Production of singlet oxygen by eosinophils activated in vitro by C5a and leukotriene B4

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Abstract Using the *trans*-methoxyvinylpyrene analogues of benzo[*a*]pyrene-7,8-dihydrodiol (MVP) as a singlet oxygen ($^1O_2$) chemiluminescence probe, we have demonstrated that guinea pig eosinophils release $^1O_2$ when activated with the physiological agonists C5a and leukotriene B_4. This release, which occurs at agonist concentrations as low as 10^{-7} M, occurs more rapidly than activation with phorbol ester (10^{-6} M), is similar in level, but is more transitory. In addition, the release of $^1O_2$ occurs in the absence of added bromide ions and represents, we propose, an important feature of eosinophil-mediated inflammatory damage.

Key words: Singlet oxygen; Eosinophil; C5a; Leukotriene B_4; Chemiluminescence

1. Introduction

There is considerable evidence to suggest the importance of eosinophils in the pathogenesis of allergic diseases, such as asthma, and in the defence against invading parasitic larvae of helminths [1–3]. Eosinophils are characteristically tissue-associated cells and it is at these sites that they release several mediators, which are though to mediate damage to host cells or cytotoxic effects on parasites. These mediators include lipid substances, such as platelet-activating factor and thromboxanes, and basic proteins, such as major basic protein (MBP) [4–6]. In addition, there is evidence demonstrating that activated eosinophils release reactive oxygen species (ROI) such as hydrogen peroxide and superoxide anion, as well as nitric oxide [7–9]. Together these reactive intermediates can cause injury to host tissues and parasitic larvae. In addition, phorbol myristate acetate (PMA)-activated human eosinophils have been shown to release singlet oxygen ($^1O_2$), however this was only in the presence of bromide ion [10] and its physiological relevance has not been investigated. Eosinophils produce $^1O_2$ via the Mallet reaction (Eq. 2) between H_2O_2 and hypobromous acid (HOBr), formed Eq. 1).

$$H_2O_2 + Br^- \xrightarrow{\text{peroxidase}} HOBr + H_2O \quad (1)$$

$$HOBr + H_2O_2 \rightarrow {}^1O_2 + HBr + H_2O \quad (2)$$

$^1O_2$ arises because, unlike hypochlorous acid (HOCl), HOBr is not as rapidly quenched by reactions with endogenous amines (Eq. 3) and is therefore free to react with H_2O_2 [11].

$$HOCl + COOH-\underset{R}{\overset{H}{C}}-NH_2 \rightarrow COOH-\underset{R}{\overset{H}{C}}-NHCL \quad (3)$$

The ability of mediators which induce eosinophil recruitment in vivo and activation in vitro to elicit $^1O_2$ production in eosinophils has not yet been investigated. We have therefore used purified guinea pig eosinophils, to investigated whether the chemoattractant molecules C5a and leukotriene B_4 (LTB_4), which we have previously shown to activate eosinophils in vitro leading to their homotypic aggregation and release of superoxide anion [12], are also able to stimulate $^1O_2$ production. A comparison with optimal activation induced by PMA, an activator of protein kinase C, was also made. We have employed the *trans*-methoxyvinylpyrene analogue of benzo[*a*]pyrene-7,8-dihydrodiol (MVP) as a $^1O_2$ chemiluminescence probe [13].

2. Materials and methods

2.1. Reagents

Hanks' solutions, HEPES and horse serum were purchased from Life Technologies Limited (Paisley, UK) and Percoll was from Pharmacia (Milton Keynes, UK). Superoxide dismutase, glucose oxidase, xanthine oxidase, lucigenin, xanthine phorbol myristate acetate, were purchased from Sigma Chemical Co., Poole, UK. C5a and LTB_4 were purchased from Bachem, UK. Myeloperoxidase was a gift from Dr Jo Cambridge (Department of Rheumatology, University College London), the 5-methoxyvinylpyrene analogue of 7,8-diol was a gift from Dr Harold Hodson (Wellcome Research Laboratories, Beckenham, UK) and L-*N*-(1-iminoethyl)-ornithine (L-NIO) was a gift from Professor Salvador Moncada (The Wolfson Institute, University College London).

