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Contribution of nitric oxide synthase to luminol-dependent chemiluminescence generated by phorbol-ester-activated Kupffer cells

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Phorbol 12-myristate 13-acetate-induced luminol chemiluminescence in rat Kupffer cells was doubled by the addition of L-arginine and significantly (up to 70%) inhibited by N^G-nitro-L-arginine and N^G-monomethyl-L-arginine, competitive inhibitors of L-arginine-dependent nitric oxide (NO) formation. The release of superoxide anion (O_2^-) by NADPH oxidase was neither affected by L-arginine nor by the inhibitors. Only very slight luminol chemiluminescence was detectable in lipopolysaccharide-pretreated Kupffer cells, a condition in which significant amounts of NO were formed but no O_2^-. In a cell-free system, significant luminol chemiluminescence only occurred when both authentic NO and the O_2^-/H_2O_2-generating system xanthine/xanthine oxidase were present. The results indicate that luminol chemiluminescence in phorbol-ester-activated Kupffer cells largely depends on L-arginine metabolism by NO synthase, requiring the concurrent formation of NO and O_2^-/H_2O_2.

INTRODUCTION

The generation of reactive oxygen species by neutrophils, eosinophils, monocytes and macrophages represents an important mechanism by which these cells mediate their antimicrobial and tumoricidal functions. For monitoring the formation of reactive oxygen species, chemiluminescence has become a widely used method. Apart from native chemiluminescence, amplifiers such as lucigenin or luminol are employed. Whereas lucigenin is considered to be specific for O_2^- (Allen, 1981; Dahlgren *et al.*, 1985; Gyllenhammar, 1987), luminol has been reported to react with O_2^-, H_2O_2, HOCl, $^{\cdot}OH$ and $^1O_2$ (Allen & Loose, 1976; Müller-Peddinghaus, 1984; Brestel, 1985; Jungi & Peterhans, 1988; Gyllenhammar, 1989).

Activated macrophages and neutrophils also release nitric oxide (NO) (McCall *et al.*, 1989; Stuehr *et al.*, 1989) upon induction of NO synthase (EC 1.14.13.39) activity with, e.g., bacterial lipopolysaccharides (LPS) and γ-interferon. NO is derived from the guanidino nitrogen of L-arginine, and its synthesis is specifically blocked by the L-arginine analogues N^G-nitro-L-arginine (L-NNA) and N^G-monomethyl-L-arginine (L-NMMA) (Hibbs *et al.*, 1987; Marletta *et al.*, 1988).

Here we report that luminol chemiluminescence in isolated Kupffer cells (liver macrophages) reflects the formation of NO by NO synthase, acting in concert with reactive oxygen species released by NADPH oxidase.

METHODS

Materials

Collagenase H, catalase, luminol (5-amino-2,3-dihydrophthalazine-1,4-dione), superoxide dismutase (SOD), RPMI 1640 medium and xanthine oxidase were purchased from Boehringer (Mannheim, Germany), phorbol 12-myristate 13-acetate (PMA; 'TPA'), D-arginine, L-arginine, L-NMMA, L-NNA, L-argininosuccinate, L-citrulline, lucigenin [10,10-dimethylbis(9,9-bisacridinium nitrate)], lipopolysaccharides (LPS), L-ornithine, urea and xanthine were from Sigma (Steinheim, Germany), NO was from Merck (Darmstadt, Germany) and fetal-calf serum from Gibco (Eggenstein, Germany).

Isolation and cultivation of rat Kupffer cells

Kupffer cells were isolated by collagenase perfusion of the liver from male Wistar rats weighing 180–220 g as described previously (Rymsa *et al.*, 1990). The liver cell suspension was freed of hepatocytes by 2-fold centrifugation at 50 *g* for 3 min. The supernatant was centrifuged at 300 *g* for 15 min. The cell pellet was resuspended in serum-free RPMI 1640 medium containing 2 mM-L-glutamine and gentamicin (0.1 mg/ml). The Kupffer cells were separated by their ability to adhere to uncoated plastic. They were kept in culture in RPMI 1640 medium supplemented with 20% (v/v) heat-inactivated fetal-calf serum in a humidified CO_2/air (1:19) atmosphere at 37 °C. The purity of the Kupffer-cell cultures was better than 98% as judged by positive peroxidase staining and phagocytosis of latex particles. LPS pretreatment of Kupffer cells was performed by addition of 0.5 μg of LPS/ml 12 h before starting chemiluminescent measurements.

