Hydroperoxide-induced chemiluminescence of the perfused lung

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(Received 19 September 1979/Accepted 22 July 1980)

Light-emission of the perfused lung is induced by t-butyl hydroperoxide, giving chemiluminescence yields that oscillate between 800 and 1500 counts/s depending on the site and position of the lung. The response of the perfused lung to infusion with different hydroperoxides gives a pattern similar to that observed with the liver microsomal fraction: ethyl hydroperoxide shows a much higher chemiluminescence yield than the tertiary (t-butyl and cumene) hydroperoxides. Alveolar oedema affected the light-emission of the perfused lung depending on the time at which oedema developed, decreasing light-emission on infusion of hydroperoxide in the oedematous lung and increasing it when oedema appeared after the maximal chemiluminescence yield was already achieved. Paraquat, administered *in vivo* or *in vitro*, augmented light-emission by approximately 2-fold. The effect of paraquat was a time-dependent process. Lung chemiluminescence, compared with liver chemiluminescence, needed higher hydroperoxide concentration to induce light-emission.

There is agreement that lipid peroxidation involves oxidative radical chain reactions that could lead to the disruption of biological membranes. Photoemission of biological systems related to oxygen-dependent chain reactions involving lipids has been termed low-level chemiluminescence, unlike the photoemission from the luciferin–luciferase system (Seliger, 1975).

Chemiluminescence from mammalian organs was first reported by Tarusov *et al.* (1961, 1962), who detected a weak light-emission from the mouse liver *in situ*. Thereafter, there have been sporadic reports on chemiluminescence, such as those of Howes & Steele (1971, 1972) and Stauff & Ostrowski (1967) on isolated microsomal fraction and mitochondria respectively. More recently, Boveris *et al.* (1978, 1980) reported light-emission from both liver *in situ* and isolated perfused liver, and pointed out the importance of organ chemiluminescence as a readily detectable, useful and continuous assay of oxidative metabolism.

The advantage of chemiluminescence reactions, over the thiobarbituric acid assay, as giving useful information on peroxidative breakdown of phospholipids, was reported by several authors in microsomal fractions (Howes & Steele, 1971, 1972; Noguchi & Nakano, 1974), mitochondria (Vladimirov *et al.*, 1969; Suslova *et al.*, 1969) and tissue homogenates (Di Luzio & Stege, 1977). The chemiluminescences detected were accompanied by

the accumulation of products of lipid peroxidation such as malonaldehyde.

In the present paper we report the characteristics of hydroperoxide-induced chemiluminescence of the perfused lung and its enhancement by paraquat. Paraquat was selected since this agent is actively accumulated by the lung, and pulmonary damage is the usual cause of death from paraquat poisoning (Copland *et al.*, 1974). One suspected mechanism of paraquat toxicity is the generation of oxidizing free-radical species and lipid peroxidation; Bus *et al.* (1974, 1977) described malonaldehyde formation in microsomal fractions induced by paraquat; this fact remains unclear in the intact organ, where the peroxidative effect induced by paraquat could be restricted by defence mechanisms not operating in the microsomal fraction.

Materials and methods

Perfusion technique

Sprague–Dawley male rats (Charles River Breeding Laboratories, Wilmington, MA, U.S.A.) weighing 180–200 g and kept on a normal diet were anaesthetized with pentobarbital sodium (60 mg/kg body wt.) by intraperitoneal injection and were ventilated through a tracheostomy by using a rodent respirator (Harvard Apparatus, Millis, MA, U.S.A.) at 60 cycles/min, 2 ml tidal volume, and 2 cmH₂O

end-expiratory pressure. Lungs were isolated according to the technique described elsewhere (Bassett & Fisher, 1978*a,b*; Fisher *et al.*, 1980). To clear lungs of blood, they were perfused by a hydrostatic pressure gradient from a reservoir at a rate of approx. 10 ml/min via a cannula inserted through the right ventricle into the pulmonary artery. The perfusate flowed freely from the transected left atrium. When the lungs appeared white (3–5 min), they were transferred into the perfusion apparatus and ventilation was continued as described above. The perfusate was infused at about 10 ml/min into the pulmonary artery with a peristaltic pump (Buchler Instruments, Fort Lee, NJ, U.S.A.) and was not recirculated. The perfusion medium consisted of Krebs–Ringer phosphate buffer, pH 7.3–7.4 (Umbreit *et al.*, 1964), plus 5 mM-glucose. The perfusate temperature was adjusted to ensure that the perfused medium through the lungs was at 37°C. Ventilation and perfusion pressures were monitored continuously with pressure transducers (Statham Instruments P23 ID) and recorded with an oscillograph (MFE Corp., Salem, NH, U.S.A.). Hydroperoxides, used as 0.2–0.4 M solutions in water, were infused with a model 341 syringe pump (Sage Instruments, Orion Laboratories, Cambridge, MA, U.S.A.) into a cannula situated in the inflow line to the pulmonary artery. The rate of infusion of the hydroperoxide solution was approx. 0.3 ml/min.