2.2. Isolation and purification of guinea pig eosinophils

Eosinophils were prepared by the method of Teixeira et al., [14]. Briefly, ex-breeder female guinea pigs (Harlan Porcellus; 700–800 g) were treated with horse serum (1 ml i.p.) every other day for 2–3 weeks and cells collected by peritoneal lavage with heparinised saline (10 IU/ml) 2 days after the last injection. The cells obtained were layered onto a discontinuous Percoll-HBSS (calcium- and magnesium-free) gradient and centrifuged ($1500 \times g$, 25 min at 20°C). Eosinophils (>95% pure as evaluated by stained smears prepared by cytocentrifuge and >98% viable as evaluated by trypan blue exclusion) were collected from the 1.090/1.095 and 1.095/1.100 g/ml density interfaces. The purified eosinophils were then washed twice in HBSS containing 0.25% BSA and resuspended prior to analysis in HBSS with BSA at a final concentration of 10^7 cells/ml.

2.3. Chemiluminescent measurement of singlet oxygen and superoxide

The methoxyvinylpyrene analogue of benzo[*a*]pyrene-7,8-dihydrodiol (MVP) was synthesised by the method of Posner et al. [13]. Samples (2 ml in 35-mm sealed culture grade dishes) were kept in a light-tight chamber at 37°C. Chemiluminescence was measured using a luminometer incorporating a gallium arsenide photomultiplier tube

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(Hamamatsu), cooled to −20°C, with a response above 10% of quantum efficiency in the wavelength range 200–900 nm. Light emission was characterised using specific enhancers of chemiluminescence: 10 μM lucigenin for superoxide, and 10 μM MVP for singlet oxygen. Chemiluminescence was measured from the time of addition of agonist every 5 min for a total time of 20 min. Counts for each time point were collected over 100 s with a 1-s gate time.

2.4. Generation of superoxide, free hydroxyl, nitric oxide, peroxynitrite and singlet oxygen

Superoxide was generated enzymatically from 0.33 U of xanthine oxidase, and acetaldehyde (10 μM) in PBS. Hydroxyl radicals were generated in a system containing 200 μM hydrogen peroxide with 10 μM Fe^{3+}/EDTA, nitric oxide from homolytic breakdown of *S*-nitrosocysteine at 37°C and pH 7.4. Peroxynitrite, prepared free of hydrogen peroxide by ozone and azide [15], and was added as a bolus. Singlet oxygen was generated from hypobromous acid and hydrogen peroxide produced using a myeloperoxidase, sodium bromide, and glucose oxidase-glucose cell-free system. Kodak Wratten filters, cut-off 703 nm (680–720) and 470 nm (460–500), were used to demonstrate $^1O_2$-mediated non- and MVP-enhanced chemiluminescence respectively [16].

3. Results

3.1. Lucigenin and MVP chemiluminescence in the presence of superoxide, singlet oxygen, nitric oxide, peroxynitrite and hydroxyl

Superoxide produced from xanthine oxidase and acetaldehyde caused a SOD-inhibitable lucigenin chemiluminescence (Fig. 1A). The addition of MVP to xanthine oxidase and acetaldehyde induced no chemiluminescent signal above background (Fig. 1). In a system producing hydrogen peroxide from glucose and glucose oxidase, neither background, MVP nor lucigenin chemiluminescence was observed (data not shown), however when myeloperoxidase was added, background (non-enhanced) chemiluminescence and MVP chemiluminescence was produced (Fig. 1b). Chemiluminescence was immediate, reached a peak after 30 s, and was enhanced 5-fold with MVP. Non- and MVP-enhanced chemiluminescence was blocked by the respective filters (data not shown). Addition of *S*-nitrosocysteine, which rapidly releases NO, or a hydroxyl radical-generating system of Fe^{3+}/EDTA did not result in chemiluminescence with MVP. Peroxide-free peroxynitrite (10 μM) caused an immediate chemiluminescence of 2500 cps which decayed to basal levels in 20 s.