Measurement of chemiluminescence

Luminol- and lucigenin-amplified chemiluminescence was measured in the instrument described by Cadenas & Sies (1984). Measurements with Kupffer cells were carried out at 37 °C 48 h after isolation of the cells. Before starting the experiments, the cells were washed three times with Hanks balanced salt solution, pH 7.4. Incubation [$(2–3) \times 10^5$ cells/culture tube] was performed in Krebs–Henseleit bicarbonate buffer, pH 7.4, supplemented with 10 mM-glucose and 20 mM-Hepes and equilibrated with CO_2/air (1:19). Luminol (50 μM), lucigenin (50 μM), PMA (2.5, 50 and 250 nM), L-arginine (0.1, 0.2, 0.5 and 2.0 mM), D-arginine (0.5 mM), L-argininosuccinate (0.5 mM), L-citrulline (0.5 mM), L-ornithine (0.5 mM), urea (0.5 mM) SOD (5 μg/ml) were added

Abbreviations used: L-NMMA, N^G-monomethyl-L-arginine; L-NNA, N^G-nitro-L-arginine; LPS, lipopolysaccharides; SOD, superoxide dismutase; PMA, phorbol 12-myristate 13-acetate ('TPA'); O_2^-, superoxide anion; $^{\cdot}OH$, hydroxyl radical; $ONOO^-$, peroxynitrite anion.

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where indicated. Luminol was dissolved in dimethyl sulphoxide (35 mM, final concn.); other compounds were dissolved in Hanks balanced salt solution. Viability of the cells was examined by counting the number of cells taking up Trypan Blue (Jauregui *et al.*, 1981). NO was determined by the oxyhaemoglobin method (Feelisch & Noack, 1987).

Chemiluminescence measurements in a cell-free system were performed at 37 °C in Krebs–Henseleit bicarbonate buffer, pH 7.4, supplemented with 20 mM-Hepes and equilibrated with CO_2/air (1:19). Luminol (50 μM), lucigenin (50 μM), NO (0.25, 0.5 and 1 μM), xanthine (150 μM), xanthine oxidase (1, 2 and 3 munits/ml), catalase (1 and 5 μg.ml) or SOD (1 and 5 μg/ml) were added where indicated. NO-equilibrated stock solutions (0.5–1.5 mM) were prepared by bubbling NO through double-distilled water for 25 min (Kelm & Schrader, 1990).

RESULTS

Luminol and lucigenin chemiluminescence in Kupffer cells

Kupffer cells in culture emitted only slight basal luminol chemiluminescence (Figs. 1*a* and 1*b*). Upon addition of PMA (2.5 nM), chemiluminescence markedly increased, reaching a maximum at about 15 min after the addition of the phorbol ester. Addition of 0.5 mM-L-arginine produced a pronounced further increase in chemiluminescence. The stimulation by L-arginine was independent of whether the amino acid was added at the maximum of PMA-induced chemiluminescence (Fig. 1*a*) or was included in the incubation medium before PMA (Fig. 1*b*). The stimulatory effect was almost maximal at 0.5 mM-L-arginine (half-maximal at about 0.2 mM). It was also present when the cells were stimulated by higher concentrations of PMA up to 250 nM (results not shown). Other urea-cycle metabolites, namely L-argininosuccinate, L-citrulline, L-ornithine and urea, as well as D-arginine, at concentrations of 0.5 mM, were without notable effect (results not shown).

