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GENERATION OF PHOTOEMISSIVE SPECIES BY MITOMYCIN C REDOX CYCLING IN RAT LIVER MICROSOMES

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Abstract—Generation of reactive oxygen species during redox cycling is thought to be involved in the chemotherapeutic action of quinone anticancer drugs. A clinically used agent which contains a quinone moiety is mitomycin C (Mit C). With isolated rat liver microsomes we detected photemissive species during Mit C-induced redox cycling. After addition of reduced glutathione (GSH) a large increase in Mit C-induced chemiluminescence was observed. The increase of photoemission in deuterium oxide as well as >90% of intensity at wavelengths >610 nm suggest that singlet oxygen is a photoemissive species generated by this system. Glutathione disulfide (GSSG) accumulates during the reaction. We propose that superoxide anion radicals formed during redox cycling of Mit C react with GSH. Generation of glutathionyl radicals followed by oxygen addition then leads to the formation of photoemissive species and GSSG.

Numerous compounds of different chemical classes can undergo one-electron reduction followed by autoxidation. This enzyme-catalyzed process, known as redox cycling, leads to the formation of the superoxide anion radical [1–3]. With quinonoid compounds, semiquinone radicals are formed which react with dioxygen.

The generation of reactive oxygen species during redox cycling is thought to be involved in the chemotherapeutic action of quinone anticancer drugs [4–6]. Such a clinically used drug is Mit C* [7, 8]. Schwartz [9] showed that Mit C was reduced in endoplasmatic reticulum by NADPH catalyzed by an enzyme system. Electron-spin resonance investigations revealed the semiquinone radical, the superoxide anion radical [10] and the hydroxyl radical [11] as intermediates during Mit C-induced redox cycling.

Free radical interactions during redox cycling elicit chemiluminescence, as was shown for the quinone 2-methyl-1,4-naphthoquinone (menadione) [12, 13]. Addition of GSH enhances the generation of photoemissive species, the bulk of which is attributed to singlet oxygen [12].

In the present work it is shown that photoemissive species are generated during redox cycling of Mit C in rat liver microsomes. Special attention was directed to the role of GSH and its contribution to chemiluminescence and oxygen uptake.

MATERIALS AND METHODS

Chemicals. Mit C was from Sigma Chemical Company (St. Louis, MO) GSH, SOD, catalase, glutathione reductase, glucose-6-phosphate dehydrogenase (grade I) and other biochemicals were from Boeringer Mannheim (Mannheim, F.R.G.). Chemicals were from Merck (Darmstadt, F.R.G.).

Preparation of microsomal fractions. Microsomal fractions were prepared from male Wistar rats (150–200 g body weight) as described in Poyer and McCay [14] and Cadenas and Sies [15]. Phenobarbital pretreatment of rats was performed by addition of phenobarbital to the drinking water (1 mg/ml) for 7 days. The microsomes were stored in 0.1 M sodium phosphate buffer (pH 7.4) at −70° until use.

Low-level chemiluminescence measurements. Low-level chemiluminescence was detected as described by Cadenas and Sies [16] using a photon counter equipped with a red-sensitive (350–800 nm) photomultiplier (EMI 9658 AM, EMI-Gencom, Plainview, NY) cooled to −25° by a thermoelectric cooler. Photoemission is expressed in counts per second. Values are differences to the background light emission. Chemiluminescence reactions were performed in a thermostatted glass cuvette equipped with a magnetic stirrer to maintain a homogeneous suspension and a lid with ports for tubings for oxygen exchange and additions. Additions to the microsomal suspension were made by thin polyethylene tubings from outside of a light-tight box when the photomultiplier was in operation. Detection of red light was performed by placing a 610 nm cut-off filter (Schott, RG 610) between the cuvette and the light guide.