3.2. Effect of PMA on eosinophil MVP chemiluminescence

Addition of PMA to eosinophils caused a time- and dose-dependent increase in MVP chemiluminescence (Fig. 2a). The chemiluminescence increased linearly, reaching a peak (200 cps) after 10 min, which was sustained to the end of monitoring (20 min). This PMA-induced increase in chemiluminescence was blocked using a 460–500-nm filter but was unaffected by SOD (Fig. 2b). In contrast, the effects of PMA on lucigenin chemiluminescence were markedly reduced in the presence of SOD (Fig. 2b).

3.3. Identification of singlet oxygen as the source of MVP chemiluminescence from PMA-activated eosinophils

To exclude peroxynitrite as a source of MVP chemiluminescence during eosinophil activation cells were pre-treated for 10 min with the nitric oxide synthase inhibitor L-NIO (100 μM). This had no effect on chemiluminescence (Fig. 3) and by inhibiting NO formation precludes peroxynitrite formation as a source of emission. The singlet oxygen quencher histidine (2 mM), but not the hydroxyl radical quencher mannitol (2 mM), decreased MVP chemiluminescence by 80%.

3.4. Stimulation of eosinophil singlet oxygen release by physiological agonists

The physiological chemoattractants C5a and LTB_4, used at a concentration of 10^{-7} M, induced a transient rise in MVP chemiluminescence which was faster than that observed with PMA, reaching a peak 5 min following agonist addition (Fig. 4), after which there was a fall in chemiluminescence to levels which were still higher than the background. Thus, the level of chemiluminescence detected was similar to that detected in response to activation with 10^{-6} M PMA, but it was not sustained.

4. Discussion

Eosinophils play an important role in defence against invading parasites and in the pathophysiology of allergic dis-

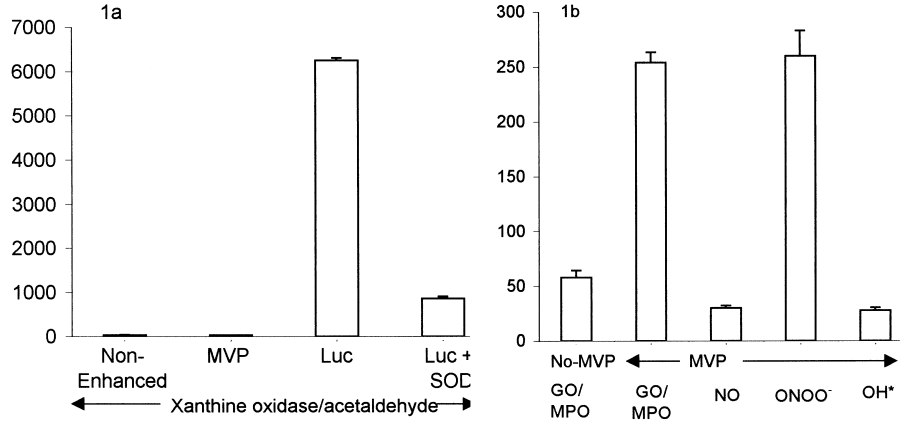

Fig. 1. a: Non-, lucigenin (LUC)- and MVP-enhanced chemiluminescence in a xanthine oxidase plus acetaldehyde superoxide-generating system. b: Non- and MVP-enhanced chemiluminescence with singlet oxygen (glucose oxidase-myeloperoxidase; GO/MPO), nitric oxide, peroxynitrite and hydroxyl radicals (OH*) generated from peroxide/Fe^{3+}-EDTA, all in a cell-free system. Each value is the mean ± S.D. count collected over 100 s with a 1-s gate time, and is representative of six separate observations. $^*P < 0.01$ vs background (23 ± 2 cps) and $^{**}P < 0.01$ vs non-enhanced.