PMA-stimulated luminol chemiluminescence was markedly inhibited by L-NNA and L-NMMA (Fig. 1*b*; Table 1). These inhibitory effects were already significant without added L-arginine, with 0.1 mM-L-NNA or L-NMMA inhibiting chemiluminescence by 40 and 43 % respectively (maximal effects). The inhibitory effects of L-NNA and L-NMMA were even more pronounced (67 and 64 % respectively) in the presence of 0.5 mM-L-arginine. Inhibition was partially (about 30 %) reversed by increasing the L-arginine concentration to 1.0 mM (Fig. 1*b*), suggesting a competitive mechanism of inhibition.

Lucigenin-amplified chemiluminescence was also stimulated by the addition of PMA; however, there were only slight changes induced by 0.5 mM-L-arginine, 0.1 mM-L-NMMA and 0.1 mM-L-NNA (Fig. 1*c*; Table 1). Lucigenin chemiluminescence was almost completely suppressed by SOD, indicating its specificity for O_2^- (results not shown).

In cells activated by pretreatment with LPS for 12 h, only very slight luminol or lucigenin chemiluminescence was measured, even after addition of 0.5 mM-L-arginine (results not shown). In the presence of 0.5 mM-L-arginine these cells released NO at a rate of about 12 nmol/h per 10^6 cells.

Luminol and lucigenin chemiluminescence in a cell-free system

Authentic NO (up to 1 μM) produced only slight luminol-dependent chemiluminescence (Table 2). Further supplementation with the O_2^-/H_2O_2-generating system xanthine/xanthine oxidase, however, resulted in the emission of significant chemiluminescence, which dramatically increased with increasing xanthine oxidase activity (Fig. 2*a*; Table 2). Addition of xanthine alone (150 μM; results not shown) or of xanthine/xanthine oxidase (150 μM; up to 3 munits/ml; Fig. 2*a*) without prior supplementation with NO produced no or only little luminol chemiluminescence. The NO–xanthine/xanthine oxidase-dependent luminol chemiluminescence was decreased by SOD (up to 71 %) and catalase (up to 34 %) and was almost undetectable in the presence of both SOD and catalase (91 % decrease; Table 2).

Authentic NO (up to 1 μM) also produced only slight lucigenin-dependent chemiluminescence (Table 2). It somewhat decreased lucigenin chemiluminescence emitted in the presence of xanthine/xanthine oxidase (Fig. 2*b*; Table 2).

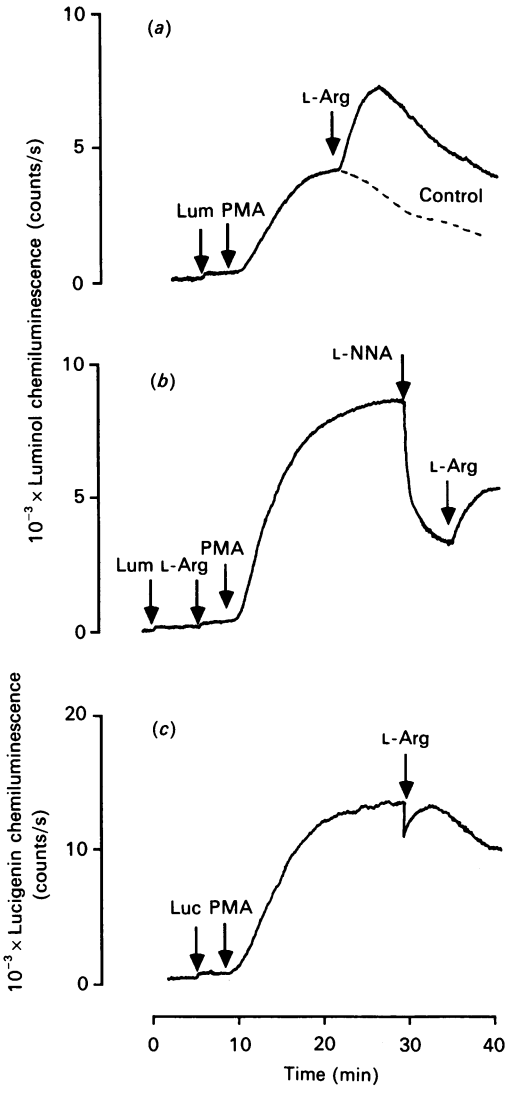

Fig. 1. Effect of L-arginine and L-NNA on luminol and lucigenin chemiluminescence in PMA-activated Kupffer cells

