Volume 261, number 2, 369–372 FEBS 08171 February 1990

The first observation of O_2^- generation in in situ lungs of rats treated with drugs to induce experimental acute respiratory distress syndrome

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Received 18 December 1989

To investigate O_2^- generation in in situ lungs of rats treated with drugs to induce experimental acute respiratory distress syndrome, phorbol myristate acetate (PMA) or endotoxin were injected into rats, who had been continuously infused with 2(methyl-6-[*p*-methoxyphenyl]-3,7-dihydroimidazo[1,2-α]pyrazin-3-one (MCLA) and the chemiluminescence in in situ lung was detected by a sensitive photon counter. In PMA-treated rats, two phases of chemiluminescence over non-specific chemiluminescence were observed. The first phase luminescence was sensitive to Cu-Zn superoxide dismutase, while the second phase chemiluminescence was less sensitive to Cu-Zn superoxide dismutase. Similar chemiluminescence was detected in the rats treated with endotoxin instead of PMA, but not in neutropenic rats.

Superoxide anion; Cypridina luciferin analog; In situ lung chemiluminescence; Experimental acute respiratory distress syndrome

1. INTRODUCTION

Isolated cells, such as amoeba [1], yeast [2], phagocytizing leukocytes [3,4] and macrophages [5] emit a low level of chemiluminescence, which could only be detected by a highly sensitive photon counter. However, the origin of the excited species produced in these cells is not clear, because of less accurate emission spectra taken in the visible region and a possible energy transfer of the original excited species to other compounds, changing the original spectrum. An increased spontaneous luminescence from the surface of perfused rat liver (or lung) has first been reported by Cadenas et al. [6] who used *tert*-butyl hydroperoxide in the perfusate as an initiator of phospholipid peroxidation. The molecular source of photoemission, however, should be difficult to identify, because of the small concentration and low rate of production of the excited species. Recently, cypridina luciferin analogs, 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-α]pyrazin-3-one (CLA) and 2-methyl-6-[*p*-methoxyphenyl]-3,7-dihydroimidazo[1,2-α]pyrazin-3-one (MCLA) were found to be very sensitive and to be specific chemiluminescence probes to detect O_2^- generated by activated leukocytes and macrophages [7,8]. It has been known that intravenous injection of the neutrophil-activating agent, phorbol myristate acetate (PMA), causes neutrophil sequestration and edema in sheep lungs but not in neutropenic sheep lungs [9]. If the lung of an experimental animal is infiltrated by activated leukocytes, by which O_2^- is generated in this organ and reacts with injected MCLA, MCLA-dependent organ chemiluminescence, over the control level, would be detected from the surface of the lung by a sensitive photon counter. The present work was undertaken to prove the O_2^- generation in in situ lungs of phorbol myristate (endotoxin)-treated rats, using MCLA as a chemiluminescence probe and a sensitive photon counter placed in front of the lung as a photon detector.

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2. MATERIALS AND METHODS

2.1. *Photon counting system*

As shown in fig.1, the system used in the present experiments was essentially the same as reported by Cadenas et al. [6] except that an R1332 photomultiplier (Hamamatsu Photonics), responsive in the range of 350–650 nm, was used instead of an RCA8850 photomultiplier fitted with a lucite rod (an apparatus for efficient light collection), and a dry-air jacket located in the front of a shutter and a window of the photomultiplier to avoid a condensation of moisture. The rat to be measured for the light emission from the lung surface was placed in a special light-tight box, as close as possible to the window of the photomultiplier (about 10 cm from the surface of the lung). The sensitivity of the photomultiplier was detected by Hasting's method and found to be 1 cps = about 1500 photon/s at a 10-cm distance from the window of the photomultiplier. Male Wistar rats, weighing between 300 and 500 g, were anesthetized with pentobarbital (50 mg/kg). The lung surface was exposed in situ by removing approximately 3 cm^2 of the thoracic wall. Two microcannulas were inserted into the right and left jugular veins, and were placed in the superior vena cava: one tube for MCLA infusion and the other for drug infusion. Furthermore, the femoral artery was also cannulated and used for monitoring arterial blood pressure (BP). The animal was maintained on a respirator for a small animal (tidal volume: 1.0–1.5 ml; rate, 80–90 cycles/min; inspiratory pressure,

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00145793/90/$3.50

20–30 cm H_2O) and a circulating water blanket at 30°C during the photon detection.