Assay conditions. All chemiluminescence assays were carried out in 0.1 M potassium phosphate buffer, pH 7.4, at 37° gassed with oxygen. Diethylenetriaminepentaacetic acid (Detapac) (0.5 mM) was present as a chelator. A final volume of the reaction mixture of 6.5 ml contained 1.5 mg microsomal protein per ml. The concentration of Mit C is given in the text. The stock solution of GSH (325 mM) contained 2 mM EDTA. Employing a NADPH-regenerating system, glucose-6-phosphate

* Abbreviations used: Mit C, mitomycin C; GSH, reduced glutathione; GSSG, glutathione disulfide; SOD, superoxide dismutase.

(10 mM) and glucose-6-phosphate-dehydrogenase (0.43 U/ml) were added prior to the addition of $NADP^+$ (0.4 mM) starting the reaction.

Measurement of oxygen uptake. Oxygen uptake was measured by a pO_2 analyzer with a Clark-type electrode inserted into a closed cuvette (1.3 ml volume), maintained at 37°. The reaction mixture in the cuvette was constantly stirred with a magnetic stirrer throughout the measuring period.

Measurement of glutathione disulfide. Aliquots of the incubation mixture (1 ml) were added to 1 ml ice-cold 1 N perchloric acid (PCA) containing 50 mM N-ethyl-maleimide (NEM). After protein precipitation and centrifugation at 5000 rpm for 5 min, 1.8 ml of the supernatant was neutralized to pH 6.2 with $KHCO_3$. Preparations of samples for the GSSG assay were made as described by Akerboom and Sies [17]. $KHCO_3$ was taken instead of KOH to adjust the pooled eluate to pH 7.25. GSSG was determined specifically with glutathione reductase, measuring the NADPH oxidation at 340–400 nm in a dual wavelength spectrophotometer (Sigma Instruments, model ZWS 11, München, F.R.G.) 5 µl EDTA (0.1 M) and 5 µl NADPH (4 mg/ml) were added to 700 µl eluate prior to 5 µl (0.143 U) glutathione reductase addition.

RESULTS

Mitomycin C-induced chemiluminescence and oxygen uptake in rat liver microsomal fractions

In the presence of NADPH-regenerating system, supplementation of microsomal suspensions with Mit C in oxygen-saturated buffer led to light emission (Fig. 1). Maximal photoemission intensity was achieved within 15–30 sec, giving a plateau level for 2 min followed by a very slow decay; light emission had decreased to 50% of maximal intensity after 18 min reaction time (not shown). The chemiluminescence emitted from microsomal fractions depended on Mit C concentration; an increase was found up to 250 µM (Fig. 2). Addition of 50 µM Mit C enhanced the maximal photoemission intensity from 305 ± 75 (mean ± SD) (N = 4) in the control to 713 ± 95 (N = 8) counts × sec^{-1} (Fig. 1). The maximal intensity was not enhanced by addition of 20 µM dicoumarol. The chemiluminescence reaction was accompanied by an increase in oxygen uptake. Figure 2 compares the oxygen uptake and the maximal photoemission intensity in dependence on the Mit C concentration. The oxygen consumption in the reaction mixture increased from 9 ± 1 (N = 4) nmole × min^{-1} × mg microsomal $protein^{-1}$ in the control to 18.8 ± 2.2 (N = 4) with 50 µM Mit C. Half-maximal oxygen uptake (34.6 nmole × min^{-1} × mg microsomal $protein^{-1}$) was reached with 200 µM Mit C (calculated from double reciprocal plot according to Lineweaver and Burk).

Effect of reduced glutathione on mitomycin C-induced redox cycling and glutathione disulfide accumulation

Addition of GSH (5 mM) to Mit C-supplemented (50 µM) microsomal fractions enhanced Mit C-dependent light emission intensity more than fourfold yielding a value of 3028 ± 186 (N = 15) counts × sec^{-1} (Fig. 1). The time course of the reaction showed a fast increase and, as for Mit C alone, a slow decay of photoemission. Supplementation of microsomal fractions with GSH alone led to maximal light emission of 1605 ± 195 (N = 11) counts × sec^{-1} (Fig. 1).

