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PARAQUAT-INDUCED CHEMILUMINESCENCE OF MICROSOMAL FRACTIONS

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Abstract—Paraquat-induced chemiluminescence of microsomal fractions requires electron donors (from NADPH through NADPH–cytochrome P-450 reductase) and oxygen; the latter is a deciding factor to observe this chemiluminescence, in accordance with the known requirements for the redox cycling of the herbicide with formation of $O_2^{\cdot-}$ and derived oxygen species. Paraquat-dependent chemiluminescence was not associated with accumulation of malondialdehyde (thiobarbituric acid reactive material), and addition of the bipyridylium compound to an ongoing lipid peroxidation reaction produced a different time course for this chemiluminescence and inhibited malondialdehyde formation.

Accumulation of the paraquat radical under anaerobiosis was not accompanied by chemiluminescence, but subsequent oxygenation of the sample produced a burst of chemiluminescence, the intensity of which was related to the time of incubation under anaerobiosis and paraquat radical accumulated.

Superoxide dismutase inhibits this chemiluminescence, thus suggesting the involvement of $O_2^{\cdot-}$; catalase was without effect.

Selective light emission at 634 and 703 nm with a minimum intensity at 668 nm points to the involvement of singlet molecular oxygen in the paraquat-elicited photoemission.

At variance with the microsomal fraction, paraquat-infused perfused liver did not show any noticeable chemiluminescence, probably due to the presence of cellular defense mechanisms.

Redox cycling of paraquat as well as certain drugs (antineoplastic compounds, iron chelates etc.) forms the molecular basis for the cytotoxicity of the herbicide by generating active oxygen species at the expense of NADPH [1]. Formation of the paraquat radical (PQ^+), in an enzymatic one-electron reduction, is the first required step followed by a reaction with molecular oxygen to yield the superoxide anion radical and subsequently, for example, hydrogen peroxide, the hydroxyl radical, the crypto-hydroxyl radical and others [2, 3]. Further attack of any of these radicals on membrane lipids is supposed to be associated with lipid peroxidation, a controversial topic in paraquat toxicity. Changes in the NADPH/$NADP^+$ cellular ratio, as determined in the perfused liver in the presence of paraquat, appear to be a deciding factor for the rapid redox cycling of paraquat. This would further alter the glutathione status of the cell, reflected by a decrease in reduced glutathione and parallel increases in mixed disulfides and glutathione disulfide levels [4]. These parameters together can be considered as an index of oxidative stress occurring during paraquat poisoning.

In this work, we make use of low-level chemiluminescence as a technique for detecting the formation of excited photoemissive species derived from oxidative reactions involving oxygen-centered radicals [5]. We have applied low-level chemiluminescence to the paraquat-infused liver under the conditions described to produce changes in the NADPH and glutathione cellular status [4] and to microsomal fractions in the presence of paraquat and an NADPH-regenerating system. Although the oxidative reactions that give rise to chemiluminescence are sometimes associated with lipid peroxidation, this report describes a chemiluminescence system which is not accompanied by peroxidation of membrane lipids.

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MATERIALS AND METHODS

Low-level chemiluminescence was measured as previously described [5] with a red-sensitive photomultiplier (EMI 9658). Selective light emission at 634, 668 and 703 nm was obtained by interference filters (Jenaer Glaswerk Schott, Mainz, F.R.G.) which were placed between the cuvette and the photomultiplier.

Preparation of microsomal fractions [6] and experimental conditions for liver perfusion [4] were described previously. In the experiments with microsomal fractions, the NADPH-regenerating system consisted of 15 mM glucose-6-phosphate, 0.35 U glucose-6-phosphate dehydrogenase/ml and 0.4–0.6 mM $NADP^+$. Assays were carried out in 0.1 M potassium phosphate buffer, pH 7.4, at 37° and under constant stirring. Unless otherwise stated, paraquat concentration was 1.0–1.2 mM.

Malondialdehyde accumulation, as thiobarbituric acid reactive material [7], was measured at 535–570 nm using a $\Delta\varepsilon$ of 156 mM^{-1} cm^{-1}. Formation of the paraquat radical was followed at 603 nm (ε_{603} = 12 mM^{-1} cm^{-1}) [8] in a dual-wavelength spectrophotometer [Sigma Instruments, model ZWS-11 (Biochemical Co., München, F.R.G.)].

Paraquat (1,1'-dimethyl-4,4'-bipyridylium dichlor-

ide) was from Fluka Chemical Co. (Buchs, Switzerland). Other biochemicals were from Boehringer (Mannheim, F.R.G.) and chemicals from Merck (Darmstadt, F.R.G.).