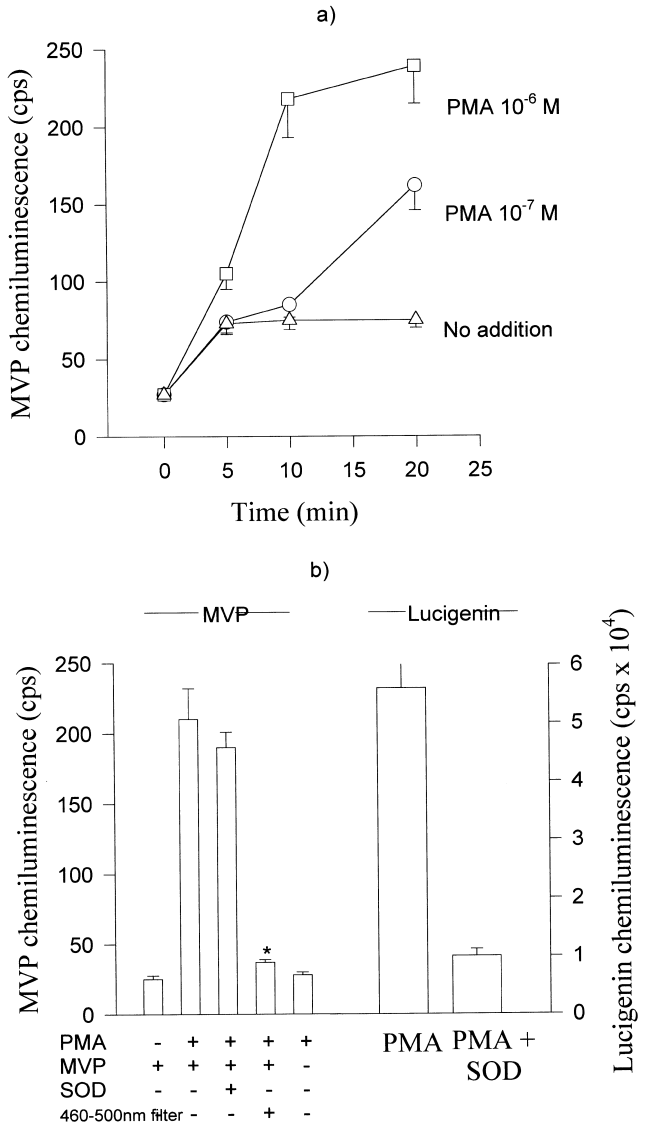

Fig. 2. Singlet oxygen and superoxide production by PMA-activated eosinophils. a: Time-dependent MVP-enhanced chemiluminescence following activation with 0.1 and 1.0 μM PMA. Each value is the mean ± S.D. chemiluminescence count collected over 100 s with a 1-s gate time, and is representative of four separate observations. b: Effect of SOD and a 460–500-nm filter on MVP- and lucigenin-enhanced chemiluminescence in PMA (1.0 μM)-activated eosinophils. Each value is the mean ± S.D. count 10 min after PMA addition, collected over 100 s with a 1-s gate time, and is representative of four separate observations.

eases, such as asthma [3]. The ability of eosinophils to secrete lipid mediators, basic proteins and reactive oxygen and nitrogen species appears to contribute to the actions of these cells [4–9]. Here we demonstrate that eosinophils produce $^1O_2$ in response to endogenous inflammatory mediators.

MVP has been cited as a specific enhancer of chemiluminescence from $^1O_2$. We have confirmed this by showing that chemiluminescence produced in the presence of glucose/glucose oxidase/myeloperoxidase was not SOD-inhibitable, and that no chemiluminescence was evident with xanthine oxidase/acetaldehyde. Isolated guinea pig eosinophils activated with nM concentrations of the physiological agonists C5a and LTB_4, or with the PKC activator PMA, produced $^1O_2$. SOD did not block the production of $^1O_2$, whilst it completely quenched superoxide detected by lucigenin chemiluminescence. Inhibition of nitric oxide synthase by pre-incubation with L-NIO, preventing formation of $ONOO^-$ which can elicit MVP chemiluminescence, did not affect the emission resulting from activation. In addition, when activated with C5a or LTB_4, eosinophil singlet oxygen production was comparable with that elicited using μM levels of PMA. However, $^1O_2$ production by the former was faster to reach a maximum, but more transient compared with PMA, which was slower in onset and sustained for a longer period. This is consistent with our previous studies evaluating the effects of similar stimuli on eosinophil aggregation and production of ROI [12,17]. Thus, under these conditions, eosinophil activation by physiological stimulators such as LTB_4 and C5a was rapid in onset and transient, whereas the effect of PMA was slower, but more sustained.

