Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 4349–4353, July 1983
Cell Biology

Inhibition by prostaglandins of leukotriene B_4 release from activated neutrophils

(chemotaxis/degranulation/superoxide/inflammation)

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Communicated by P. Roy Vagelos, March 31, 1983

ABSTRACT **Chemoattractant *N*-formylmethionylleucylphenylalanine (fMet-Leu-Phe) in the presence of cytochalasin B stimulates the release of leukotriene B_4 (LTB_4), superoxide (O_2^-), and *N*-acetylglucosaminidase from elicited rat peritoneal and human peripheral neutrophils [PMN (polymorphonuclear leukocytes)]. Prostaglandins E_1 and E_2 (PGE_1 and PGE_2) inhibit LTB_4 release from PMN in a dose-related manner with an IC_{50} of 1×10^{-8} M. This action is associated with increased levels of cyclic AMP. The inhibitory activity of a variety of PGs on LTB_4 production by rat peritoneal PMN parallels their affinity for PGE receptors in other tissues. O_2^- release is also suppressed by low levels of PGE_1 and PGE_2 in a dose-related manner and this inhibition is enhanced by theophylline. In contrast, lysosomal enzyme release is only minimally affected by physiological levels of PGs. These data are consistent with an action of PGs at the level of the PG receptor on LTB_4 and O_2^- release from the fMet-Leu-Phe-stimulated rat peritoneal PMN. In addition, the fMet-Leu-Phe-induced adherence of PMN to endothelial cells and inhibition of this phenomenon by PGs may now be explained by PG-mediated inhibition of LTB_4 formation.**

The influx and activation of neutrophils [PMN (polymorphonuclear leukocytes)] are essential for induction of inflammatory processes (1, 2). The chemoattractant *N*-formylmethionylleucylphenylalanine (fMet-Leu-Phe) and products of complement activation such as $C5_a$ recruit and activate the PMN to release active oxygen species and undergo degranulation (2, 3). Such actions in concert with increased levels of prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2) lead to the cardinal symptoms of acute inflammation (3–5). Leukotriene B_4 (LTB_4), a product of the 5-lipoxygenase pathway, is itself a potent chemoattractant for PMN (6) and promotes their accumulation and adherence to the vascular endothelium proximal to the area of inflammation (3, 7). Whether these actions of LTB_4 supplement or mediate the effects of other chemoattractants remains unknown.

Pharmacological levels of PGs inhibit superoxide (O_2^-) production induced by fMet-Leu-Phe in the presence of cytochalasin B (2) by an action purported to depend on increased cyclic AMP production (8). A similar cyclic AMP-dependent inhibition of lysosomal enzyme release has been observed by using large amounts of PGs (2, 8). Pharmacological levels of PGI_2 (2.8×10^{-6} M) have also been reported to cause a partial inhibition (45%) of LTB_4 release from human PMN activated with serum-treated zymosan (9).

The studies in this report indicate that fMet-Leu-Phe, in the presence of cytochalasin B, triggers not only the release of O_2^- and *N*-acetylglucosaminidase (NAGase) but also the release of endogenously formed LTB_4 from elicited rat peritoneal and human peripheral PMN. PGs affect these events differently. Physiological concentrations of PGE_1 and PGE_2 were found to totally suppress the release of LTB_4 and effectively inhibit O_2^- production, the latter particularly in the presence of theophylline, but minimally suppress lysosomal enzyme release, even in the presence of theophylline.

MATERIALS AND METHODS

Reagents. fMet-Leu-Phe, cytochalasin B, and *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide were obtained from Sigma; CPD-A1 human blood was from the Greater New York Blood Program (New Brunswick, NJ); and 250- to 350-g Sprague–Dawley rats were from Charles River Breeding Laboratories. LTB_4 [(5*S*,12*R*)-5,12-dihydroxy-6,8,10,14-icosatetraenoic acid] was obtained from J. Rokach (Merck Frosst) (10); MMM-I-135 (10,10-difluoro-13-dehydroprostacyclin) was a gift from J. Fried (University of Chicago) (11).

The incubation buffer contained 130 mM NaCl, 5.5 mM KCl, 0.6 mM Na_2HPO_4, 0.6 mM NaH_2PO_4, 10 mM glucose, 1.0 mM $CaCl_2$, and 25 mM Tris·HCl adjusted to pH 7.4 (12).