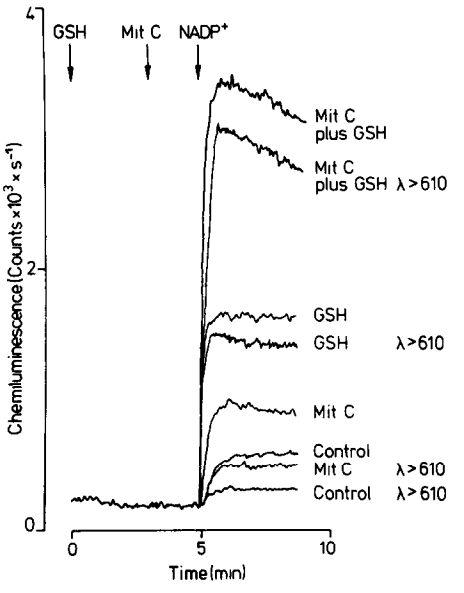

Fig. 1. Mitomycin C-induced chemiluminescence of rat liver microsomal fractions with and without glutathione and measurement of red photoemission. The microsomal fractions (1.5 mg protein × ml^{-1}) were incubated in 0.1 M potassium phosphate buffer, pH 7.4 at 37°, gassed with dioxygen, containing a NADPH-regenerating system. Detapac (0.5 mM) was present as a chelator. Arrows indicate the time-points of GSH (5 mM), Mit C (50 µM) and $NADP^+$ (0.4 mM) addition. Reactions were started with $NADP^+$. Control: no additions of GSH and Mit C. Other traces: addition of either GSH or Mit C or Mit C plus GSH as indicated on the rifht side of the figure. A glass cut-off filter (Schott RG 610) was used to obtain red photoemission ($\lambda > 610$).

White catalase (40 µg/ml) had no effect on chemiluminescence, photoemission decreased with SOD (20 µg/ml) to baseline photoemission (Table 1). Addition of 20 µM dicoumarol did not alter light emission (Table 1). In the presence of GSH, the Mit C-induced chemiluminescence was augmented more than the oxygen consumption (Fig. 2); in comparison with the fourfold enhancement of light emission only 20% more oxygen was consumed (23.4 ± 1 (N = 6) nmole × min^{-1} × mg microsomal $protein^{-1}$). Catalase or SOD diminished this higher oxygen uptake to 18.5 and 16.4 nmole × min^{-1} × mg microsomal $protein^{-1}$ respectively. It can be calculated that in the presence of 50 µM Mit C and 5 mM GSH the chemiluminescence reaction proceeds with an efficiency of 1.52 ± 10^6 photons × sec^{-1} × mg microsomal $protein^{-1}$, assuming a quantum efficiency of the photomultiplier of about 2×10^3, calculated by the luminol standard reaction [18]. The oxygen uptake occurred at a rate of 0.39 nmole × sec^{-1} × mg microsomal $protein^{-1}$. Comparison of the chemiluminescence with the oxygen uptake allows one to

Table 1. Photoemission during mitomycin C-induced redox cycling of rat liver microsomes

Additions	Chemiluminescence (counts × sec^{-1})		
	H_2O*	H_2O†	D_2O*
Control	305 ± 75	115 ± 50	315 ± 50
GSH	1,605 ± 195	1,365 ± 92	3,075 ± 177
Mit C‡	713 ± 95	250 ± 50	650 ± 70
Mit C plus GSH‡	3,028 ± 186	2,820 ± 76	5,400 ± 990
Mit C plus GSH + SOD§	0	0	n.d.‖

Conditions as in Fig. 1. Values of chemiluminescence are maximal intensities after addition of $NADP^+$ (0.4 mM). Mit C, 50 μM; GSH, 5 mM; SOD, 20 μg/ml. Detection of red photoemission was performed with a cut-off filter (Schott RG 610) ($\lambda > 610$). D_2O, 100%; dicoumarol, 20 μM. Data represent means ± SD of 3–10 measurements.

