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The first application of a chemiluminescence probe, 2-methyl-6-[*p*-methoxyphenyl]-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (MCLA), for detecting O_2^- production, in vitro, from Kupffer cells stimulated by phorbol myristate acetate

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The purpose of this study was to investigate the generation of superoxide anion radical, O_2^-, by Kupffer cells in vivo in rat liver. Phorbol myristate acetate (PMA) was infused into perfused rat liver which had been continuously infused with 2-methyl-6-[*p*-methoxyphenyl]-3,7-dihydroimidazo-[1,2-*a*]pyrazin-3-one (MCLA, a highly sensitive luminescence reagent for O_2^-) and the MCLA luminescence from the liver surface was detected with a sensitive photon counter. Under identical conditions, but without MCLA infusion, PMA and nitro blue tetrazolium were infused to verify generation of O_2^- by formation of formazan deposition. Based on MCLA luminescence and formazan deposition, both dependent on the O_2^- reaction, we have concluded that liver Kupffer cells generate O_2^- in vivo in response to stimuli such as PMA. Further, the generation of O_2^- by Kupffer cells may be by the same mechanism as displayed by macrophages.

Kupffer cell; Phorbol myristate acetate superoxide; Organ chemiluminescence; 2-Methyl-6-[*p*-methoxyphenyl]-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one

1. INTRODUCTION

It is generally accepted that foreign materials taken up from the digestive system are mainly degraded by non-parenchymal cells, particularly by Kupffer cells. Kupffer cells, as resident macrophages of the liver have many typical functions in common with phagocytic leukocytes. It has been reported that isolated Kupffer cells respond to stimuli such as opsonized zymosan [1,2], phorbol myristate acetate (PMA) [1,2] and platelet activating factor [2] with increased oxygen consumption to produce superoxide, O_2^-. Furthermore reactive oxygen species generated by phagocytic cells upon activation have been suggested to cause tissue injury during sepsis, endotoxic shock and ischemia-reperfusion [3,4]. Therefore, considerable interest has been focused on the consequences of activation of Kupffer cells during these pathophysiological conditions. However, such investigations are limited due to lack of sensitive and reliable methods for determing active oxygen radical species in the liver and their cellular source, since liver is histologically and functionally a heterogeneous organ. Okuda et al. first reported applying a luminol enhanced chemiluminescence technique to the perfused rat liver during ischemia-reperfusion [5]. Luminol has often been used to amplify emission by activated granulocytes and macrophages [6]. However, luminol chemiluminescence is not a specific indicator of O_2^- generation, since oxidation of luminol by a wide variety of oxidants leads to its characteristic luminescence. Recently, 2-methyl-6-[*p*-methoxyphenyl]-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (MCLA) has been proved to be a very sensitive and specific luminescence probe to detect O_2^- generated by activated leukocytes and macrophages [7,8]. Takahashi et al. first applied MCLA-dependent organ chemiluminescence technique to the in situ rat lung treated with PMA and detected O_2^- generation in that model, despite the fact that hemoglobin should greatly absorb the MCLA-dependent luminescence [9]. In view of this, we describe here the first application of the MCLA-dependent organ chemiluminescence technique to the hemoglobin-free perfused rat liver and report the time course of O_2^- production by rat liver Kupffer cells stimulated by PMA.

2. MATERIALS AND METHODS

2.1. *Animals*

All of the animal experiments were performed observing the ethical principles provided by the Experimental Animal Laboratory of

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Gunma University School of Medicine. Male Wistar rats (SLC; Hamamatsu, Japan), weighing 250–300 g, were fed on standard laboratory chow and tap water ad libitum. Before experiments, the rats were heparinized with sodium heparin (1,000 unit/kg by intraperitoneal injection) and anaesthetized with sodium pentobarbital (50 mg/kg by i.p. injection).