RESULTS AND DISCUSSION

It is known that microsomal fractions in the presence of NADPH or an NADPH-regenerating system and Fe (also contaminating Fe in the buffer) undergo lipid peroxidation, as shown by an accumulation of malondialdehyde and an increase in chemiluminescence intensity (Fig. 1A) [9, 10] during a slow oxygen-consuming reaction (7–8 nmoles/min/mg protein). It is believed that this lipid peroxidation reaction needs the concurrence of reduced cytochrome P-450 and H_2O_2 to give the perferryl ion, with the capacity of peroxidizing membrane lipids [11].

Paraquat in the absence of reducing equivalents did not elicit any chemiluminescence, whereas in their presence a chemiluminescence signal, different in time course from that typical of lipid peroxidation, is observed (Fig. 1B). Moreover, this light emission was found not to be associated with accumulation of thiobarbituric acid reactive material during the period of time measured. Oxygen uptake, on the other hand, was accelerated by paraquat and proceeded at rates 6–20-fold higher than that of the peroxidation reaction, and within 1.5–3.0 min the solution became anaerobic. Paraquat-induced chemiluminescence and oxygen uptake correlated with paraquat concentrations and seemed to reach a saturation level at about 1.5–2.0 mM paraquat (data not shown).

Conversely, when paraquat was added to microsomes already undergoing lipid peroxidation (Fig. 1C), chemiluminescence was abruptly enhanced and accompanied by an enhanced oxygen uptake; it is to be noted that from the moment of paraquat addition malondialdehyde accumulation was inhibited.

The inhibition of malondialdehyde formation by paraquat observed here was previously reported by other workers [11–13] and it was also demonstrated that this inhibition of lipid peroxidation was not due to the creation of anaerobic conditions by paraquat, but rather to a diverted electron flow during paraquat metabolism [11]. Thus, in the absence of paraquat, electrons will be used for the formation of a perferryl complex with the capacity to peroxidize, whereas in the presence of paraquat most of the electrons from NADPH–cytochrome P-450 reductase flow to paraquat with formation of PQ^+ and subsequent redox cycling.

Effect of oxygen on paraquat-induced chemiluminescence

The requirements for paraquat-dependent chemiluminescence are electron donors (from the NADPH-regenerating system through NADPH–cytochrome P-450 reductase) and oxygen. Regarding the former, microsomes obtained from phenobarbital-pretreated rats showed a higher chemiluminescence intensity than controls; however, similar photoemission intensities in both cases were observed when calculations were made in terms of their NADPH–cytochrome P-450 reductase activities (data not shown). Oxygen, on the other hand, is a deciding factor in observing this chemiluminescence: (a) assays carried out under anaerobiosis failed to give any photoemissive signal; and (b) the duration of the light emission reaction, if sufficient reducing equivalents were present, depended on the oxygen supply.

Fig. 2A shows chemiluminescence and formation

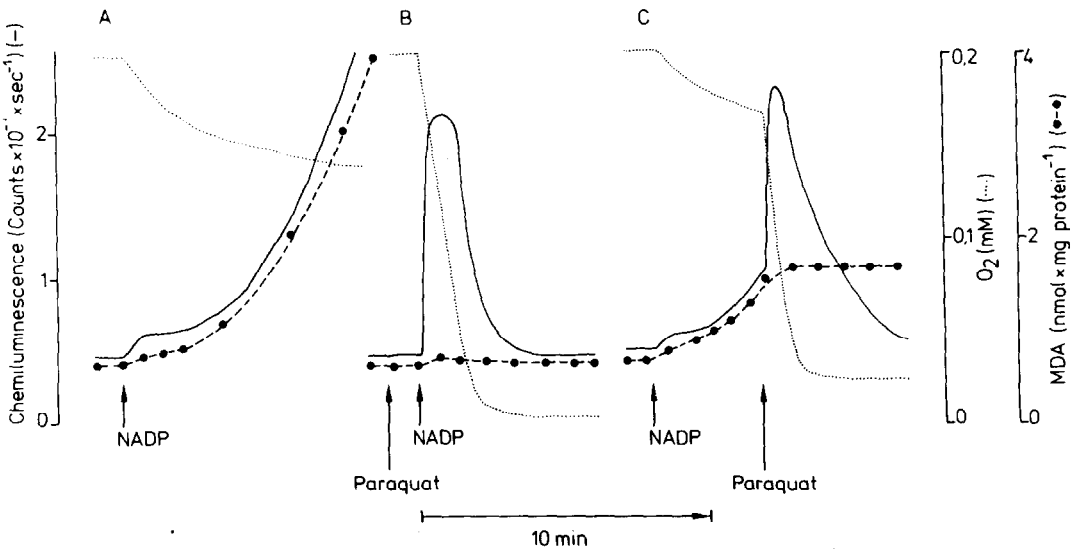

Fig. 1. Chemiluminescence, oxygen uptake and malondialdehyde (MDA) accumulation, of microsomal fractions in the absence and presence of paraquat. Rat liver microsomal fraction (0.5–0.6 mg protein/ml) was incubated in 0.1 M potassium phosphate buffer, pH 7.4, at 37°, with NADPH-regenerating system (as described in Materials and Methods). (A) Reaction started upon addition of $NADP^+$. (B) Paraquat (1 mM) was present prior to the addition of $NADP^+$. (C) Paraquat (1 mM) is added during an ongoing lipid peroxidation reaction.