* No optical cut-off filter inserted.

† With cut-off filter ($\lambda > 610$).

‡ Same value was observed also in the presence of 20 μM dicoumarol.

§ Same value was observed in the absence of GSH.

‖ Not determined.

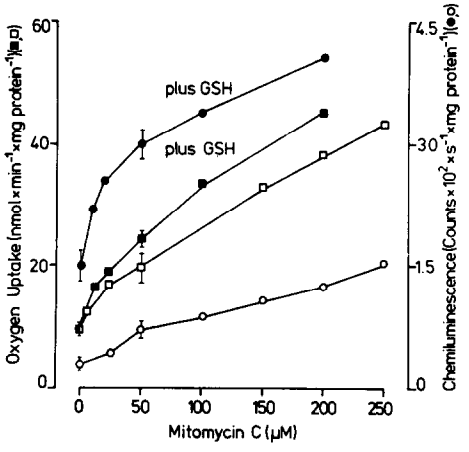

Fig. 2. Relationship between mitomycin C-induced chemiluminescence and the rate of mitomycin C-mediated oxygen uptake in the presence and absence of reduced glutathione. Values of chemiluminescence are maximal intensities after addition of $NADP^+$ (0.4 mM) (see Fig. 1). Values of oxygen uptake rate are taken 30 sec after addition of $NADP^+$. Data for 0 and 50 μM Mit C points are means ± SD of 4–15 experiments. All other points are means of 2–3 experiments.

estimate an efficiency of about 6.5×10^{-9} photons per oxygen molecule consumed for the Mit C + GSH supported redox reaction and 1.9×10^{-9} photons per oxygen molecule consumed for Mit C alone.

During the GSH-mediated (5 mM) redox cycling of 50 μM Mit C 62.5 nmole GSSG × mg microsomal $protein^{-1}$ accumulated within a reaction period of 7 min; absence of Mit C led to a lower GSSG production (Table 2).

The nature of light emission

Placement of a 610 nm cut-off filter revealed that in the presence of GSH >90% of the Mit C-induced light emission was at wavelengths beyond 610 nm, suggesting the bimolecular singlet oxygen light emission at 634 and 703 nm (Fig. 1). Replacement of water by deuterium oxide, which increases the lifetime of singlet oxygen in the incubation mixture [19], led to an 1.8-fold increase in the GSH-mediated Mit C-induced chemiluminescence, again indicative of singlet oxygen (Table 1). Under control conditions, as well as in the presence of Mit C alone, only 40% of light emission was beyond 610 nm (Fig. 1); replacement of water by deuterium oxide in this case showed no increase in light emission (Table 1).

Menadione-induced chemiluminescence and menadione-mediated oxygen uptake in the presence and absence of reduced glutathione

Confirming previous results [12] the menadione induced chemiluminescence recorded from incubations of rat liver microsomal fractions was approximately 1400 counts × sec^{-1}. GSH enhanced this photoemission to 13,300 counts × sec^{-1}. The menadione-mediated oxygen uptake was 77 nmole × min^{-1} × mg microsomal $protein^{-1}$ in the presence and absence of GSH. In the absence of GSH the menadione-induced chemiluminescence was enhanced after addition of 20 μM dicoumarol by about 15% (data not shown).

DISCUSSION

Here we describe the generation of photoemissive species during the Mit C-induced one-electron reduction-oxidation process in rat liver microsomal fractions (Fig. 1). The observed dependence of chemiluminescence of Mit C concentrations correlates with the oxygen uptake (Fig. 2). A large increase in Mit C-induced chemiluminescence was detected after the addition of GSH (Fig. 1). Wefers and Sies [20] reported that GSH reacts with superoxide anion radicals giving glutathionyl radicals. Thiyl radicals may interact with dioxygen forming glutathione peroxy sulphenyl radicals [21] which can yield the nonradical products GSSG, glutathione sulfonate and dioxygen, where the latter is (in part) singlet

