobin. Hemolysis was measured after centrifugation of aliquots of RBC suspensions and expressed as per cent hemoglobin in the supernatants with respect to the total hemoglobin content. Hemoglobin concentration was determined according to Drabkin and Austin [20]. The presence of methemoglobin was evaluated by the absorbance at 630 nm [8] or hemolysed samples after dilution in 0.2 M phosphate buffer pH 6.5 and expressed as per cent total hemoglobin concentration. The assays of hemolysis and methemoglobin formation, as well as glutathione peroxidase activity, were performed

after removing daunomycin by gel filtration on a Sephadex G-25 superfine column.

Glutathione and membrane sulphydryl groups. The erythrocyte content of reduced and oxidized glutathione (GSH and GSSG) was assayed spectrophotometrically as reported by Anderson [21].

The analysis of membrane sulphydryl groups was performed on either intact erythrocytes or isolated membranes, treated or not with daunomycin in the presence of glucose or NAD(P)H as substrate. After incubation with daunomycin of intact cells, plasma membranes were isolated as reported above. Then, aliquots of these membrane suspensions at concentration corresponding to 50% (v/v) HRBCs were washed extensively until the supernatant of samples containing daunomycin was nearly colourless. The membranes were then solubilized by addition of 400 μL of a solution containing 25 mM sodium deoxycholate, 250 mM Na_2SO_4 and 125 mM sodium borate (pH 8.5), then of 100 μL 33% SDS (w/v) and 400 μL 29 mg/mL sodium deoxycholate. Titration of membrane sulphydryl groups with 75 μg/mL DTNB was then performed as reported previously [22]. The number of sulphydryl groups was calculated from the increase in absorbance at 412 nm using a molar absorbance of 13,600. Suspensions of membranes exposed to daunomycin after their isolation from untreated erythrocytes were subjected to the same procedure. Controls performed with membranes to which daunomycin had been added to an extent producing the appearance of a pale orange colour showed that residual daunomycin in the membranes did not interfere with the assay. For each sample the concentration of sulphydryl groups was referred to the protein membrane content [23].

RESULTS

Inhibition of catalase by 3-AT in intact HRBCs or isolated membrane suspensions upon exposure to daunomycin

Fifty per cent (v/v) suspensions of intact human RBCs, containing 50,000 U/mL endogenous catalase, were incubated with different concentrations of daunomycin (1–10 mM) for 1 hr at 37° in the presence of 5 mM glucose and 50 mM 3-AT. A linear time-dependent, irreversible inhibition of catalase activity was observed, depending on the daunomycin concentration (Fig. 1). The rates of catalase inhibition were determined from the slopes of the time-course plots shown in Fig. 1. The rate observed with 5 mM daunomycin (triangles), namely 0.44 ± 0.03 %/min, was higher than the minimal detectable value and thus was determined with an error of approximately 7%. The rate observed with 10 mM daunomycin (circles), namely 1.90 ± 0.07 %/min, was significantly higher: $P < 0.001$ from Student's *t*-test; whereas 1 mM daunomycin (squares) did not cause catalase inactivation. Moreover, the enzyme activity was unaffected in the absence of daunomycin and/or 3-AT, giving unambiguous evidence for H_2O_2 production.

Prior conversion of hemoglobin to its carboxymonoxy derivative completely prevented catalase inactivation by 50 mM 3-AT at all the daunomycin concentrations tested (Fig. 1 legend). These data would indicate HbO_2 as the main site of H_2O_2 production by daunomycin activation in intact HRBCs.