2.2. *Reagents*

MCLA was dissolved in saline just before use, and the concentrations ranging between 300 and 1000 μM were based upon ϵ_{430nm} = 9600 $M^{-1} \cdot cm^{-1}$ [8]. PMA from Sigma Chemicals was dissolved to 5 mg/ml in dimethyl sulfoxide, which was then divided into 0.04-ml aliquots for storing at −80°C and diluted to an adequate concentration with saline before use. Endotoxin, lipopolysaccharides from *E. coli*-serotype 0111:B4, was purchased from Sigma Chemicals. This was dissolved to 1 mg/ml in saline before use.

2.3. *Enzymes*

Bovine erythrocyte Cu-Zn superoxide dismutase (SOD, grade III) and milk xanthine oxidase (grade III) were obtained from Toyobo and Sigma Chemicals, respectively. SOD and xanthine oxidase were dissolved to 10 mg/ml and 10^5 units/ml in saline, respectively, just before use.

2.4. *Preparation of leukocyte-depleted rats*

This was done according to the method described by Shasby et al. [10]. 1.75 mg of nitrogen mustard/kg was intravenously injected to rats, who suffered from leukopenia (<500 cells/mm^3) 72 h after the injection.

3. RESULTS AND DISCUSSION

Since MCLA, when reacted with O_2^-, has a rather broad emission spectrum in the visible region with a peak at 465 nm, biological pigment, especially hemoglobin, should greatly lower the MCLA-dependent organ chemiluminescence. When MCLA

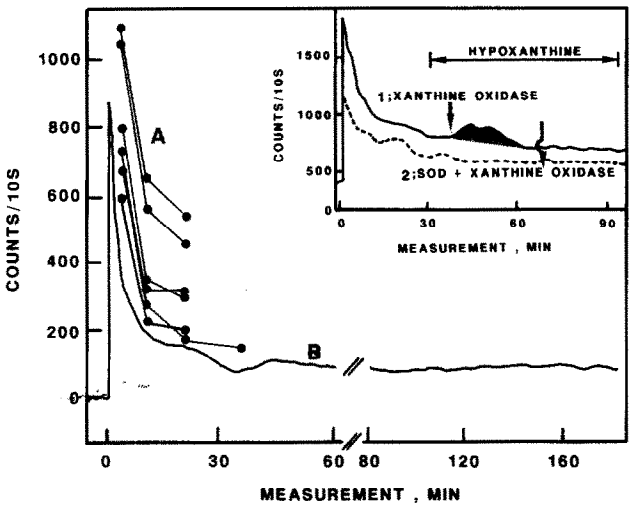

Fig.2. MCLA-dependent chemiluminescence in peripheral blood (A) and from the lung surface (B). (A) Two ml of 1 mM MCLA/kg was intravenously injected and 0.5 ml of peripheral blood was drawn into a heparinized syringe at 5, 12, 20, 30 min after injection. The blood sample was promptly used for the chemiluminescence assay. (B) 0.5 ml of 300 μM MCLA was injected in bolus form, followed by a continuous infusion of 300 μM MCLA at a flow rate of 0.5 ml/h. (Inset) MCLA-dependent chemiluminescence from the lung surface of the xanthine oxidase-hypoxanthine-injected rat. The MCLA infusion was carried out as in fig.2B. During the continuous MCLA infusion, an additional compound, 1.5 mM hypoxanthine, was continuously infused at a flow rate of 1 ml/h, while 1.0×10^5 units of xanthine oxidase (——) or a combination of 1.0×10^5 units of xanthine oxidase and 10 mg of SOD/kg (– – –) was injected in bolus form at the time indicated by the arrow.