Table 2. Glutathione disulfide accumulation during redox cycling of mitomycin C in the presence of reduced glutathione

Additions	nmole GSSG × mg microsomal protein^{-1}
Blank control	17.0 ± 1.0
GSH	42.5 ± 6.4
GSH plus Mit C	80.5 ± 1.0

Conditions as in Fig. 1. See also Materials and Methods. The reaction was started with $NADP^+$ (0.4 mM). Aliquots were taken after 7 min reaction time. GSH (5 mM) was present in all incubation mixtures. Mit C, 50 μM. Blank control: microsomes without starting the regenerating system by $NADP^+$. Data represent means ± SD, N = 7.

oxygen [20]. Detection of more than 90% light emission at wavelengths beyond 610 nm and the increase of maximal photoemission in a buffer system with deuterium oxide suggest that singlet oxygen is a photoemissive species generated by this system (Fig. 1, Table 1).

We also propose that superoxide anion radicals formed during redox cycling of Mit C can interact with GSH giving glutathionyl radicals. The initiation of the GSH radical reaction chain leads to the formation of GSSG, glutathione sulfonate and singlet oxygen. The presence of 50 μM Mit C in a GSH-supplemented microsomal suspension led to a 2.5-fold increase in GSSG accumulation (Table 2), indicative of the capacity in redox cycling of this quinone anticancer drug. SOD abolishes photoemission because the interaction of superoxide anion radicals with GSH and the following reaction chain is prevented by the enzymatic acceleration of the dismutation reaction. Although some superoxide anion radical generating quinones are able to inhibit SOD directly at μM concentrations and may potentiate their own toxicity by this effect, Mit C is without effect on SOD [22]. No alteration in chemiluminescence was found at concentrations up to 250 μM. The lower oxygen uptake after addition of SOD could be explained by the partial regeneration of dioxygen and the hinderance of the GSH radical reaction chain. Any system producing superoxide anion radials would be expected to produce hydrogen peroxide. Therefore, addition of catalase diminishes oxygen consumption by oxygen regeneration but not the GSH-mediated chemiluminescence, because the radical reaction chain leading to photoemissive species is not hindered.

In previous studies, using menadione as a model compound [12] it was shown that intact perfused rat liver or isolated hepatocytes exhibit low-level chemiluminescence. In comparison to Mit C, menadione enhanced the GSH-mediated photoemission and oxygen uptake rate of rat liver microsomes to a higher extent. Menadione contains an electrophilic center at the 3 position and a small part of the photoemission is caused by the chemical reaction of GSH with menadione forming a conjugate [12]. Although substantial quantities of menadione-conjugate are formed, the major route of GSH depletion is via the oxidative process [23] leading to GSSG and excited oxygen species. After additon of Mit C to GSH-supplemented microsomes no increase in chemiluminescence was found. In contrast to menadione, Mit C contains no free position at the quinone ring (position 3 is occupied) and therefore the formation of the Mit C-GSH-conjugate cannot occur.

The detection of photoemissive species during Mit C-induced redox cycling in the presence of GSH may be of toxicological significance. It is possible that such reactive photoemissive oxygen species are involved in the mechanism of action of this quinone anticancer drug. Lown and coworkers had shown that the damage of DNA by Mit C and other quinone antibiotics, dependent on oxygen, was inhibited by addition of SOD and catalase, suggesting an involvement of superoxide anion radicals and hydrogen peroxide in DNA damage [24, 25]. The damage is enhanced when the quinone agent has the ability to bind to DNA [26].

In comparison to the menadione-induced chemiluminescence [12], the Mit C-induced chemiluminescence was not enhanced by addition of dicoumarol, an inhibitor of the two-electron reducing enzyme DT-diaphorase which is present in microsomal fractions in a small amount. Further, it is of potential clinical interest that Mit C is not reduced by the enzyme carbonyl reductase, the two-electron transferring quinone reductase in man which protects from the quinone induced oxidative stress in cells [27].

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