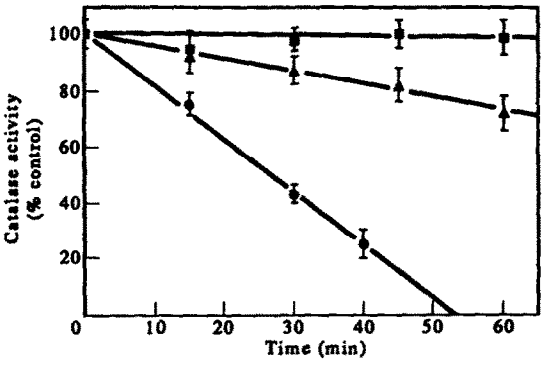

Fig. 1. Time-course of the inhibition of HRBC catalase activity upon exposure of cell suspensions to daunomycin and 3-AT. Fifty per cent (v/v) HRBCs in PBS, containing 50,000 U/mL catalase, were exposed at 37° in the presence of 5 mM glucose and 50 mM 3-AT to different concentrations of daunomycin. Circles: HbO_2-HRBCs with 10 mM daunomycin. Triangles: HbO_2-HRBCs with 5 mM daunomycin. Squares: HbO_2-HRBCs with 1 mM daunomycin. Similar time-courses to that designated by squares showing not appreciable enzyme inhibition in 1 hr incubation (see "Assay" in Materials and Methods and "ND" in the footnotes of Table 3) were given by the following samples: HbCO-HRBCs with 1, 5 or 10 mM daunomycin; HbO_2-HRBCs without daunomycin; HbO_2-HRBCs without 3-AT but in the presence of daunomycin at all the concentrations tested; HbO_2-HRBCs in the absence of both daunomycin and 3-AT. Each value is the mean from six separate experiments and the SD is reported only when greater than the symbol size.

However, when suspensions of isolated erythrocyte membranes, at a concentration corresponding to 50% (v/v) HRBCs and added with 10,000 U/mL bovine liver catalase, were exposed to daunomycin in the presence of 50 mM 3-AT and 5 mM NAD(P)H, redox activation of drug was also evidenced by the inactivation of catalase. In this case, the time-course of inhibition of the enzyme activity was also linear (not shown): $r = 0.95$–0.99. The rates of catalase inhibition as determined from the time-courses observed in the presence of different concentrations of daunomycin are shown in Table 1. No inhibition was observed in the absence of daunomycin and/or 3-AT. Moreover, when the isolated membranes were pretreated with NEM, received 10,000 U/mL catalase and were treated with daunomycin in the presence of NAD(P)H and 3-AT, the inhibition of catalase activity was negligible, suggesting a crucial role in daunomycin activation by the plasma membrane of membrane sulphydryl groups, which were blocked by NEM by 90–95% (Table 1). Slight catalase inhibition was observed only with 10 mM daunomycin at a rate which still appeared much lower than in the case of non-NEM-pretreated membranes.

On the other hand, Table 1 also shows that

Table 1. Relationship between membrane sulphydryl groups and production of H_2O_2 via redox activation of daunomycin by HRBC plasma membranes

Samples (mM)	Rate of catalase inhibition (%/min)	Sulphydryl groups (nmol/mg proteins)
Non-NEM-pretreated membranes		
0.0	ND	49.8 ± 3.2
1.0	0.40 ± 0.03†	48.5 ± 8.2
5.0	1.88 ± 0.20*	43.6 ± 5.4§
10.0	2.54 ± 0.03‡	46.8 ± 7.5§
NEM-pretreated membranes		
0.0	ND	3.8 ± 1.8
1.0	ND	NT
5.0	ND	NT
10.0	0.38 ± 0.06	NT

Suspensions of isolated membranes at concentration corresponding to 50% (v/v) RBC, after 1 hr exposure to different concentrations of daunomycin in the presence of 5 mM NAD(P)H, were assayed for the reactivity of the sulphydryl groups with DTNB as described in Materials and Methods. DTNB assay was also performed on membrane suspensions pretreated with 20 mM NEM and incubated as the control sample without daunomycin.

For the measurement of H_2O_2 production, the membrane suspensions pretreated or not with NEM, were exposed to daunomycin in the presence of 50 mM 3-AT, 5 mM NAD(P)H and 10,000 U/mL bovine liver catalase for 1 hr. At intervals % of catalase activity was tested which was plotted as a function of time and the rate of catalase inhibition was calculated as described in Materials and Methods. Control samples without 3-AT showed no catalase inhibition.

ND, not detectable: no time-dependent catalase inhibition was observed; 100 ± 5% catalase activity was measured during the whole incubation period.

* Significantly different from †: $P < 0.001$ (Student's *t*-test).

‡ Significantly different from * and †: $P < 0.01$ and $P < 0.001$, respectively.

