Evidence for Two Sources of Arachidonic Acid for Oxidative Metabolism by Mouse Peritoneal Macrophages*

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The products of arachidonic acid oxidations by resident mouse peritoneal macrophages have been found to depend upon the nature of the stimulus. For example, soluble membrane-mediated inflammatory stimuli such as phorbol myristate acetate and lipopolysaccharide stimulated the formation of prostaglandin E$_2$ via the cyclooxygenase pathway. In contrast, zymosan, a particulate, phagocytoidal inflammatory mediator, stimulated leukotrienes C$_4$ and B$_4$, synthesis via the lipoxygenase pathway in addition to stimulating prostaglandin E$_2$ synthesis. Thus, the release of leukotrienes is not necessarily linked to the release of prostaglandins in a cell that has the enzymatic capability of producing both mediators. This suggests that the prostaglandin synthetase system can obtain substrate arachidonic acid from a source different from that for leukotriene synthesis.

The release of unesterified arachidonic acid from cellular phospholipids is considered to be the initial requirement for the synthesis and secretion of prostaglandins from intact cells (1). A similar mechanism is presumed to occur for the synthesis and secretion of leukotrienes. The membrane origin of phospholipid precursors, in juxtaposition to the endoplasmic reticulum containing the PG biosynthetic enzymes, may satisfactorily explain the availability of substrate AA for oxygenation by cyclooxygenase, the initial step in PG synthesis. However, since 5-lipoxygenase, the initial enzyme in the leukotriene pathway, is a soluble enzyme and thus presumably resides in the cytosol, it is not clear whether this enzyme utilizes AA from the same phospholipid source.

The mechanism by which the phospholipase produces unesterified AA for oxidation by the cyclooxygenase and 5-lipoxygenase, the initial enzymes in the prostaglandin and leukotriene pathways, has not been elucidated. In fact, there is not full agreement on which phospholipases are involved. Historically, phospholipase A$_2$ has been considered the enzyme involved in phospholipid hydrolysis (2). However, in platelets, the combined action of phospholipase C and a diacylglycerol lipase releases AA (3). It is of course possible that both pathways are operative in a given cell.

Mouse peritoneal macrophages have the capacity to synthesize and secrete AA oxidation products derived from both cyclooxygenase- and lipoxygenase-catalyzed pathways. We and others have reported that resident mouse peritoneal macrophages produce large amounts of PG$_E_2$ and 6-keto-PGF$_{1a}$, the stable product of PG$_E_2$, when exposed to phagocytic particles such as zymosan (4), soluble agents such as PMA (5), or lipopolysaccharide (6), as well as antigen-antibody complexes that interact with specific cell surface receptors (7). Recently, zymosan was shown to stimulate the production of a slow reacting substance of anaphylaxis from resident mouse peritoneal macrophages, as defined by assay on guinea pig ileum, having the chemical characteristics of LTC$_4$ (8). These cells contain two phospholipase A$_2$ activities, one with an optimal pH at 4.5 and a calcium-dependent enzyme with a pH optimum of 8.5 (9). In addition, a phospholipase C has also been identified (10). Despite the uncertainty of which phospholipase system releases AA, there is a general belief that the unesterified AA would serve as substrate for oxygenation by both the cyclooxygenase and lipoxygenase pathways. However, the present studies, which are the first to demonstrate that PGs can be released alone as well as in conjunction with LTs, suggest the existence of two independent sources of substrate AA.

EXPERIMENTAL PROCEDURES

Materials

Animals—Female CFW-1 mice (15–25 g) and male Hartley guinea pigs (400–500 g) were purchased from Charles River Laboratories, Wilmington, MA. The mice and guinea pigs were maintained on standard pellet diets and water ad libitum.

Chemicals—M-199 medium, other tissue culture reagents and HIPS were purchased from Grand Island Biological Co. The porcine serum was inactivated by heating at 56 °C for 30 min. Twelve-well tissue culture cluster plates (2.4 x 1.7 cm wells) were purchased from Linbro Division, Flow Laboratories. Zymosan was from ICN, K&K Laboratories, and was prepared as previously described (11). Latex beads (0.8 μm in diameter) were from Dow Chemicals.

At the termination of the experiments, the cells were washed with phosphate-buffered saline. One ml of 0.1% Triton X-100 in phosphate-buffered saline was added and the lysed cells were removed from the plate. Lactic dehydrogenase activity was determined in both the media and cell lysates by copper-neocuprine colorimetry coupled to the reduction of NAD (14). The protein content was determined by the method of Lowry et al. (13).