(*a*) Luminol (Lum; 50 μM), PMA (2.5 nM) and L-arginine (L-Arg; 0.5 mM) were added at the times indicated. 'Control' indicates the time course of chemiluminescence without addition of L-arginine. (*b*) Luminol (Lum; 50 μM), L-arginine (L-Arg, 0.5 mM) and PMA (2.5 nM) were first added, then L-NNA (0.1 mM). The inhibitory effect of L-NNA was partially reversed by 0.5 mM-L-arginine. (*c*) Lucigenin (Luc; 50 μM), PMA (2.5 nM) and L-arginine (L-Arg; 0.5 mM) were added at the times indicated.

DISCUSSION

The present work describes a novel effect of L-arginine metabolism on PMA-induced luminol chemiluminescence in Kupffer cells. Luminol chemiluminescence was markedly en-

hanced by the addition of L-arginine and significantly inhibited by L-NNA and L-NMMA (Figs. 1*a* and 1*b*; Table 1), competitive inhibitors of L-arginine metabolism by NO synthase. Even without supplementation with L-arginine, both inhibitors decreased luminol chemiluminescence by about 40%, and, in the presence of 0.5 mM-L-arginine, the percentage of inhibition increased to almost 70%. Thus it is concluded that a large part of luminol chemiluminescence in PMA-activated Kupffer cells is dependent on the catalytic activity of NO synthase.

The catalytic activity of NO synthase alone, however, is not sufficient to account for L-arginine-dependent luminol chemiluminescence. This is concluded from the observation that, in Kupffer cells activated by LPS instead of PMA, only very little luminol chemiluminescence was detectable, even in the presence of L-arginine. LPS-pretreated Kupffer cells, like other macrophages, form significant amounts of NO (Billiar *et al.*, 1989), whereas NADPH oxidase is not catalytically active (Birmelin *et al.*, 1986; Rellstab & Schaffner, 1989), as confirmed in our experiments on lucigenin chemiluminescence. Hence, products of the catalytic activity of both NO synthase and NADPH oxidase are simultaneously required to give rise to the L-arginine-dependent luminol chemiluminescence in PMA-activated Kupffer cells.

An interplay of NO and O_2^-/H_2O_2 in producing luminol chemiluminescence is also suggested by the (cell-free) experiments with authentic NO and the O_2^-/H_2O_2-generating system xanthine/xanthine oxidase (Fig. 2*a*; Table 2). Significant luminol chemiluminescence was only emitted when both NO and O_2^-/H_2O_2 were simultaneously present. The exact chemical

Table 1. Effects of L-arginine, L-NNA and L-NMMA on luminol and lucigenin chemiluminescence in PMA-activated Kupffer cells

Luminol (50 μM), lucigenin (50 μM) and L-arginine (0.5 mM) were added at the start of the experiments, whereas L-NNA (0.1 mM) and L-NMMA (0.1 mM) were added at the maximum of PMA-induced chemiluminescence. Results are means ± S.E.M. for four to six separate incubations of two or three preparations. The values in parentheses are percentages with respect to the control.

[L-Arginine] (mM)	Inhibitor	10^{-3} × Maximal chemiluminescence	
		Luminol	Lucigenin
–	–	4.2 ± 0.4 (100)	12.9 ± 1.8 (100)
–	L-NNA	2.5 ± 0.3 (60)	11.8 ± 2.0 (91)
–	L-NMMA	2.4 ± 0.2 (57)	12.3 ± 2.1 (95)
0.5	–	8.6 ± 0.7 (205)	12.7 ± 2.1 (98)
0.5	L-NNA	2.8 ± 0.4 (67)	12.8 ± 1.7 (99)
0.5	L-NMMA	3.1 ± 0.5 (74)	13.0 ± 1.8 (101)