Photon counting

Chemiluminescence was measured in a photon counter as described by Boveris *et al.* (1978, 1980). An RCA 8850 photomultiplier responsive in the range 300–650 nm with an applied potential of 1.8 kV was fed into a Princeton Applied Research (Princeton, NJ, U.S.A.) model 1121 amplifier–discriminator adjusted for single photon counting and connected to both a frequency counter (Heathkit IB-1100; Heath Co., Benton Harbor, NH, U.S.A.) and a recorder. Collection of light from the organ was established by using a Lucite rod [poly(methyl methacrylate)] (22 cm long × 5 cm diam., and 19.6 cm² surface) as optical coupler. The perfused lung was placed in a special light-tight box as close as possible to the end of the light-guide. Since the light-guide was of greater diameter than the lungs, the chemiluminescence detected varied with lung size and the position of the lung in front of the light-guide; we attempted to maintain these variables nearly constant. Chemiluminescence measurements were expressed in arbitrary units, counts/s, with 1 count/s corresponding to about 10³ photons/s (Boveris *et al.*, 1980).

Chemicals

t-Butyl hydroperoxide was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Cumene hydroperoxide ($\alpha\alpha'$ -dimethylbenzyl hydro-

Fig. 1. Hydroperoxide-induced chemiluminescence of the perfused lung

(a) The infusion rate of t-butyl hydroperoxide was kept constant and is indicated in the upper ordinate. (b) Dependence of chemiluminescence on the infusion rate of t-butyl hydroperoxide (Bu^tOOH). Points indicate maximal chemiluminescence yield. The concentration of hydroperoxide in the perfusate is indicated in the upper abscissa. The rate of lung perfusion was constant at 10 ml/min. Other assay conditions were as described in the Materials and methods section.

peroxide) and ethyl hydroperoxide were purchased from Matheson, Coleman and Bell (Norwood, OH, U.S.A.) and Ferrosan (Malmo, Sweden) respectively. Paraquat (Methyl Viologen or 1,1'-dimethyl-4,4'-bipyridylum dichloride) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Results

Hydroperoxide-induced chemiluminescence of the perfused lung

Infusion of t-butyl hydroperoxidase into the perfused lung, at a rate of 125 $\mu\text{mol}/\text{min}$, promoted light-emission (Fig. 1a). The chemiluminescence

Fig. 2. Effect of different hydroperoxides on lung chemiluminescence

Assay conditions were as described for Fig. 1(a) and in the Materials and methods section. Abbreviations used: Bu'OOH, t-butyl hydroperoxide; CuOOH, cumene hydroperoxide; EtOOH, ethyl hydroperoxide.

signal commenced 1 min after starting the infusion of the hydroperoxide and increased rapidly, reaching a constant value in approx. 4 min. The light-emission was a function of the rate of t-butyl hydroperoxide infusion (Fig. 1b); half-maximal stimulation of light-emission was obtained with 4 μmol of t-butyl

Fig. 3. Effect of alveolar oedema on lung chemiluminescence

Experimental conditions were as described in the Materials and methods section. Chemiluminescence (CL) and ventilation pressure (P_{TR}) were recorded simultaneously. Increased ventilation pressure was taken as an indication of alveolar oedema. (a) Control, no oedema; (b) chemiluminescence recorded in a lung where pulmonary oedema had already developed; (c) pulmonary oedema developed when the maximal chemiluminescence yield was already achieved.

hydroperoxide/min and maximal chemiluminescence yields required infusion of $100\mu\text{mol}$ of hydroperoxide/min.

Both primary (ethyl) and tertiary (t-butyl and cumene) hydroperoxides were effective in promoting light-emission on infusion into the perfused lung (Fig. 2). When infused in similar concentrations, ethyl hydroperoxide gave a 4-fold higher chemiluminescence yield than the two tertiary hydroperoxides. The time required to obtain maximal chemiluminescence yield was approximately the same for all three hydroperoxides. The greater response of the perfused lung to ethyl hydroperoxide infusion suggests that this hydroperoxide is a better substrate for lung cells.