Labeling of Cellular Phospholipids with $[^3H]$Arachidonic Acid and the Assay for $[^3H]$PG$_E_2$ and $[^3H]$LTs, Synthesis and Secretion

The macrophages were incubated with 1.5 μCi of $[^3H]$AA in 1 ml of M-199 containing 1% HIPS. After 16–20 h, the radioactively labeled cells were washed twice with 2 ml of M-199 containing 1% HIPS and various additions were made in 1 ml of this tissue culture medium. After the incubation period, usually 3 h, the media were collected, buffered at pH 4.7 with 0.1 M sodium acetate, and extracted with two 3-ml aliquots of freshly distilled diethyl ether. The aqueous phases were then re-extracted with 3.75 ml of chloroform/methanol (1:2), 1.25 ml of chloroform, and 1.25 ml of water, as previously described (8). The ether phases were evaporated to dryness under a stream of nitrogen at 40 °C. The residues from the ether extractions were...
dissolved in 0.2 ml of ethyl acetate/methanol (3:1) and aliquots (usually 0.1 ml) were applied to lanes (1 x 20 cm) of a thin layer chromatography plate. Aliquots of the aqueous phase (usually 0.1 ml) were similarly spotted. The plates were developed with ethyl acetate/methanol/acetic acid (95:6:1), dried, then redeveloped in the same direction with hexane/ethyl ether/acetic acid (60:40:1). The origin (the cellulose spotting gel) containing the LTC4 from the chromatography of the aqueous phase and the PGE2 zones from the chromatogram of the ethyl ether extracts were removed and the radioactivity was determined in 8 ml of Aquasol with a Packard 3255 liquid scintillation spectrometer. The percentage of recovery of [3H]PGE2 and [3H]LTC4, (prepared by high pressure liquid chromatography from extracts of macrophage culture media) was 80 ± 7 and 69 ± 2 (n = 3), respectively.

High Pressure Liquid Chromatography Analysis for [3H]LTC4 Synthesis

Ethyl ether extracts were evaporated to dryness under nitrogen and chromatographed on two micro-Porfos columns (Waters Associates) connected in series. The eluting solvent was hexane/ethanol/acetic acid (95:5:0.1) pumped at a flow rate of 2 ml/min.

Radioimmunoassay for PGE and Muscle Contractile Assay for LTC4

In these experiments, 6 identical cultures/observation were prepared and incubated as described above except that [3H]AA was omitted. After 3 h, the media from duplicate cultures were removed, combined, and buffered at pH 4.7 with 0.1 M sodium acetate buffer. The acidified media were extracted with three successive 2-ml aliquots of ethyl ether. The ether phases were evaporated to dryness under nitrogen. The residues were dissolved in methanol and the PGE2 content was determined by radioimmunoassay as previously described (11, 16) without further purification. The extracted aqueous phases were combined (total volume = 6 ml) and concentrated to approximately 1-ml volumes with a Savant Speed-Vac concentrator. The volumes of the aqueous phases were precisely measured and the amount of LTC4 in the total sample was determined by a bioassay. The recovery of synthetic LTC4 through this procedure was greater than 90%.

Male Hartley guinea pigs weighing 400 to 500 g were killed by cervical dislocation followed by exsanguination. The ileum was excised and flushed free of its contents with cold modified Krebs-Ringer solution. Strips 2 cm in length were suspended in 10-ml tissue baths containing modified Krebs-Ringer solution with atropine sulfate and pyrilamine maleate, each at a final concentration of 1 x 10^-6 M. The tissue was washed at 3-min intervals throughout all experiments and was aerated with 96% oxygen and 4% carbon dioxide. The temperature was maintained at 37 °C with an Excal 100 circulator. Resting tension was adjusted to 1.0 g following a 20-min equilibration period.

The concentration of the slow reacting substance of anaphylaxis generated by mouse peritoneal macrophages was determined by a 2 + 2 dose bioassay (17). The concentration of LTD4, used for the standard curve was chosen to fall in the linear portion of its concentration-response curve. The volumes of samples used to obtain the test curve were determined to give approximately the same degree of response as was found in the standard curve. A total of 16 doses, administered in a Latin Square pattern, was used to determine the concentration of the slow reacting substance of anaphylaxis in a given sample. The shift in the log dose between the standard LTD4 curve and the sample test curve was read graphically and used to calculate the concentration of biologically active material in the sample.

RESULTS

The cultures of mouse peritoneal macrophages used in these studies have been shown both by morphological and functional criteria to contain greater than 96% mononuclear phagocytes. These cells were strongly adherent, highly phagocytic, possessed membrane Fc receptors and secreted lysozyme (11). The macrophage cultures used in these studies incorporated 65-75% of the [3H]AA. The predominantly labeled component was phosphatidylcholine with smaller amounts of label found in phosphatidylethanolamine and triglyceride (11). The ingestion of zymosan particles by the radiolabeled macrophages resulted in the synthesis and secretion of both [3H]LTC4 and [3H]PGE2 as shown in Fig. 1. In a similar manner, the divalent metal ionophore A-23187 stimulated the cellular production of both of these arachidonic acid oxygenation products in a dose-dependent manner. In contrast, the soluble agents LPS and PMA promoted exclusively the synthesis and secretion of [3H]PGE2 with no effect on the production of [3H]LTC4. Latex beads, 1 mg/ml, were readily phagocytized by the cells but did not promote the synthesis and secretion of either [3H]PGE2 or [3H]LTC4. Cell viability was not affected by these agents under these conditions as assessed by the retention of the cytoplasmic enzyme lactic dehydrogenase.