Preparation of Cells. Rat PMN were obtained by peritoneal lavage with 40 ml of 0.9% NaCl/10 mM EDTA, pH 7.0, 16–24 hr after aseptic induction (13). PMN were recovered by centrifugation and the pellet was washed once in calcium-free incubation buffer, subjected to lysis buffer (14) for 5 min, rewashed in incubation buffer, and resuspended at a concentration of 5×10^6 cells per ml.

Human peripheral PMN were isolated from fresh citrated human blood as described (15) and lysed as were the rat cells. Both preparations were >95% PMN.

Incubation Conditions. Two-milliliter aliquots of PMN suspension were preincubated with calcium for 3 min at 37°C and then PGs or solvent was added for 5 min. Cytochalasin B (5 μg/ml) was next added for 5 min, followed by addition of 10 μM fMet-Leu-Phe for 5 min. Dimethyl sulfoxide was at a final concentration of 0.7%. The incubations were terminated by centrifugation at $260 \times g$ for 5 min at 0°C. The supernatants were analyzed for (5*S*)-5-hydroxy-6,8,11,14-icosatetraenoic acid (5-HETE), LTB_4, and enzymes. Cell pellets were assayed for cyclic AMP.

NAGase. NAGase assays used a modification of the method

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Abbreviations: LTB_4, leukotriene B_4 (5*S*,12*R*)-5,12-dihydroxy-6,8,10,14-icosatetraenoic acid; PG, prostaglandin; PGI_2, prostacyclin; O_2^-, superoxide; NAGase, *N*-acetylglucosaminidase; fMet-Leu-Phe, *N*-formylmethionylleucylphenylalanine; PMN, neutrophils; 5-HETE, (5*S*)-5-hydroxy-6,8,11,14-icosatetraenoic acid; 5-HPETE, (5*S*)-5-hydroperoxy-6,8,11,14-icosatetraenoic acid; MMM-I-135, 10,10-difluoro-13-dehydroprostacyclin; AA, arachidonic acid; NSAID, nonsteroidal antiinflammatory agent.

of Woollen *et al.* (16). Briefly, 0.1 ml of 2.25 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in a citrate/NaH_2PO_4 buffer (pH 4.5) and 20 μl of supernatant were incubated for 3 hr at 37°C and 0.12 ml of 0.2 M glycine buffer (pH 10.0) was added to stop the reaction. Absorbance was measured at 405 nm. Approximately 15% of the NAGase contained in the PMN was released when treated with 10 μM fMet-Leu-Phe in the presence of cytochalasin B.

Determination of Cyclic AMP. Pelleted cells were resuspended in 1 ml of 10% trichloroacetic acid and were stored overnight at 4°C. After centrifugation the supernatants were extracted with diethyl ether and the aqueous phase was dried with nitrogen. Cyclic AMP content of the redissolved residues was determined by radioimmunoassay.

Determination of 5-HETE and LTB_4. The incubation media were acidified to pH 3.5 with HCl and were extracted with 4.5 ml of freshly distilled ether. The ether extracts were washed twice with 0.5 ml of water and dried with a nitrogen stream at 20°C and the residues were methylated with diazomethane in ether containing 8% methanol. The methyl esters were separated by using a Waters HPLC system monitoring 280-nm absorption (for LTB_4) and 229-nm absorption (for 5-HETE). Hexane/2-propanol, 91:9 (vol/vol), was pumped at 1.0 ml/min through two Waters μPorasil columns in series. Retention times were 9 min and 18 min for 5-HETE and LTB_4, respectively. Maximal fMet-Leu-Phe stimulation (mean ± SEM) released 78 ± 9 pmol of 5-HETE and 506 ± 30 pmol of LTB_4, respectively, from 1×10^7 PMN after correction for 75% recovery. Identification of the products was verified by combined gas chromatographic/mass spectral analyses of the trimethylsilyl derivatives and comparison with authentic standards.