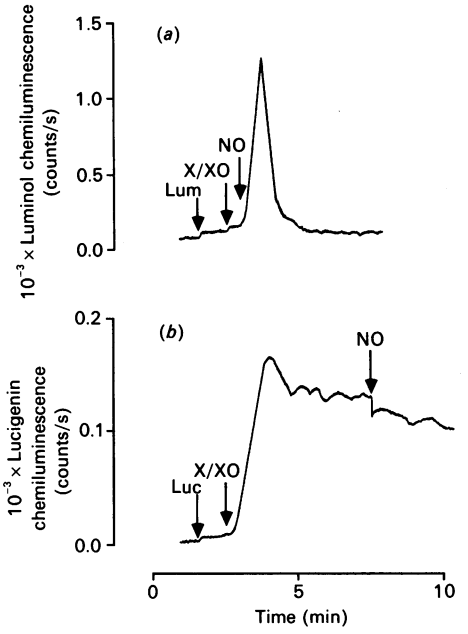

Fig. 2. Luminol and lucigenin chemiluminescence in a cell-free system consisting of authentic NO and xanthine/xanthine oxidase

(*a*) Luminol (Lum; 50 μM), xanthine/xanthine oxidase (X/XO; 150 μM; 3 munits/ml) and NO (0.5 μM), and (*b*) lucigenin (Luc; 50 μM), xanthine/xanthine oxidase (X/XO; 150 μM; 3 munits/ml) and NO (0.5 μM) were added at the times indicated.

Table 2. Dependence of luminol and lucigenin chemiluminescence on NO and xanthine/xanthine oxidase in a cell-free system: inhibitory effects of SOD and catalase

Xanthine (150 μM), luminol (50 μM), lucigenin (50 μM), xanthine oxidase, SOD and catalase were first added, then authentic NO. Data represent maximal levels (see Fig. 2). Results are means ± S.E.M. for three to five separate experiments. Abbreviation: n.d., not determined.

[NO] (μM)	Xanthine oxidase (munits/ml)	10^{-3} × Maximal chemiluminescence (counts/s)	
		Luminol	Lucigenin
0.25	–	0.02 ± 0.01	0.01 ± 0.01
0.5	–	0.04 ± 0.01	0.03 ± 0.01
1.0	–	0.06 ± 0.02	0.05 ± 0.02
–	3	0.07 ± 0.03	0.18 ± 0.03
0.5	1	0.23 ± 0.05	0.06 ± 0.03
0.5	2	0.56 ± 0.11	0.10 ± 0.02
0.5	3	1.27 ± 0.10	0.16 ± 0.03
0.5	3 plus SOD (1 μg/ml)	0.88 ± 0.06	0.09 ± 0.01
0.5	3 plus SOD (5 μg/ml)	0.37 ± 0.03	0.06 ± 0.04
0.5	3 plus catalase (1 μg/ml)	0.84 ± 0.27	n.d.
0.5	3 plus catalase (5 μg/ml)	0.84 ± 0.22	n.d.
0.5	3 plus SOD/catalase (5 μg/ml, each)	0.11 ± 0.04	n.d.

species giving rise to chemiluminescence will have to be analysed in detail. Since luminol chemiluminescence was significantly suppressed by SOD, a possible intermediate would be the peroxynitrite anion ($ONOO^-$), formed from O_2^- and NO (Blough & Zafiriou, 1985), known to decompose to strong oxidants [$^{\cdot}OH$ and nitrogen dioxide radical ($^{\cdot}NO_2$)] (Beckman *et al.*, 1990). Scavenging of O_2^- by NO to form $ONOO^-$ may also explain the decrease in lucigenin-dependent chemiluminescence observed after the addition of NO to the xanthine/xanthine oxidase system (Fig. 2*b*). On the other hand, the fact that NO–xanthine/xanthine oxidase-dependent luminol chemiluminescence was not only diminished by SOD, but also by catalase (Table 2), suggests an additional involvement of H_2O_2. One possibility would be that H_2O_2 participates as the second reactant (co-oxidant) in a sequential oxidation of luminol, as has been proposed for hypochlorite (HOCl) and H_2O_2 in producing luminol chemiluminescence in neutrophils (Lind *et al.*, 1983; Brestel, 1985).

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