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REACTIVE OXYGEN PRODUCTION ASSOCIATED WITH ARACHIDONIC ACID METABOLISM BY PERITONEAL MACROPHAGES

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Summary: The nature of the calcium-dependent chemiluminescence observed in peritoneal macrophages after exposure to the calcium ionophore A23187 or during the phagocytosis of zymosan has been investigated. Eicosatetraynoic acid, an inhibitor of the lipoxygenase and cyclooxygenase pathways of arachidonic acid metabolism, inhibited the calcium-dependent chemiluminescence whereas indomethacin, a selective inhibitor of the cyclooxygenase pathway, did not. Arachidonic acid induced chemiluminescence only in phagocytosing cells, whilst 15-HPETE, an intermediate of the lipoxygenase pathway, generated a similar, transient chemiluminescent response in either unstimulated or phagocytosing cells. The results suggest that the lipoxygenase pathway may be a significant source of the reactive species of oxygen that give rise to chemiluminescence. Prostaglandin E_1 inhibited the chemiluminescence induced by zymosan and A23187, but did not affect that generated in response to 15-HPETE or arachidonic acid, suggesting that the inhibition is directed at a step either connected with or occurring prior to the release of free arachidonic acid by the cells.

The phagocytosis of zymosan particles by macrophages is accompanied by the production of reactive oxygen species (1,2) that can be detected as chemiluminescence in the presence and absence of luminol (3). The species of oxygen produced (O_2^-, H_2O_2, $\cdot OH$ and perhaps ΔO_2) play an important role in the bacterial killing that occurs during phagocytosis (4,5). We have reported previously that two modes of chemiluminescence can be distinguished during phagocytosis: one, a glucose-dependent component that is only fully expressed when both Ca^{2+} and Mg^{2+} are present in the medium; and the other, a glucose-independent component that requires Ca^{2+} but not Mg^{2+} (6) and can be induced by the Ca^{2+} ionophore A23187 independently of phagocytosis (6,7). Macrophages also produce and release prostaglandins and thromboxanes after the addition of A23187 in the presence of Ca^{2+} (7) or in response to inflammatory (8,9) and phagocytic (10,11) stimuli. The formation of prostaglandin E_2 by the cells indicates that free arachidonic acid has been released from endogenous membrane-associated lipid pools as a result of the action of a phospholipase (Fig. 1; 12). Prostaglandins, particularly the E-type, are potential regulators of a

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variety of immune cell functions including lymphocyte proliferation (13-15), thymocyte differentiation (16,17) and the cellular interactions that result in inflammation (18); they may also act as negative feedback inhibitors of several macrophage functions, including chemiluminescence (19-22).

We have investigated the possibility that the Ca^{2+}-dependent chemiluminescence and prostaglandin production may arise from a common sequence of metabolic events. Both the cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism contain reactions where oxygen is incorporated into an intermediary metabolite and then released (Fig. 1). Both, therefore, have the potential to generate chemiluminescence.

Experimental: Thioglycollate-elicited peritoneal macrophages were harvested from 6-7 week old male CBA mice 4 days after intraperitoneal injection of 1.0 ml of Brewer's thioglycollate medium (23). The cells were resuspended in Eagle's minimal essential medium buffered with 20 mM Hepes, pH 7.2. Chemluminescence was measured in a Packard Tricarb liquid scintillation counter operating in the out-of-coincidence mode at 37°C. In all experiments, 10^6 macrophages were incubated in a total volume of 2.5 ml either in the absence or presence of luminol as indicated in the legends, and vials were preheated to 37°C and dark-adapted for at least 60 min in the counter. Emitted light was recorded continuously by a chart recorder connected to a rate-meter attached to the counter. In addition, counts per preset time interval were recorded by automatic printout. Indomethacin, luminol, soybean lipoxygenase and arachidonic acid were obtained from Sigma Chemical Co., St. Louis, Mo. Prostaglandin E_1 was a gift of Dr. J. Pike (Upjohn Co., Mich.). A23178 was provided by Dr. R. Hamill (Eli Lilly, Ind.) and eicosatetraynoic acid was a gift from Dr. B. Peskar (Dept. of Pharmacol., Univ. of Freiburg).

Results and Discussion

Ability of arachidonic acid to induce chemiluminescence. Only low, background levels of chemiluminescence were observed when arachidonic acid was added to unstimulated peritoneal macrophages. In contrast, a rapid increase in chemiluminescence occurred when arachidonic acid (30-40 μM) was added to macrophages actively phagocytosing zymosan (Fig. 2); even larger responses were obtained if sequential additions were made to the phagocytosing cells. An essentially similar result was obtained when either Ca^{2+} or glucose, or both, were omitted from the incubation medium. This result suggests that, of the pathways of arachidonic acid metabolism that become activated during phagocytosis (Fig. 1), one, at least, is capable of generating reactive oxygen metabolites.