RESULTS AND DISCUSSION

A survey of granulocytes from various sources revealed that the rat peritoneal PMN, elicited with casein (14), most consistently produced 5-HETE and LTB_4 in response to fMet-Leu-Phe stimulation. Comparisons of the effects of various stimulators (data not shown) on the release of 5-lipoxygenase products by the rat peritoneal cells showed the ionophore A23187 to be most effective. Phorbol myristate acetate was without effect. fMet-Leu-Phe in the presence of cytochalasin B, despite a smaller stimulation, was selected over the ionophore because of the more physiological nature of its response. fMet-Leu-Phe, a formylated peptide related to chemotactic products elaborated by bacteria, is known to bind to a specific receptor on the PMN (17). The obligatory requirement for the presence of cytochalasin B for maximal fMet-Leu-Phe-induced LTB_4 formation is in accord with a similar requirement for release of O_2^- (2) and degranulation products (18, 19).

The amounts of LTB_4 and 5-HETE liberated by PMN stimulated with fMet-Leu-Phe and cytochalasin B increased linearly with increasing cell density up to the maximum studied, 1×10^7 PMN per ml (data not shown). At 5 μg of cytochalasin B per ml, the amount of LTB_4 produced increased markedly with increasing fMet-Leu-Phe concentration up to 1 μM. Similarly, at 10 μM fMet-Leu-Phe, the amount of LTB_4 produced increased with cytochalasin B concentration until a plateau was reached at 5 μg/ml.

PGE_1 and PGE_2 are both potent inhibitors of the production of LTB_4 and 5-HETE by stimulated rat peritoneal PMN; dose–response relationships are shown in Fig. 1 *A* and *B*. A concentration of 5×10^{-7} M of either PGE_1 or PGE_2 completely suppresses the production of these arachidonic acid (AA)-derived metabolites. The IC_{50} for both PGE_1 and PGE_2 for the inhibition of LTB_4 and 5-HETE production (1×10^{-8} M) is in the physiological range and is similar to their dissociation constants from known PGE receptors (20–22). A comparison of the efficacies of various PGs in diminishing the release of 5-lipoxygenase products reveals a remarkable similarity to their affinities for the PGE receptor in the rat kidney (22). $PGE_1 \approx PGE_2 > PGA_1 \approx PGA_2 > PGF_{2\alpha} > PGF_{1\alpha} > PGD_2$ (Table 1). The significant inhibition by PGI_2 and more substantial inhibition by the stable PGI_2 mimic MMM-I-135 (11) may imply the coexistence of a PGI_2 receptor on the PMN. To ascertain the significance of the findings in rat for man, human peripheral PMN were examined in a similar manner. Both PGE_1 and PGE_2 inhibited LTB_4 and 5-HETE production totally, as with the rat peritoneal PMN; the IC_{50} values were in the range of 1×10^{-7} M (Fig. 1 *C* and *D*).

Actions of PGs, where studied, have been shown to be mediated via a membranous receptor coupled to adenylate cyclase (20, 21). The stimulatory effect of fMet-Leu-Phe on the human PMN is associated with a rapid rise in cyclic AMP levels (8). In our studies with rat peritoneal PMN, a time frame was selected that would permit the measurement of LTB_4 with the realization that such a burst of cyclic AMP would necessarily precede this event. Despite this fact, it was felt that total cyclic AMP levels, even at the 5-min interval used, would give a reasonable representation of the cyclic AMP increment, particularly when the metabolism of this nucleotide by cyclic AMP phosphodiesterase was inhibited by theophylline.

As shown in Table 2, theophylline increased cyclic AMP from a basal level of 4.6 to 7.6 pmol per 10^7 cells. PGE_1 had a similar effect, which was enhanced by theophylline. In each instance, no significant increase in LTB_4 was noted. fMet-Leu-Phe triggered only a 20% rise in cyclic AMP but increased LTB_4 from a nondetectable level to 351 pmol per 10^7 cells. Inclusion of theophylline in the fMet-Leu-Phe incubation resulted in a doubling of cyclic AMP over control values, but, in this instance, LTB_4 was depressed to a value that was one-half of that elicited by fMet-Leu-Phe alone. Low levels of PGE_1 and PGE_2 (data for PGE_2 not shown) caused dose-related inhibition of LTB_4 production and simultaneous increases in cyclic AMP levels; both phenomena were enhanced by theophylline. The action of theophylline alone to increase intracellular cyclic AMP levels and the concomitant depression in fMet-Leu-Phe-stimulated LTB_4 production is not easily rationalized, with current dogma associating the action of this peptide with increased cyclic AMP production (8). Either the stimulatory effect of the peptide on LTB_4 release must be dissociated from increased cyclic AMP levels or the cyclic AMP relevant to the fMet-Leu-Phe response must not be exposed to phosphodiesterase. In either event, it is clear that the action of PGE_1 alone and its enhancement by theophylline to increase cyclic AMP levels are associated with a decreased release of LTB_4. These findings support the earlier conclusion in this report implicating an action of PGs on LTB_4 production at the level of the PG receptor.