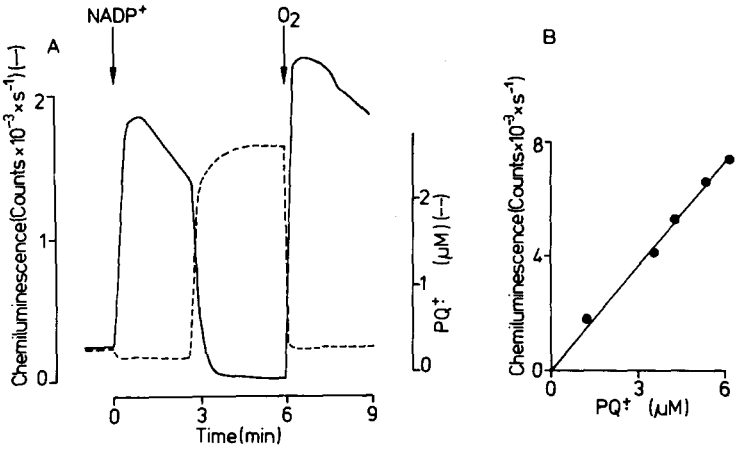

Fig. 2. Relationship between chemiluminescence and the accumulation of paraquat radical during anaerobiosis. (A) Conditions as in Fig. 1B were employed along with parallel incubations for PQ^{+} measurements. (B) After different periods of anaerobiosis, oxygen was allowed into the cuvette and chemiluminescence recorded and plotted against the concentration of PQ^{+} (paraquat radical) that has accumulated.

of the paraquat radical (PQ^{+}) as a function of time. It can be seen that when oxygen is depleted (as in the assay conditions of Fig. 1B), chemiluminescence intensity decays and the formation of the paraquat radical starts. Conversely, a further reaction with oxygen is accompanied by chemiluminescence and a rapid decay of the paraquat radical formed. A prolonged time under anaerobiosis will lead to a higher accumulation of the paraquat radical and the chemiluminescence intensity observed upon oxygenation is linearly related to the amount of paraquat radical accumulated (Fig. 2B).

The oxygen dependence of paraquat-induced chemiluminescence is shown in Fig. 3, with a maximal chemiluminescence intensity of about 70–100 μM oxygen. At low oxygen concentrations (below 0.2 mM) paraquat displayed a higher chemiluminescence intensity, and oxygen uptake proceeded rapidly and reached anaerobiosis, in spite of the constant gassing of the sample. On the other hand, higher oxygen concentrations (beyond 0.2 mM) exhibit a rather lower light emission intensity.

Oxygen species involved in paraquat-induced chemiluminescence

$O_2^{\cdot}$ production during the redox cycling of paraquat seems essential for observing light emission, as seen by the inhibition of the latter in the presence of superoxide dismutase (Fig. 4) (half-maximal inhibitory effect at about 1.1 μg/ml of the enzyme); oxygen uptake proceeded as usual during the superoxide dismutase inhibited chemiluminescence reaction, thus suggesting that redox cycling was not halted. H_2O_2, on the other hand, which could be formed during the spontaneous dismutation of $O_2^{\cdot}$, is not

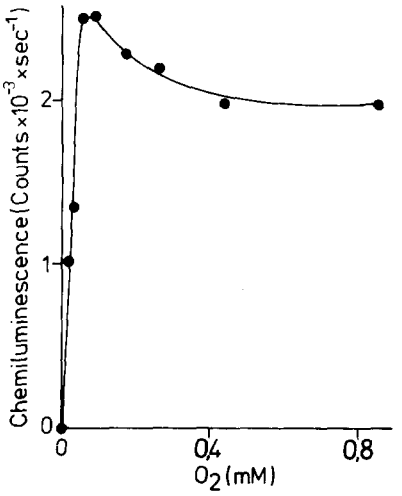

Fig. 3. Oxygen dependence of paraquat-induced chemiluminescence. Conditions as in Fig. 1B. Different oxygen concentrations were obtained by gassing with a gas mixture of O_2 and N_2 through a gas proportioner at a variable ratio.