As shown in Fig. 2, the time course for both zymosan-stimulated [3H]LTC4 and [3H]PGE2 synthesis and release was linear for at least 2 h of incubation. PMA, 0.1 µM, did not stimulate [3H]LTC4 synthesis at any time during a 3-h incubation period, whereas the PMA-induced [3H]PGE2 synthesis was linear for 1 h. In addition, 0.1 µM PMA did not inhibit zymosan-induced synthesis of [3H]LTC4.

In order to ensure that the [3H]AA labeling of cellular phospholipids for measuring the release of tritium-labeled products was truly reflective of endogenous production, PGE2 was measured by radioimmunoassay and LTC4 was measured by its contractile activity on segments of guinea pig ileum. All four agents, zymosan, A-23187, LPS, and PMA, stimulated the synthesis and secretion of PGE2 (Table 1). In agreement with the radioactive labeling technique, zymosan and A-23187 also stimulated LTC4 synthesis. Furthermore, LPS and PMA...
Prostaglandin and Leukotriene Synthesis in Macrophages

The release of AA from cellular phospholipids is generally considered to be the rate-limiting step in cellular PG synthesis (2, 18). Therefore, it would be predicted that any agent that stimulates the release of substrate AA in a cell capable of synthesizing both PGs and LTs would cause the formation of products from both pathways. Although the present findings with zymosan and A-23187 would support this concept, the observation with PMA and LPS, which stimulate PGE2 but not LTC4 synthesis, requires the reevaluation of this single substrate-source concept. The alternate possibility that 5-lipoxygenase, unlike cyclooxygenase, requires activation for oxygenation of AA in intact cells cannot be excluded. However, studies by Hsueh et al. (19) in rabbit alveolar macrophages showing that the action of PMA is additive to maximal stimulatory doses of zymosan in affecting the release of PGs favors the concept that there are two sources of substrate AA.

The present observation that resident mouse peritoneal macrophages synthesize and secrete the potent chemoattractant LTB4 emphasizes the potential importance of the lipoxygenase pathway in the pathogenesis of inflammation, since this lipoxygenase product has been shown to cause the accumulation of polymorphonuclear leukocytes to an inflammatory site (20, 21). The coordinate induction pattern for LTC4 and LTB4 synthesis in response to the action of the inflammatory mediators studied, i.e. the ability of zymosan, but not of PMA or LPS, to stimulate leukotriene synthesis, further suggests a two-substrate AA concept, since the reaction of AA with 5-lipoxygenase yields 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid, the common precursor for both LTC4 and LTB4.

Cyclooxygenase, the initial enzyme resulting in PG synthesis, is associated with the endoplasmic reticulum (22, 23). In contrast, 5-lipoxygenase, the initial enzyme in the leukotriene pathway, is isolated from rat basophilic leukemia cells as a soluble enzyme (24). However, the exact cellular location within the macrophages has not been established. The finding of Hsueh et al. (19) that PMA is additive to the maximum doses of zymosan in stimulating the release of PGs from rabbit alveolar macrophages led to the suggestion that there are two phospholipases in the cell, one in the membrane and one in the lysosome. The ability of LPS and PMA to selectively stimulate PGE2, but not LTC4, synthesis and secretion suggests that these stimulants interact with the plasma mem-
brane to activate a phospholipase resulting in the release of AA for the selective oxidation by the cyclooxygenase residing in the endoplasmic reticulum. Both PMA and LPS have been shown to bind to detergent-solubilized fractions of chicken fibroblast and ovine erythrocyte membranes, respectively (25–27). The failure of PMA and LPS to promote leukotriene synthesis suggests that the AA released is at a site distal to the 5-lipoxygenase.

In contrast, zymosan particles are phagocytized and internalized into lysosomal-phagocytic vesicles within the cell, and a phospholipase A₂ activity with a pH optimum of 4.5 has been suggested to be lysosomal (9). Thus, a lysosomal phospholipase may release AA in the vicinity of both 5-lipoxygenase and cyclooxygenase. Clearly, the exact mechanism of the release of leukotrienes is not necessarily linked to the release of PGs.

The studies of this report provide the first evidence that the release of leukotrienes is not necessarily linked to the release of PGs in a cell that has the enzymatic capability of producing both mediators. Since products of the leukotriene pathway can have vastly different biological actions, and in some instances, opposite to those of the PG pathway, a common source of AA available for the formation of such a plethora of mediators is not in accord with their role as cell regulators. Finer control of cell function might be predicted to occur under conditions where the release of PGs is not necessarily associated with the release of leukotrienes.

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