2.2. *Reagents*

MCLA from Tokyo Kasei, Co. Ltd., was dissolved in saline just before use. The MCLA concentration was based upon $\varepsilon_{430} = 9{,}600$ $M^{-1} \cdot cm^{-1}$ [8]. PMA from Sigma Chemicals was dissolved at 5 mg/ml in dimethyl sulfoxide, which was then divided into 0.02 ml aliquots for storing at −80°C. PMA in dimethyl sulfoxide was diluted to the desired concentrations by Krebs-Hensleit bicarbonate buffer at pH 7.4 (KHB). Hypoxanthine was a product of Wako pure chemicals and was used without purification. Nitro blue tetrazolium (NBT) was purchased from Sigma Chemicals. SOD from bovine erythrocytes and xanthine oxidase (XOD, grade III) were obtained from Sigma Chemicals.

2.3. *Formazan deposition*

Experiments were followed according to the method described by Nishida et al. [8], except that KHB was used instead of Hanks balanced-salt solution as perfusion buffer. Briefly the liver was perfused successively with KHB for 5 min to wash out blood and then with the same buffer containing 0.5 μg/ml PMA, 50 mg/ml NBT for a further 10 min and finally with the same buffer for 5 min to wash out the remaining NBT in the sinusoidal space. The liver perfusion was performed at a flow rate of 30 ml/min in a non-recirculating system with continuous 95% O_2 + 5% CO_2 bubbling. The excised liver was then fixed in 10% formalin, embedded in paraffin, sectioned, stained with Kernechtrot and examined under a light microscope.

2.4. *Measurement of chemiluminescence*

The photon counting system used in the present study was essentially the same as described by Takahashi et al. [9], except that the system was connected to a perfusion system (Fig. 1). The rat liver was exposed and cannulated through the portal vein and perfusion was initiated with KHB, immediately followed by transecting the inferior vena cava and abdominal aorta. The perfusion buffer which had been continuously gassed with a mixture of 95% O_2 + 5% CO_2 was infused using a peristaltic pump at a constant flow rate of 30 ml/min (non-recirculating model) at 37°C. One mM hypoxanthine or 1 μM SOD in a flask, 1 μM MCLA in syringe pump A and xanthine oxidase (0.25 unit/ml) in syringe pump B were used for the O_2^- detecting system, while PMA or 1 μM SOD in the same flask and 1 μM MCLA in syringe pump A were used for the experimental system. In all experiments, the MCLA was continuously infused by syringe pump A at a flow rate at which 1 μM MCLA could be achieved in the sinusoidal space of the rat liver for the O_2^- detecting. The rat for measuring photon emission from the liver surface was placed in a special light-tight box as close as possible to the window of the photomultiplier (at about 10 cm distance from the surface of the liver) and was covered with a black sheet with a 1 × 1 cm hole to exclude luminescence from other organs. Only the luminescence from the rat liver surface could be detected through the hole.

3. RESULTS AND DISCUSSION

To know whether or not O_2^- generated by hypoxanthine plus xanthine oxidase reacts with MCLA in perfused rat liver resulting in photon emission, a mixture of hypoxanthine and MCLA was continuously infused and xanthine oxidase was then infused via syringe pump B for 5 min to perfused liver in the light-tight box of the photon counting system (Fig. 1) for detecting photon emission from liver surface. As shown in Fig. 2, slightly increased luminescence above natural emission (a non-specific luminescence) which can result due to autoxida-