Further evidence that inflammatory cells can produce $^1O_2$ has been provided by Steinbeck et al. [18] who used 9,10-diphenylanthracene-coated beads as a $^1O_2$ trap, and demonstrated $^1O_2$ generation by phagocytic neutrophils, which accounted for 20% of the oxygen consumption. It is recognised that $^1O_2$ is an important cytotoxic species in cell killing mediated by monocytes, neutrophils and eosinophils (reviewed in [19]). The ability of $^1O_2$ to avidly attack cellular targets protected from other less damaging species supports this [20]. The fact that physiological modulators of inflammatory cells can induce production of $^1O_2$ is significant not only to parasite killing, but also in damage to host targets such as lung epithelial cells in asthmatic patients. We are at present addressing this in animal models of allergic airway disease and parasite killing in vitro.

The mechanism by which eosinophils produce $^1O_2$ is proposed to involve H_2O_2 and hydrohalogenated acids [21], however $^1O_2$ can also be produced from the interaction of H_2O_2 with either peroxynitrite or NO [22,23]. Eosinophils produce both NO and superoxide, however the addition of SOD to activated cells did not change the $^1O_2$ production, implying that peroxynitrite was not a source of $^1O_2$.

In summary, we have demonstrated for the first time that eosinophils when activated by physiological effectors release $^1O_2$, and that this occurs in the absence of added bromide.

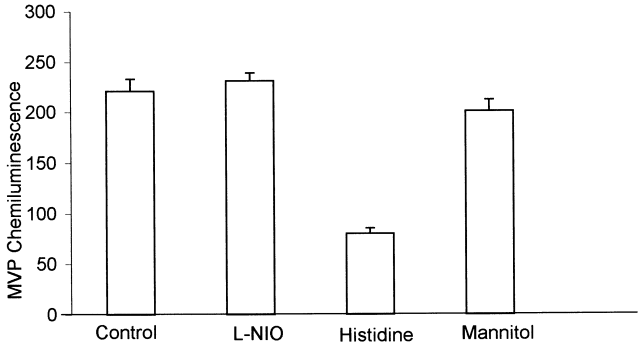

Fig. 3. Effect of peroxynitrite, singlet oxygen and hydroxyl scavengers on MVP chemiluminescence in PMA-activated eosinophils. Cells were preincubated either with L-NIO or with histidine and mannitol added 10 min after PMA activation, the period of maximum chemiluminescence. Each value is the mean ± S.D. count 10 min after PMA addition, collected over 100 s with a 1-s gate time, and is representative of four separate observations.

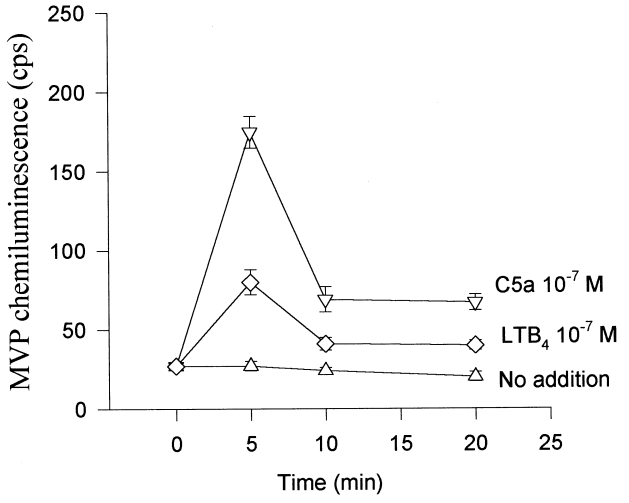

Fig. 4. Singlet oxygen production by C5a- or LTB_4-activated eosinophils. Each value is the mean ± S.D. MVP chemiluminescence count for each time point, collected over 100 s with a 1-s gate time, and is representative of four separate observations.

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