Effect of alveolar oedema on chemiluminescence of the perfused lung

Ventilation pressure during control periods of lung perfusion was approx. $5\text{--}7\text{cmH}_2\text{O}$ at end-inspiration. Lungs occasionally became oedematous during perfusion. With spontaneous development of alveolar oedema, ventilation pressure increased rapidly and pressures at end-inspiration exceeded $20\text{cmH}_2\text{O}$ (Fig. 3c). The presence of pulmonary oedema exerted different effects on chemiluminescence of the perfused lung depending on the time during perfusion at which pulmonary oedema developed: oedema that developed before the start of the infusion of t-butyl hydroperoxide markedly lowered

the chemiluminescence yield (Fig. 3b), whereas oedema that developed when the maximal light-emission value was already obtained promoted an increase in chemiluminescence (Fig. 3c).

Possible reasons for the lower chemiluminescence yield under the conditions in Fig. 3(b) are impairment of the pulmonary cells, affecting the metabolism of the hydroperoxide, or altered distribution of the hydroperoxide in the lung circulation. On the other hand, the enhancement of light-emission in parallel to the appearance of oedema shown in Fig. 3(c) could be promoted by optical causes, i.e. an increase in the transparency of the organ under such conditions. The development of oedema in isolated lungs may have been in part due to absence of protein from the pulmonary perfusate; the incidence of oedema did not appear causally related to the infusion of hydroperoxide or paraquat.

Paraquat and lung chemiluminescence

The effect of paraquat on the chemiluminescence of the perfused lung was investigated with two different approaches. In the first approach, paraquat was administered to the animal at the intraperitoneal dose of 50mg/kg body wt.; about 2 h later chemiluminescence in the perfused lung was measured. Lungs from the paraquat-injected rat showed 2-fold higher hydroperoxide-induced chemiluminescence yield than the control (Fig. 4). Light-emission after withdrawal of the hydro-

Fig. 4. Chemiluminescence from the lung of rat previously injected with paraquat

Rats were injected with paraquat (60mg/kg body wt.) and the lung was removed 2 h later for assay of chemiluminescence. Experimental conditions were as described for Fig. 1(a) and in the Materials and methods section.

peroxide appeared to decrease more rapidly in the lung of the paraquat-injected rat compared with the control.

In the second approach, paraquat was infused into the perfused lung through the cannula situated in the inflow line to the pulmonary artery, to give a concentration in the pulmonary perfusate of either 0.3 or 3.0 mM. Lungs were infused with the bipyridyl for either 15 or 30 min before the infusion of the *t*-butyl hydroperoxide. The infusion with paraquat was carried out in the dark. This method allowed us to record the dark-current base-line of the perfused lung while infusing paraquat and thus investigate if paraquat itself increased the spontaneous light-emission of the organ.

No changes were found in the base-line or the hydroperoxide-induced chemiluminescence when paraquat in the pulmonary perfusate was at a concentration of 0.3 mM (results not shown). This

result agrees with the lack of effect on the production rate of lactate plus pyruvate by isolated perfused lung reported by Bassett & Fisher (1978b), at this paraquat concentration. With 3 mM-paraquat (a concentration that does produce metabolic changes in the perfused lung) there was no increase in the base-line chemiluminescence, but on infusion of the hydroperoxide there was a markedly enhanced chemiluminescence when compared with the control (Fig. 5). The enhancing effect of paraquat was time-dependent: the 30 min perfusion period gave higher chemiluminescence yield than did 15 min perfusion. The effect of paraquat, at the higher dose we have used, seems to be time-dependent rather than concentration-dependent, probably because lung actively accumulates the bipyridyl.

We observed that the hydroperoxide-enhanced chemiluminescence of the perfused lung was reduced by about 30% [controls ($n = 5$), 720 ± 38 counts/s; ascorbic acid-treated ($n = 5$), 500 ± 25 counts/s; $P < 0.01$; values are means $\pm$ S.E.M.] in rats pretreated with a dose of ascorbic acid (2.5 mg/kg body wt.), here suggested in an antioxidant activity, to modify chemiluminescence. Of course, this observation needs further study regarding the dose-response relationship of lung chemiluminescence and ascorbic acid, since this substance is well known by its dual effect as pro- and anti-oxidant, depending on its concentration (Willis & Kratzig, 1975). Ascorbic acid may function as an intracellular free-radical scavenger (Nishikimi, 1975), which may account for its inhibition of the hydroperoxide-induced chemiluminescence.