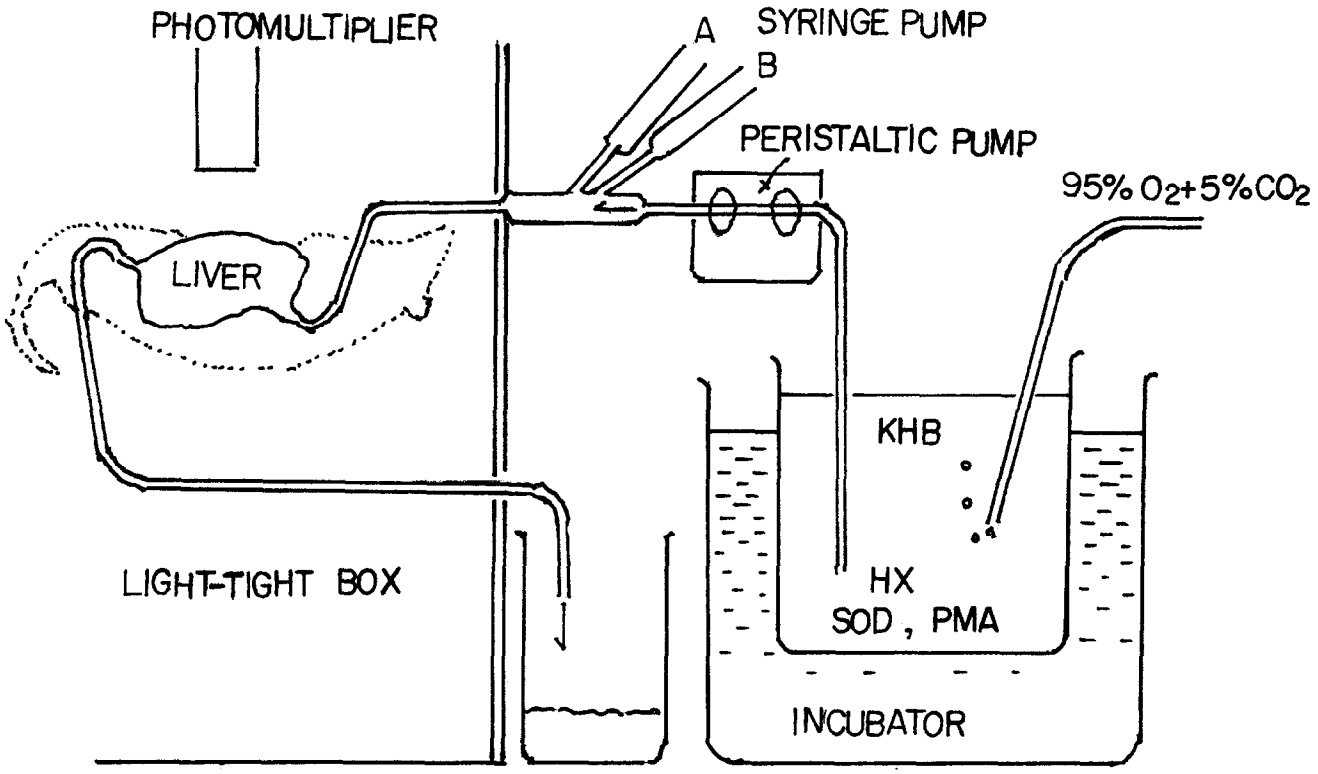

Fig. 1. Perfusion system used for the measurement of liver surface luminescence. HX = hypoxanthine.