Effect of metabolic inhibitors on chemiluminescence. Pretreatment of the cells with indomethacin, which blocks the cyclooxygenase pathway, either failed to inhibit chemiluminescence or, at low concentrations, had a stimulatory effect (Table 1). Eicosatetraynoic acid, however, which inhibits both the lipoxygenase and cyclooxygenase reactions (24), inhibited chemiluminescence due to phagocytosis by 25% and that due to A23187 by more than 70%. Preincubation of cells with arachidonic acid, on the other hand, had no significant effect on the

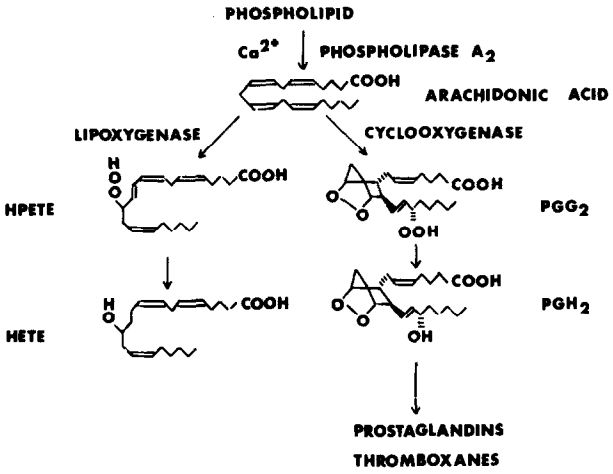

Fig. 1. Pathways of arachidonic acid metabolism, showing the major products formed from the action of two enzymes, cyclooxygenase and lipoxygenase, on arachidonic acid released from endogenous phospholipids.
PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; HPETE, hydroperoxyeicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid.

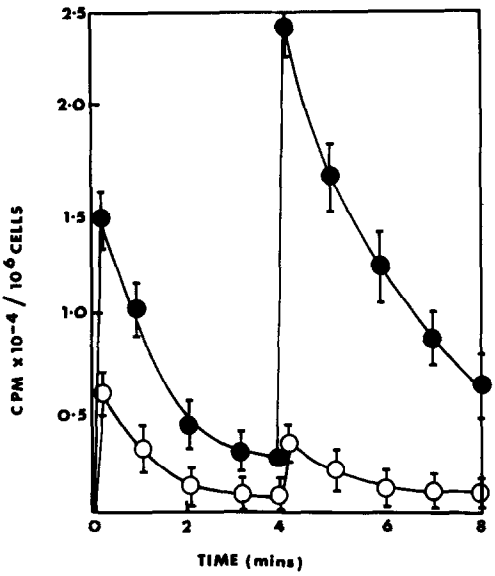

Fig. 2. Chemiluminescence observed after addition of arachidonic acid to mouse peritoneal macrophages. Cells (10^6/2.5 ml) were dark-adapted for 1 hr at 37°C in Eagle's buffer containing 5 mM glucose, 1.8 mM Ca^{2+} and 0.8 mM Mg^{2+}. Zymosan (0.4 mg/ml) was either present (●) or absent (○) for the last 40 min of the dark adaption period. At 0 and 4 min, arachidonic acid (40 μM) was added and chemiluminescence was recorded continuously for 8 min. No luminol was present. Counts represent the means ± S.E.M. from 4 separate experiments.

chemiluminescence subsequently evoked by either A23187 or zymosan. (The short-lived stimulation of chemiluminescence produced by arachidonic acid in the presence of zymosan was not seen in this experiment as arachidonic acid was added to resting cells and measurements of chemiluminescence were made at 5 min. intervals after zymosan addition). It is possible, therefore, that the lipoxygenase pathway of arachidonic acid metabolism is involved in the generation of chemiluminescence.

The effect of 15-HPETE addition to macrophages. This possibility was studied further by using 15-hydroperoxyeicosatetraenoic acid (15-HPETE), generated in vitro, as a substrate. The addition of this intermediate of the lipoxygenase pathway to non-phagocytosing or phagocytosing cells, but not to the complete medium without cells, produced a sharp peak of chemiluminescence that declined after 3-4 min (Fig. 3). A significant response was produced at concentrations as low as 5 μM. A control preparation, produced with boiled soybean lipoxygenase, failed to elicit chemiluminescence in non-phagocytosing cells. There