The inhibitory effects of PGs on fMet-Leu-Phe-induced NAGase release do not exceed 40% in either the rat peritoneal or human PMN (Fig. 1 *A–D*), nor did theophylline enhance NAGase release from the former cell source. In contrast, O_2^- release from rat peritoneal PMN is significantly inhibited by PGE_1 and PGE_2 (data for PGE_2 not shown) and this effect is markedly enhanced by theophylline (Table 2). Thus, as with LTB_4, the suppressive action of PGs on O_2^- may well be mediated at the level of the PG receptor. The actions of theophylline alone and in combination with PGE_1 to suppress O_2^- production parallel our studies with LTB_4 in that they are associated with increased levels of cyclic AMP.

The precise target of PGE_1-induced cyclic AMP action within the PMN is not known, although a cyclic AMP-induced kinase

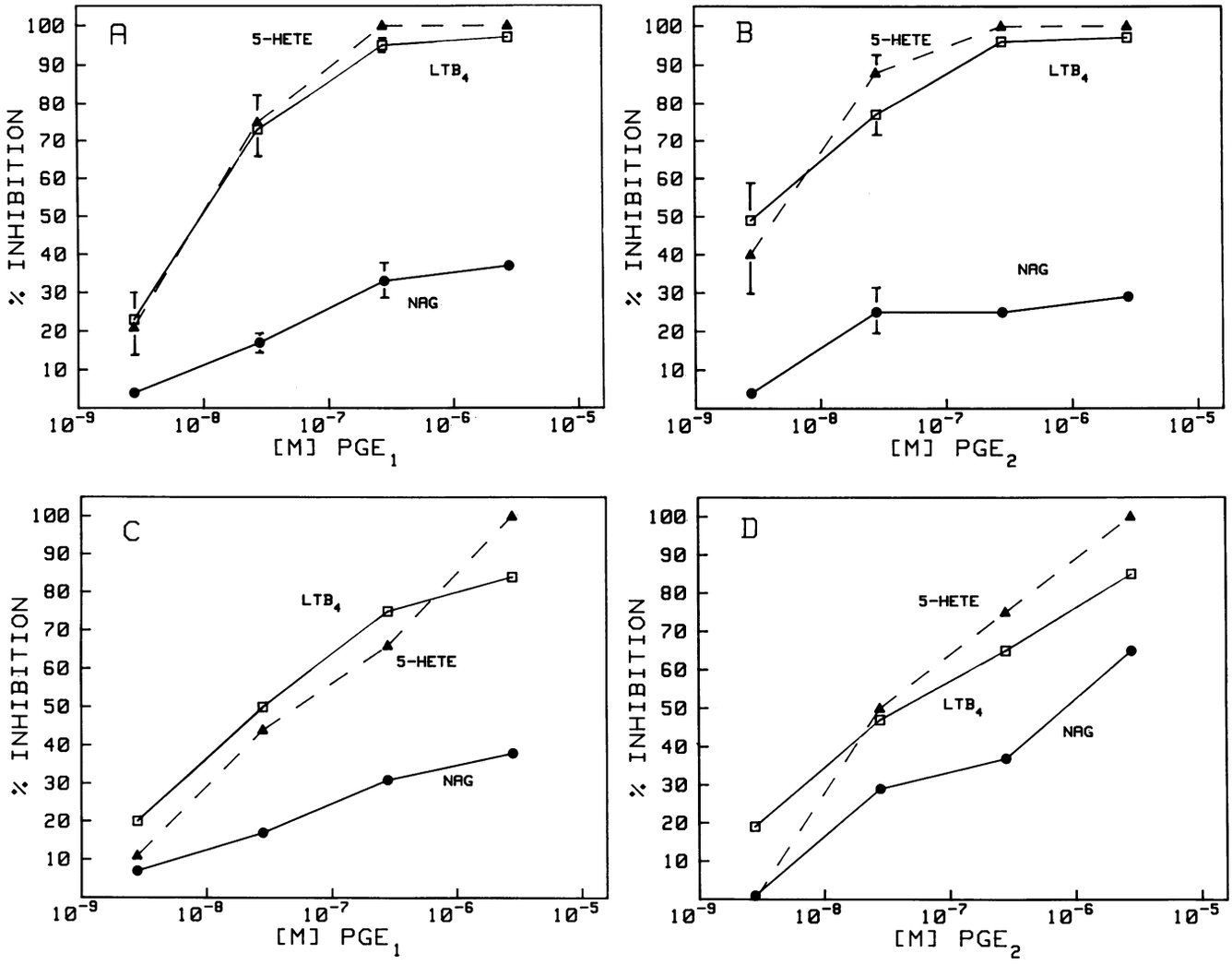

FIG. 1. Inhibition of fMet-Leu-Phe-induced release of LTB_4, 5-HETE, and NAGase (NAG) from rat peritoneal PMN by PGE_1 (*A*) and PGE_2 (*B*) and from human peripheral PMN by PGE_1 (*C*) and PGE_2 (*D*). The conditions are given in Table 1. The data with rat PMN are the result of 10 experiments; the vertical bars represent the standard errors. The data with human PMN are from one of two experiments.

step seems likely. Nevertheless, some insight into the enzymes involved is possible on the basis of available data. LTB_4 is the fourth product of the enzymatic sequence PL·AA $\xrightarrow{a}$ AA $\xrightarrow{b}$ 5-HPETE $\xrightarrow{c}$ LTA_4 $\xrightarrow{d}$ LTB_4 (PL is phospholipid). Inhibition of LTB_4 production alone does not distinguish between an action of PGs at step d or at earlier steps in this sequence. However, the data showing that inhibition of 5-HETE precisely parallel those of LTB_4 (Fig. 1) and the derivation of 5-HETE directly from 5-HPETE by peroxidatic reduction require one to focus on the initial rate-limiting steps (step a or b) as ultimate targets of PG action. 