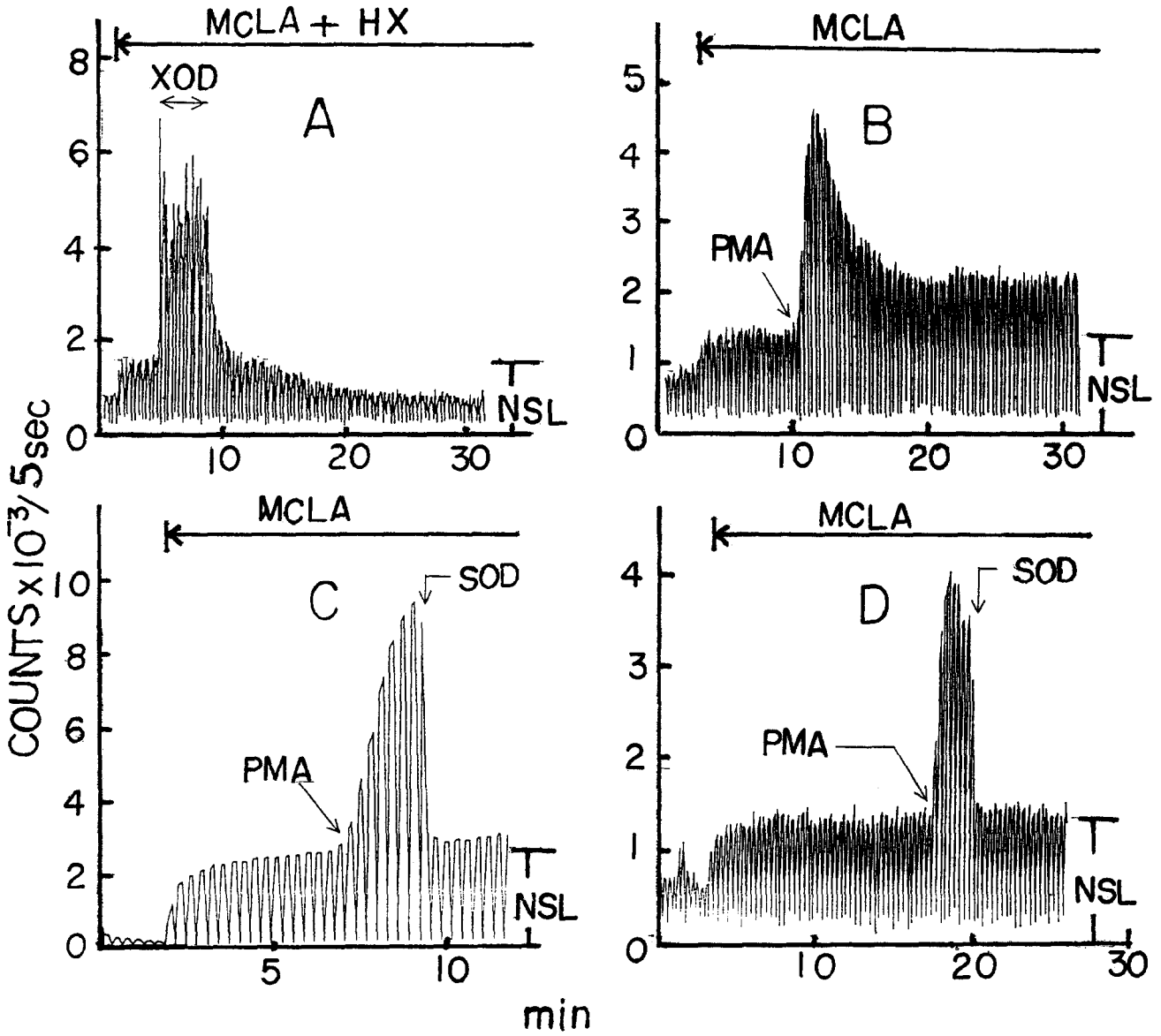

Fig. 2. Time course of chemiluminescence intensities from perfused rat liver surfaces before and after MCLA infusion withe xanthine oxidase + hypozanthine (A) or with PMA (B, C or D). The experimental conditions were as described in section 2. The arrow indicates the time at which PMA or SOD infusion was started. HX = hypoxanthine; NSL = non-specific chemiluminescence level.

tion of MCLA induced by traces of metals in the buffer and is almost insensitive to a O_2^- scavengers such as SOD [8], appeared just after infusion of MCLA and hypoxanthine. As shown in the same figure, an infusion of xanthine oxidase to the experimental rat, at the time at which constant intensity for the non-specific luminescence was obtained, caused a strong burst of luminescence which continued during the enzyme infusion but ceased when the enzyme infusion was stopped. Since xanthine oxidase with a high molecular weight is not expected to penetrate into the liver cells, the enzyme could react with hypoxanthine in blood vessels yielding O_2^- which evokes MCLA dependent luminescence. Thus our chemiluminescence method is very specific for the detection of O_2^- generated in blood vessels. Under the same experimental conditions as those in Fig. 2A, except that PMA was used instead of hypoxanthine and xanthine oxidase, a strong burst of luminescence appeared just after PMA infusion, reached maximum and gradually decreased followed by a continuous emission over the non-specific emission (Fig. 2B). When SOD was infused at the time at which a strong luminescence was observed after PMA infusion, the luminescence promptly fell to the level of non-specific luminescence (Fig. 2C,D). These observations indicate that O_2^-, released into blood vessels from PMA-activated Kupffer cells in the rat liver, reacts with MCLA yielding a strong luminescence, which could be quenched by SOD (O_2^-