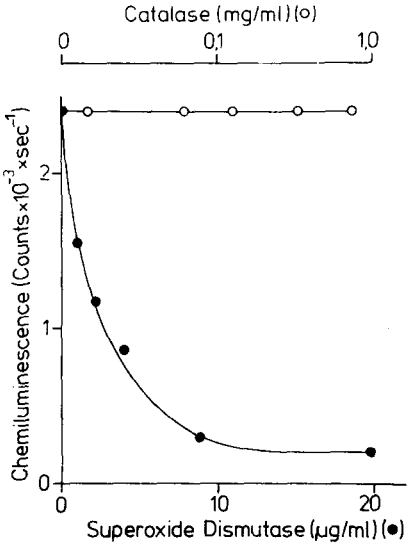

Fig. 4. Effect of superoxide dismutase and catalase on paraquat-induced chemiluminescence. Assay conditions as in Fig. 1B. Superoxide dismutase or catalase were added prior to $NADP^{+}$.

Table 1. Relationship between the light intensity emitted at 634, 668 and 703 nm during paraquat-induced chemiluminescence*

Relative light emission		
634 nm	668 nm	703 nm
1.09	0.13	1.00

* Assay conditions as described in Fig. 1B. Values are corrected for the peak transmission of the filter and the efficiency of the photomultiplier, and they are expressed as relative to that at 703 nm.

related to light emission; this is seen by the lack of an effect of catalase, up to a concentration of 1.0 mg/ml (corresponding to about 65,000 U/ml).

Regarding the nature of light emission, it seems to fulfil the requirements for singlet oxygen dimol emission as described by Deneke and Krinsky [14], i.e. similar relative intensities of the 634 and 703 nm peaks, with little light emitted 668 nm. The first requirement is fulfilled, but the value at 668 nm remains higher (10–15% of the intensity at 634 nm) than that expected (1–2%, Ref. 14) (Table 1). This difference could be due to the complexity of the system, compared with the pure system of HOCl/H_2O_2 [14] apparently generating only singlet molecular oxygen. In this regard, a report on spectral analysis of the microsomal fraction during lipid peroxidation [9] identified singlet oxygen as the main source of photoemission, but the 668–670 nm region represented even 30–40% of the maximal relative intensity at 634 nm. The time course of the chemiluminescence reaction described here in the presence of paraquat (Fig. 1B) was not different when light emission was followed at 634 or 703 nm.

In summary, electron flow through the NADPH–cytochrome P-450 reductase has two alternative pathways: in the absence of paraquat, this electron transport leads to lipid peroxidation and chemiluminescence (Fig. 1A). In the presence of paraquat, electrons are channelled to paraquat reduction to form the paraquat radical and support further redox cycling. The inhibitory effect of superoxide dismutase on paraquat-induced chemiluminescence (Fig. 5) along with the reported stimulatory effect of the enzyme on NADPH-induced lipid peroxidation of microsomes [11] suggest that the mechanisms supporting both chemiluminescence systems are distinct. Singlet oxygen generated during peroxidation reactions is believed to arise from the self-reaction of secondary peroxy radicals [5, 9], whereas in paraquat-induced chemiluminescence other sources of singlet oxygen would be operative. Although at present we cannot give a molecular mechanism for the formation of singlet oxygen upon paraquat redox cycling, the strong inhibitory effect of superoxide dismutase suggests that the spontaneous dismutation of $O_2^{\cdot}$, yielding singlet oxygen, could play a role in photoemission [15]. In this regard, the inhibitory effect of dismutase also gives further support to the hypothesis that the enzymatic dismutation of $O_2^{\cdot}$ is not a source of singlet oxygen. Although the nonenzymatic dismutation of $O_2^{\cdot}$ is considered a source of singlet oxygen [15], recent evidence against this has been brought forward [16].

Chemiluminescence of the perfused liver infused with paraquat

Because of the metabolic parameters affected during paraquat infusion in the perfused liver [4], it could be assumed that this oxidative stress could be reflected as an increase in low-level chemiluminescence. However, we report a negative result, since the perfused liver infused with paraquat did not lead to any noticeable chemiluminescence, a result in agreement with previous observations in the perfused lung [17]. Conversely, an enhancement of ethane production by the perfused liver was observed upon infusion with paraquat in the absence of substrates in the perfusion medium [18].

The lack of photoemission by the perfused liver may be related to the presence of defenses in the cytosol of liver cells against oxygen radical formation (superoxide dismutase, glutathione peroxidase, catalase etc.); these protective systems are not all present in the isolated microsomal fractions. Absence of photoemission in the intact organ does not mean that the sources of light emission here described do not occur, but rather suggests that they are quenched by existing cellular components.

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