§ Not significantly different from the control: $P \gg 0.05$.

NT, not tested.

Values are means of three separate experiments ± SD.

1 hr exposure of non-NEM-pretreated isolated membranes to the different concentrations of daunomycin in the presence of NAD(P)H appeared not to affect the membrane sulphydryl groups, as evaluated by measurement of their reactivity with DTNB, after membrane incubation with the drug. In addition, similar results (not shown) were obtained with intact HRBCs exposed to 1, 5 and 10 mM daunomycin for 1 hr at 37°. In fact, after isolation of the membranes, 48.8 ± 9.2 nmol sulphydryl/mg protein were titrated in the three cases with respect to 45.4 ± 3.5 nmol/sulphydryl/mg measured in untreated HRBCs.

Quantitative evaluation of H_2O_2

The complete prevention by HbCO of daunomycin-dependent H_2O_2 production in intact cells (see above) is apparently in conflict with H_2O_2 generation by isolated membrane suspensions. This contradictory result may be due to the different sensitivity of the H_2O_2 assay in the two systems. In order to confirm this hypothesis and obtain more quantitative measurements of the H_2O_2 produced by daunomycin, calibration tests were carried out: samples of 10,000 U/mL purified catalase or 50% (v/v) HRBCs containing 50,000 U/mL endogenous catalase were exposed to known fluxes of H_2O_2 under the same experimental conditions used for daunomycin. In both cases, the time-courses of the irreversible inhibition of catalase activity were similar to those obtained with daunomycin and r 0.98–0.99 was calculated. Moreover, as shown by the two plots in Fig. 2 (triangles and circles), the relationship between the rates of catalase inhibition and those of H_2O_2 generation was linear: $r = 0.98$.

On the other hand, as shown by the calibration plots, by means of 10,000 U/mL pure catalase (Fig. 2, triangles) and 50,000 U/mL HRBC-contained enzyme (circles) different ranges of H_2O_2 fluxes were detected, namely 0.2–2.4 and 3.3–11.0 μM/min, respectively. The differences in catalase concentration did not account for the observed differences. In fact, the range of the H_2O_2 fluxes detected by 50,000 U/mL pure catalase (Fig. 2, squares), i.e. 0.4–3.0 μM/min, was similar to that shown by 10,000 U/mL of the enzyme. Therefore, the finding that fluxes of H_2O_2 lower than 3 μM/min were not detected by means of 50,000 U/mL endogenous catalase in 50% (v/v) HRBCs must be due to factors other than enzyme concentration. Interestingly, the range of fluxes of H_2O_2 detected by 50,000 U/mL intracellular catalase approached that shown by the same concentration of pure enzyme when glucose was omitted from the experiments (Fig. 2, diamonds) or when HRBCs were depleted of 90–95% GSH (Fig. 2, stars): H_2O_2 fluxes detected by catalase under these conditions were 1.6–11.0 and 1.3–4.3 μM/min, respectively. These results implicate glutathione peroxidase as a competitive scavenger of H_2O_2, which could reduce thereby sensitivity of the catalase inhibition assay.