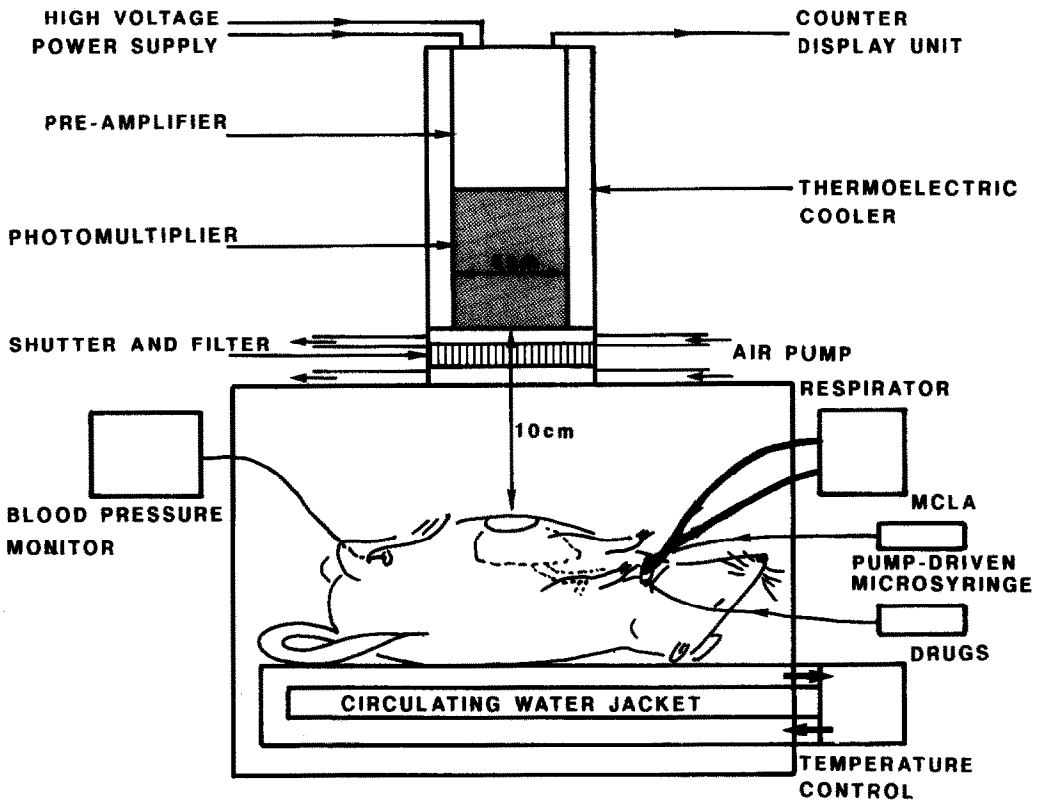

Fig.1. Singlet photon-counting apparatus and additional systems for the measurement of lung surface chemiluminescence. The lung surface was placed in front of a window of the photomultiplier.

was injected into a non-treated rat, a strong burst of luminescence, over the natural luminescence, was detected from the lung surface. Then, the luminescence decreased and remained almost constant during continuous infusion of MCLA. The lung surface chemiluminescence (LSC) in the early stages was closely related to the MCLA-dependent luminescence in the peripheral blood of the rat (fig.2).

In contrast to such an LSC (a non-specific chemiluminescence), a single injection of xanthine oxidase to rats, who had been continuously infused with hypoxanthine, caused a slight, but detectable short-lasted LSC over the non-specific chemiluminescence. Expectedly, simultaneous injection of xanthine oxidase and SOD, instead of xanthine oxidase alone, did not cause the short-lasted LSC over the non-specific chemiluminescence. These data are shown in fig.2 (inset).

When 100 μg of PMA/kg were injected into rats at the time at which LSC remained almost constant under continuous MCLA infusion, LSC increased slightly at 15–20 min (first phase) and obviously at 45–60 min (second phase) after the PMA injection. Such a luminescence pattern was observed in 8 of 12 different experimental rats. A typical chemiluminescence pattern for the PMA-treated rats is shown in fig.3A. A single injection of 10 mg of SOD to the rats, who had been treated with PMA, at the time at which the first phase luminescence was observed, caused a rapid and obvious decrease of the luminescence (fig.3B). However, more SOD was required to diminish the second phase luminescence (fig.3C). Similarly, endotoxin, another drug which induces experimental acute respiratory distress syndrome as well as PMA [11], when injected into rats, caused SOD-sensitive LSC over the non-specific luminescence level (fig.4A,B). On the other hand, little or no LSC over the non-specific luminescence was observed in rats with leukopenia during the continuous infusion of endotoxin and MCLA (fig.4C).

Histopathological examination of lung tissues isolated from the rats at 45 min after the administration of 100 μg PMA/kg indicated a great infiltration of granulocytes and edema formation in the organs (data not shown).