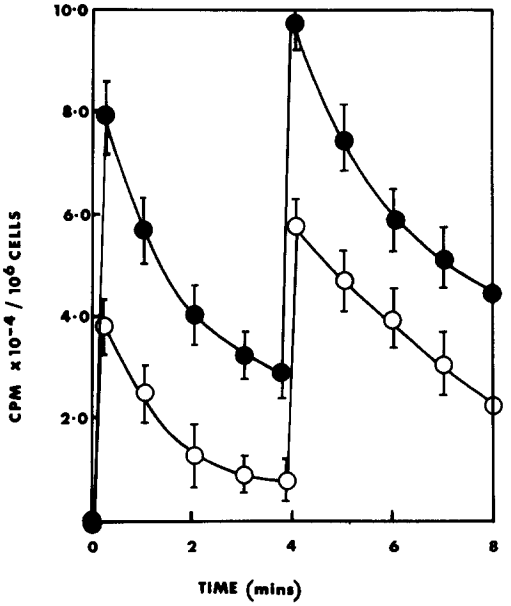

Fig. 3. Chemiluminescence observed after addition of 15-HPETE to mouse peritoneal macrophages. 15-HPETE was prepared by addition of soybean lipoxygenase (E.C. 1.13.1.13) to a sonicated suspension of arachidonic acid in 0.2M Tris/HCl buffer (38,39). The formation of the conjugated tetraenoic acid was monitored at 30°C by following the absorbance at 234 nm. Control incubations without enzyme or in the presence of boiled enzyme were carried out and monitored under identical conditions. For chemiluminescence experiments, conditions were as described in Fig. 2. At 0 and 4 min, 15-HPETE (40 μM) was added and counts were recorded for 8 min. Cells were preincubated in either the presence (●) or absence (○) of zymosan (0.4 mg/ml) for 40 min before HPETE addition. Counts represent the mean ± S.E.M. from 4 separate experiments.

Table 1: Effects of inhibitors and prostaglandin E_1 on chemiluminescence induced in mouse peritoneal macrophages.

Experiments were performed as described in Fig. 2 except that the Eagle's medium contained 0.05 ml of luminol-saturated foetal calf serum. Inhibitors were added 30 min prior to the addition of either A23187, zymosan or 15-HPETE. Chemiluminescence was followed continuously for 5 min in the A23187 and 15-HPETE experiments and for 60 min, at 5 min intervals, in the zymosan experiments. Prostaglandin E_1 was prepared in 70% ethanol and diluted in buffer; A23187, eicosatetraynoic acid and indomethacin were prepared as stock solutions in DMSO and diluted in buffer. Neither ethanol nor DMSO at these concentrations had an effect on chemiluminescence. Values represent the means ± S.E.M., with numbers of experiments in parenthesis. nt = not tested.

Addition	Concentration (μM)	% Chemiluminescence induced by:		
		A23187	Zymosan	15-HPETE
None	-	100	100	100
Eicosatetraynoic acid	30	25.3 ± 3.2 (4)	73.2 ± 2.0 (4)	nt
Arachidonic acid	30	91.1 ± 1.7 (4)	95.8 ± 3.6 (3)	nt
Indomethacin	1	133.2 ± 4.3 (3)	115.1 ± 6.7 (3)	nt
	30	95.1 ± 2.2 (3)	93.0 ± 9.4 (5)	nt
Prostaglandin E_1	20	20.3 ± 2.6 (4)	63.2 ± 3.1 (4)	96.3 ± 5.1 (3)

is a striking similarity between the short-term response of phagocytosing macrophages to arachidonic acid and their response to 15-HPETE: repeated additions of either substrate evoked chemiluminescent peaks that became larger than the initial response and both responses were produced independently of the presence of either glucose or Ca^{2+} in the medium.

Effect of prostaglandin E_1 on chemiluminescence. Of the chemiluminescence generated during phagocytosis, 40% was inhibited by prostaglandin E_1 whilst that produced by the addition of A23187 was blocked by 80% (Table 1). However, a similar concentration of prostaglandin E_1 had no significant effect on the chemiluminescence induced by 15-HPETE (Table 1).

These experiments make it possible to distinguish between the steps catalyzing the release and those catalyzing the metabolism of arachidonic acid as possible sites for the inhibitory action of the E-type prostaglandins on macrophage chemiluminescence. Inhibition appears to be exerted at a reaction occurring prior to the initiation of arachidonic acid metabolism. Because free arachidonic acid must be released from endogenous phospholipids following A23187 addition to account for the prostaglandin E_2 synthesis observed (7), it is this reaction, catalysed by a phospholipase, that is likely to be under the negative feedback control of the E-type prostaglandins. This view is supported by the observation that prostaglandin E_1 and adenosine 3',-5'-cyclic monophosphate (c-AMP) both inhibit membrane phospholipase activity in platelets (25,26). In peritoneal macrophages, c-AMP increases after the addition of prostaglandin E_1 (27,28). In addition, we have shown recently that, of a range of nucleotides examined, inhibition of A23187-induced chemiluminescence is relatively specific for prostaglandins of the E series and for butyryl derivatives of c-AMP (28).

The intermediates and products of the lipoxygenase pathway of arachidonic acid metabolism may participate in a range of immunological phenomena (29-36). Direct evidence for stimulation of this pathway in macrophages comes from the observation that the Slow Reacting Substance of Anaphylaxis (a product of the lipoxygenase pathway) is released from mononuclear cells after treatment with A23187 (37). We suggest here an additional functional role for this pathway in phagocytic cells: hydroperoxy intermediates of unsaturated fatty acids, which have the capacity to act as potential sources of highly-reactive free radicals, may be generated early in the phagocytic sequence and may play an important role in the generation of the cells' microbicidal activity. Modulation of this activity may be achieved through the action of prostaglandins of the E series.

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