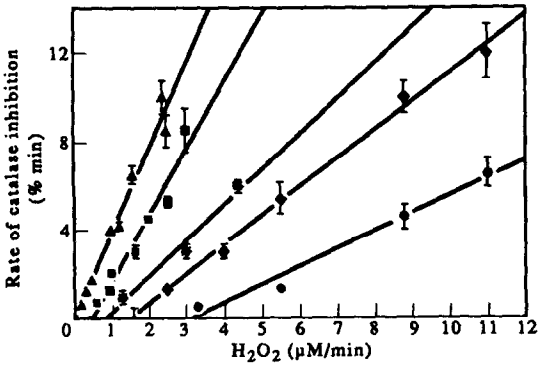

Fig. 2. Calibration plots of the rates of H_2O_2-3-AT-dependent inactivation of purified bovine liver catalase or of endogenous erythrocyte catalase as a function of the rates of H_2O_2 generation by benzylamine–amine oxidase. Catalase samples were incubated at 37° in PBS in the presence of 3-AT and known fluxes of H_2O_2 provided by 10 mM benzylamine plus 0.2–12 U/mL purified bovine serum amine oxidase; the rates of H_2O_2 generation by the enzyme were expressed as μM/min. Triangles: 10,000 U/mL bovine liver catalase. Squares: 50,000 U/mL bovine liver catalase. Circles: 50,000 U/mL endogenous catalase in 50% (v/v) HRBCs exposed to H_2O_2 fluxes in the presence of glucose. Diamonds: the same in the absence of glucose. Stars: 50,000 U/mL endogenous catalase in 50% (v/v) HRBC exposed to H_2O_2 fluxes after depletion of GSH either in the absence or in the presence of glucose. The rate of inhibition of catalase activity is expressed as %/min. Each value was determined from the slope of the time-course plots of the catalase activity decrease measured after 15, 30, 45, 60 min exposure to enzymatically produced H_2O_2. The values represent the ratio $100 - A_t/t$, where A_t indicates the % of residual catalase activity at time t with respect to the control at 0 time (100%); values are means of three separate experiments and the SD is reported only when greater than the symbol size.

In this respect it has to be emphasized that after 1 hr treatment of 50% (v/v) HRBCs with increasing daunomycin concentrations no alteration of glutathione peroxidase activity or GSH and GSSG contents were observed in the presence of 5 mM glucose, with or without 50 mM 3-AT (Table 2). This was in agreement with the finding reported in Table 1 that membrane sulphydryl groups were unaltered and indicated that glutathione peroxidase was able in any case to scavenge H_2O_2 efficiently. Accordingly, treatment of erythrocytes with daunomycin under the experimental conditions used caused an extent of hemolysis of less than 5% in either the absence or the presence of 3-AT; under these conditions methemoglobin formation was 6–10%. In control experiments, 1 hr exposure of HRBCs to fluxes of H_2O_2 generated by the benzylamine–amine oxidase system in the range 1.5–11 μM/min produced no hemolysis and very slight methemoglobin formation (<4%).

Therefore, taking into account that treatments with daunomycin resulted in no effect as far as the glutathione content and glutathione peroxidase activity were concerned (Table 2), and that the same results were obtained (not shown) after 1 hr exposure of HRBCs to enzymatically generated H_2O_2 fluxes which caused rates of catalase inhibition similar to those of the different drug concentrations, the calibration plot in Fig. 2 (circles) was adopted to estimate the rate of H_2O_2 generation by HRBC suspensions exposed to daunomycin. On the other hand, the calibration plot of Fig. 2 (triangles) was used for quantitative evaluation of H_2O_2 production by isolated erythrocyte membranes. In Table 3 the values of H_2O_2 fluxes in intact erythrocytes and in isolated membrane exposed to different daunomycin concentrations are reported. As far as the intact cells are concerned, exact quantitative values of H_2O_2 produced were obtained only when 50% v/v HbO_2-HRBCs were exposed to 5 or 10 mM daunomycin, while values of rate of H_2O_2 production not detectable lower than 3.4 ± 0.3 μm/min were ascribed to all the samples of 50% (v/v) HRBCs in which no catalase inactivation was observed. As far as isolated membranes are concerned, the rates of H_2O_2 generation upon treatment with daunomycin increased linearly between 1 and 10 mM daunomycin, irrespective of NADH or NADPH as the electron donor. Nevertheless, it is noteworthy that at all the daunomycin concentrations these values were less than 3 μM/min and that, consequently, such fluxes of H_2O_2 could not be detected in 50% (v/v) HbCO-HRBCs due to the sensitivity of the method.

DISCUSSION

Evidence is already available for redox activation of anthracycline by erythrocytes and involvement of oxyhemoglobin in the reaction. Addition of Adriamycin or daunomycin to red blood cells resulted in stimulation of hexose monophosphate shunt activity which appeared to be dependent on the presence of oxyhemoglobin [2]. The ESR spectrum of the hydroxyl radical-generated DMPO spin adduct formed following interaction between Adriamycin and HbCO-HRBCs was decreased to 50% of that produced in normal erythrocytes [3]. A direct reaction of Adriamycin with oxyhemoglobin has been proposed to give rise to Adriamycin semiquinone formation [24]. On the other hand, one-electron reduction of the anthracycline carminomycin was shown to occur in purified human erythrocyte membranes [25]. In addition, we demonstrated recently a redox activation of daunomycin by the outer side of the erythrocyte membrane on the basis of external location of the ESR-detectable daunomycin semiquinone and superoxide anion radicals [1].