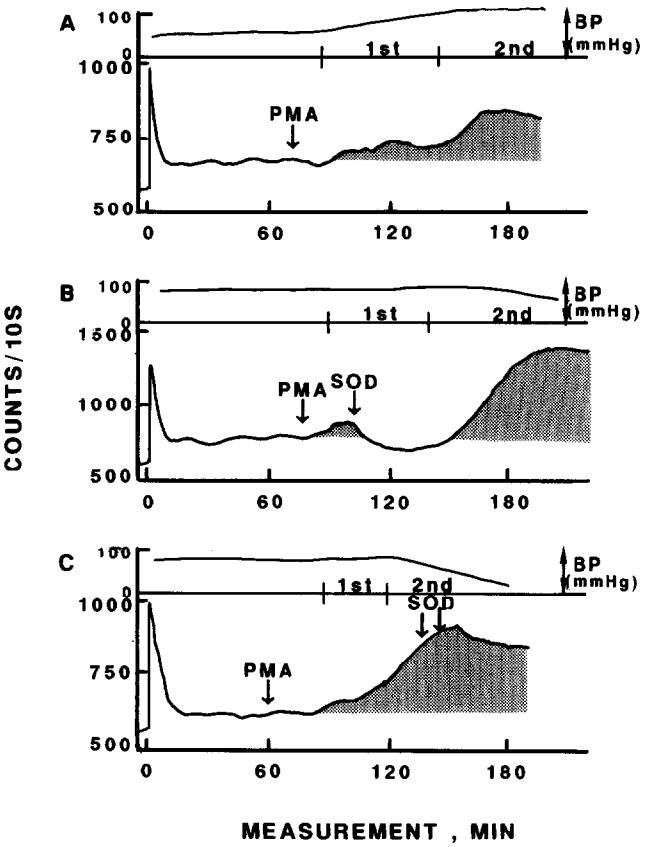

Fig.3. MCLA-dependent chemiluminescence from the lung surface of PMA-treated rats (A) and the effect of SOD on the luminescence (B,C). The MCLA infusion method was essentially the same as in fig.2B. PMA (100 μg/kg) or SOD (10 mg/kg) was injected in bolus form at the time indicated by the arrows.

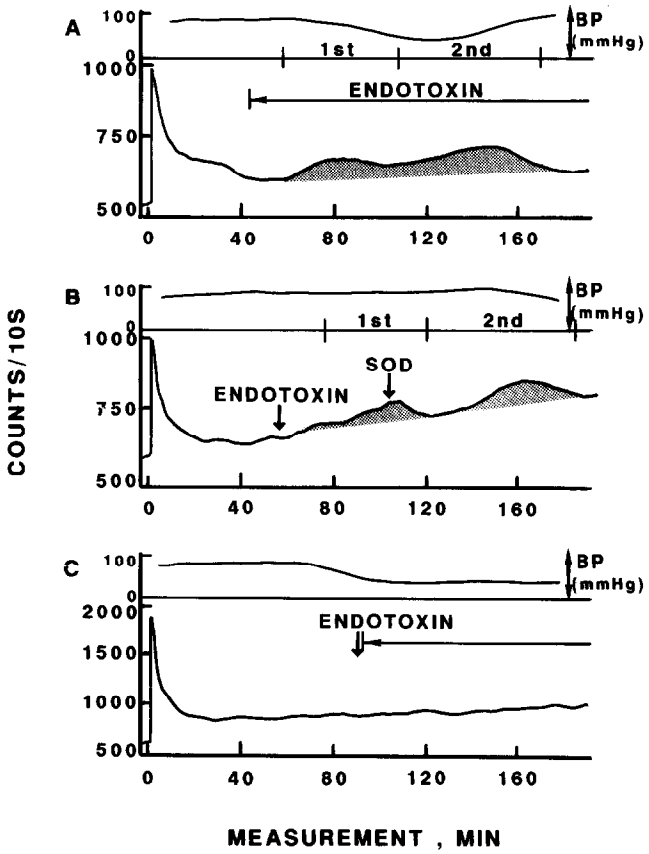

Fig.4. MCLA-dependent chemiluminescences from lung surfaces of endotoxin-treated rats without (A,B) and with leukopenia (C). The MCLA infusion method was essentially the same as in fig.2B. (A) Endotoxin was continuously infused at a rate of 2.5 mg/kg per h, during the time indicated. (B) Endotoxin (2.5 mg/kg) or SOD (10 mg/kg) was injected in bolus form at the times indicated by the arrows. (C) Endotoxin (2.5 mg/kg) was first injected and then administered continuously at a rate of 2.5 mg/kg per h during the times indicated.

These results clearly indicate that LSC over the non-specific light originates from the MCLA + O_2^- reaction and suggests that activated leukocytes are the source of O_2^- generation. The first phase chemiluminescence, which is highly sensitive to SOD, is probably involved in O_2^- generated by activated leukocytes adhered to endothelial cells in blood vessels. On the other hand, the second phase chemiluminescence which is less sensitive to SOD (seen in PMA-treated rats), is mainly attributable to O_2^- generated by activated leukocytes in acute edematous lung.

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