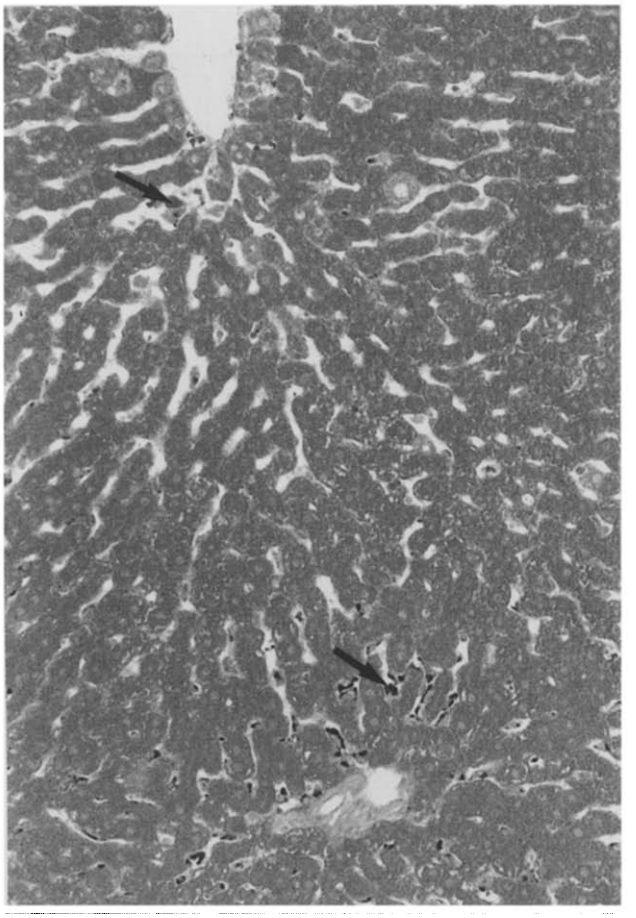

Fig. 3. Light micrograph showing formazan deposition (←) after perfusion of the liver with nitro blue tetrazolium and phorbol myristate acetate.

dependent luminescence). PMA is a stimulator of O_2^- generation from resting neutrophils, macrophages, Kupffer cells [11] and eosinophils [12]. Under our experimental conditions, neutrophils and eosinophils in blood should be almost completely washed out by perfusion buffer. Thus Kupffer cell, a resident macrophage in the liver would be the only candidate as O_2^- generating cell in the perfused liver. To ensure this, both PMA and NBT were perfused for detecting O_2^- generation by formazan precipitation as described in Section 2. In perfused rat liver after PMA treatment, faint formazan deposition was found in Kupffer cells throughout the liver (Fig. 3).

Recently, Bautista and Spitzer, who used a cytochrome *c* reduction assay to detect O_2^- in perfusate of PMA treated rat liver, have reported that PMA slightly increased O_2^-, probably generated by Kupffer cells [2]. For the O_2^- detection in vitro, the MCLA-chemiluminescence method is 95 times more sensitive than the cytochrome *c* assay method [13]. Thus the chemiluminescence method applied here is a highly sensitive approach for detecting O_2^- in vivo, generated by stimulated Kupffer cells. Therefore the MCLA method may be applied to identify the nature of stimuli which can activate Kupffer cells in vivo.

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