In the work reported here, the relative importance of cytosol and plasma membrane pathways in the redox activation of daunomycin by human erythrocytes was evaluated in terms of H_2O_2 production by using the catalase aminotriazole trapping technique. This method is based on the H_2O_2-dependent inactivation of catalase by 3-AT. Under the restriction condition that H_2O_2 fluxes are constant and slow, 3-AT reacts with the catalase–H_2O_2 Compound I, and forms a very stable ternary complex [12–14]. The rate of inhibition of catalase is dependent upon the steady-state concentration of

Table 2. Glutathione state and glutathione peroxidase activity in daunomycin-exposed intact HRBCs

Treatment		Total glutathione	GSSG	GSH
3-AT	Daunomycin (mM)	(μmol GSH/g Hb)	(nmol GSH/g HB)	(μmol NADPH/min/g Hb)
−	—	7.2 ± 0.6	70.1 ± 9.1	22.5 ± 4.5
+	—	7.3 ± 0.6	83.2 ± 9.4	19.9 ± 4.2
−	1.0	7.0 ± 0.9	60.5 ± 7.2	20.7 ± 4.0
+	1.0	7.3 ± 0.4	81.3 ± 8.9	20.8 ± 3.8
−	5.0	6.8 ± 0.2	71.0 ± 6.9	24.5 ± 4.9
+	5.0	6.5 ± 0.1	63.4 ± 7.1	26.8 ± 5.2
−	10.0	6.4 ± 0.8	73.3 ± 6.9	26.0 ± 5.0
+	10.0	6.3 ± 0.8	65.6 ± 7.5	24.2 ± 4.8

Fifty per cent (v/v) HRBCs in PBS pH 7.4 were exposed for 1 hr at 37° to different concentrations of daunomycin in the presence of 5 mM glucose, with or without 50 mM 3-AT.

Total glutathione, GSSG and glutathione peroxidase activity were measured as reported in Materials and Methods.

Values are means of two separate experiments ± SD.

Table 3. H_2O_2 fluxes generated by interaction of daunomycin with intact HRBCs via HbO_2 and plasma membrane pathways

Daunomycin (mM)	H_2O_2 (μM/min)		
	HbO_2-HRBCs	HbCO-HRBCs	Isolated membranes
0.0	ND	ND	ND
1.0	ND	ND	0.11 ± 0.01‡
2.5	—	—	0.32 ± 0.02§
5.0	3.7 ± 0.4*	ND	0.53 ± 0.03‖
10.0	5.5 ± 0.6†	ND	0.74 ± 0.04¶

Fifty per cent (v/v) HbO_2-HRBCs or HbCO-HRBCs, as well as suspensions of isolated membranes at concentrations corresponding to 50% (v/v) RBC added with 10,000 U/mL bovine liver catalase, were exposed to different concentrations of daunomycin in the presence of 50 mM 3-AT, and 5 mM glucose or NAD(P)H as substrate. The rates of catalase inhibition were determined as described in Materials and Methods. Control samples without 3-AT showed no catalase inhibition. H_2O_2 generation rates (μM/min) were obtained by referring the rates of catalase inhibition to appropriate calibration curves.

ND, not detectable: no time-dependent catalase inhibition was observed (Fig. 1 and Table 1), while the slowest appreciable time-course of the decrease in catalase activity ranging between approximately 12 and 20% inhibition during 1 hr incubation approached with a rate of 0.27 ± 0.07%/min.

* Very different from ND: 27% catalase inhibition was obtained during 60 min incubation at a rate of 0.44 ± 0.03%/min (Fig. 1). The value was inside the range of detection of H_2O_2 fluxes detectable by 50,000 U/mL endogenous catalase in 50% (v/v) HRBC in the presence of glucose.

† Significantly different from * ($P < 0.001$, $N = 6$): 100% catalase inhibition was obtained during 53 min incubation at a rate of 1.90 ± 0.07%/min (Fig. 1).