Discussion

Since chemiluminescence integrates oxidative radical reactions that are related to lipid and organic peroxide formation, it can be used as an indirect assay to assess the involvement of increased lipid peroxidation in physiological and pathological situations. The possible involvement of the lung in these reactions is of interest, since the lung is a major target organ for damage from oxidizing agents such as O_2 , O_3 , NO_2 and paraquat (Mustafa & Tierney, 1978). The isolated perfused lung model was utilized for this study; the viability of this preparation, for at least 1–2 h perfusion, has been demonstrated in previous studies by linear rates of glucose utilization, maintenance of high energy charge, appropriate responses to inhibitors and uncouplers of oxidative phosphorylation, constant physiological parameters for ventilation and perfusion, and normal ultrastructure (Bassett & Fisher, 1976a,b, 1978b; Fisher *et al.*, 1980). The present results show that chemiluminescence is induced in the lung during infusion of hydroperoxides and can be measured at the lung surface. It is evident that differences exist

Fig. 5. Effect of paraquat on hydroperoxide-induced chemiluminescence of lung

(a) Control; (b) lung perfused with paraquat (concentration in the pulmonary perfusate 3 mM) during 15 min before infusion of hydroperoxide; (c) the same as (b) after 30 min of perfusion with 3 mM-paraquat. Assay conditions were as described for Fig. 1(a) and in the Materials and methods section.

between the metabolic role of endogenously generated and externally added hydroperoxide; however, the present system may qualify as model lipid hydroperoxides in metabolic studies, as demonstrated by Sies & Summer (1975).

Hydroperoxide-induced chemiluminescence of the perfused liver (Boveris *et al.*, 1978, 1980) and lung (the present paper), under the same experimental conditions, shows several interesting differences. The concentration of hydroperoxide to produce half-maximal chemiluminescence yields was much higher in the lung, indicating a lower efficiency for metabolism of organic hydroperoxides. On the other hand, chemiluminescence yields with various hydroperoxides were different in lung and liver: lung showed maximal effect on infusion of ethyl hydroperoxide, resembling the pattern obtained with microsomal fractions of rat liver (Boveris *et al.*, 1978) and submitochondrial particles (Cadenas *et al.*, 1980a), whereas the liver showed greater effect with cumene hydroperoxide (Boveris *et al.*, 1980), as observed with rat liver mitochondria (Boveris *et al.*, 1978) and the model system cytochrome *c*/hydroperoxide (Cadenas *et al.*, 1980b). This could point to a major participation of the microsomal fraction in the light-emission by the lung, though further experimental evidence with pulmonary subcellular fractions will be necessary to corroborate this conclusion.

It is worth noting that we failed to detect an increase of spontaneous chemiluminescence in the lung of the paraquat-injected rat as well as in the paraquat-perfused lung. This might be due to different reasons: (a) that our photomultiplier was not sensitive enough to detect small variations of light emission that could correspond to an early oxidative damage of the lung by paraquat; (b) that sufficient time was not allowed, after the treatment with paraquat, to show variations that could be detected by chemiluminescence techniques; or (c) that paraquat does not result in significant lipid peroxidation in lung because lung lipids contain mostly saturated fatty acids (Montfoort *et al.*, 1971). But it remains that light-emission arising from an initial reaction between the lung and hydroperoxide was substantially augmented in the presence of paraquat. One possible mechanism is the intracellular reduction of the viologen, actively accumulated by the lung in very short times (Rose *et al.*, 1974), followed by reoxidation with concomitant production of O_2^- (Farrington *et al.*, 1973); a higher steady-state concentration of O_2^- might react with the infused hydroperoxide (Peters & Foote, 1976), yielding chemiluminescent singlet molecular oxygen. The increased chemiluminescence found with the administration of paraquat *in vivo* was confirmed by the perfusion *in vitro*, indicating that the effect of the viologen was upon the lung tissue itself. The degree of paraquat-induced

chemiluminescence in the presence of hydroperoxide was a function of the duration of perfusion, consistent with previous reports indicating that uptake of the bipyridyl by lung slices is a time- and energy-dependent process (Rose & Smith, 1977). The results suggest that the mechanism for the toxic effect of the viologen on the lung is due in part to free-radical generation, possibly involving singlet oxygen (Bus *et al.*, 1974).

The present preliminary report on lung chemiluminescence cannot identify the nature of the light-emission; although formation of chemiluminescent singlet oxygen through a self-reaction of *t*-butyl peroxy radicals (Pryor, 1978) might account partially for the light-emission observed, other chemiluminescence reactions, such as the ones comprising a dioxetane mechanism (Hasting & Wilson, 1976; Foote, 1976), cannot be excluded. In order to resolve this, a spectral analysis of the light-emission observed from the organ will be necessary; this will require a more sensitive photomultiplier with a better response beyond 600 nm.

With regard to the metabolic source of the light-emission, we are able to say that the *t*-butyl hydroperoxide-induced chemiluminescence involves hydroperoxide-metabolizing systems other than catalase, since the latter enzyme does not react with tertiary hydroperoxides (Sies & Summer, 1975).

This research was supported by U.S. Public Health Service grants HL-SCOR-15061, TW-02457 and HL-15013. E. C. is a U.S. Fogarty International Research Fellow. A. B. F. is an Established Investigator of the American Heart Association.

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