‡ Very different from ND: 24% catalase inhibition was obtained during 60 min incubation at a rate of 0.40 ± 0.03%/min (Table 1).

§ Significantly different from ‡: $P < 0.001$ ($N = 4$).

‖ Significantly different from §: $P < 0.001$ ($N = 4$).

¶ Significantly different from ‖: $P < 0.001$ ($N = 4$).

Compound I, which is controlled by the concentration of H_2O_2.

The use of such a method with erythrocytes offers several advantages: the measurement of the residual cellular catalase activity is a relatively simple procedure; furthermore, the large dilution of the samples, demanded by the high catalytic constant and the high intracellular concentration of the enzyme, makes the method applicable to systems containing molecules with high light absorptivity. Finally, the high specificity of 3-AT toward catalase [12–14] reduces the risk of possible interferences.

The time-courses of catalase inhibition by 3-AT were linear (Fig. 1) under the condition here

reported, in line with the data of Cohen and Somerson [15]. Furthermore, the results of Fig. 2 showed that the rate of inhibition of catalase by 3-AT and H_2O_2 was linearly dependent on the rate of H_2O_2 generation. The results also showed that the presence of another H_2O_2-scavenging enzyme, i.e. glutathione peroxidase, has to be considered to reduce the flux of H_2O_2 in intact HRBCs. In fact, this enzyme by consuming H_2O_2 with high efficiency decreases the intracellular steady-state concentration of Compound I [26, 27] and consequently reduces the rate of endogenous catalase inhibition by 3-AT. Accordingly, the calibration plots obtained with HRBCs depleted of glutathione, or in the absence of glucose, were different from those obtained with glucose-enriched cells and cells not depleted of glutathione.

The role of hemoglobin in the activation of daunomycin was unequivocally confirmed by the lack of 3-AT-dependent catalase inhibition in HbCO-containing HRBCs. Two mechanisms have been proposed in the literature for the activation of daunomycin by hemoglobin. Anthracyclines could undergo cyclic oxidation–reduction acting as a mediator of electron transfer from NADPH via semiquinone to HbO_2 [2], or they could directly react with HbO_2 leading to the formation of methemoglobin, semiquinone and molecular oxygen [24]. Our data could not give information on the mechanisms but only on the quantitative measurements of such activation. A lower limit for the hemoglobin-dependent pathway of H_2O_2 production upon exposure of HRBCs to daunomycin may be calculated as at least 50% of the total production; in fact, 5.5 ± 0.6 μM/min H_2O_2 was detected in HbO_2-HRBCs, while less than 3.4 ± 0.3 μM/min H_2O_2 must be produced by HbCO-HRBCs on the basis of the sensitivity limit of the assay (Fig. 2, Table 3). On the other hand, the membrane-dependent H_2O_2 production was found to account for 13–14% of the total production (Table 3). The values of H_2O_2 produced by isolated membranes in the presence of daunomycin were in accordance with the values of O_2^- generated in intact erythrocytes on the outer side of the membrane as we measured by ESR spectroscopy [1]. In fact, taking into account the 2:1 O_2^-/H_2O_2 stoichiometry of the O_2^- dismutation reaction, values of 0.08 and 0.49 μM/min H_2O_2 were calculated to be produced by the cells exposed to 1 and 5 mM daunomycin, respectively, on the basis of ESR measurements of O_2^- [1].

The data reported above support the conclusion that H_2O_2 can be generated by daunomycin in HRBCs largely via a cytosolic pathway in which hemoglobin has an important role. The production of this active oxygen species did not result in cell damage as evaluated by the negligible extent of hemolysis and methemoglobin formation. In addition, no decrease was observed in either GSH or glutathione peroxidase activity as well as in membrane sulphydryl groups. This is in line with the lack of cytotoxic effect observed on RBCs upon loading with Adriamycin [28]. Nevertheless, the low fluxes of H_2O_2 generated by the membranes could be able to damage extracellular targets, keeping in mind that it was established that the drug activation site is located on the outer side of the membrane [1], and much lower concentrations of antioxiradical scavenger are present in the extracellular fluids as compared to the cell content.

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