5-Lipoxygenase has been suggested to require stimulation for the production of 5-HETE and LTB_4 by human PMN, based on the requirement for ionophore A23187 for the incorporation of externally added AA into these products (24). However, one cannot exclude a requirement for prior incorporation of AA into phospholipid pools, which, in turn, must be activated for leukotriene formation. Thus, the suppressive action of PGs on LTB_4 synthesis may be at either the 5-lipoxygenase or the level of release of AA from phospholipid pools, or both.

There is a large body of evidence showing LTB_4 to be a potent chemoattractant for PMN and a factor in their adherence to the vascular endothelium (7). Unlike most cells, PMN are reported to be highly deficient in their ability to synthesize PGs (25). Thus, any *in vivo* actions of PGs on PMN, as suggested from the data in this report, would appear to require an extracellular source. Because an early step in the inception of acute

Table 1. Inhibition of fMet-Leu-Phe-induced LTB_4 release from PMN by PGs and the PGI_2 mimic MMM-I-135

PMN	IC_{50}, μM								
	PGE_2	PGE_1	PGA_1	PGA_2	PGI_2	$PGF_{2\alpha}$	$PGF_{1\alpha}$	PGD_2	MMM-I-135
Rat peritoneal	0.0051 (4)	0.0074 (7)	0.67 (2)	1.3 (2)	16 (2)	20 (2)	>70 (2)	>70 (2)	0.13 (2)
Human peripheral	0.043 (2)	0.086 (2)							

Incubations contained 5×10^6 PMN per ml of incubation buffer. The PMN were preincubated for 5 min with the PG, then incubated 5 min with 5 μg of cytochalasin B per ml, and then incubated 5 min with the stimulator, 10 μM fMet-Leu-Phe. The number of experiments is indicated in parenthesis.

Table 2. Relationship between cyclic AMP levels and mediator-induced events in the rat peritoneal PMN

Treatment	Without theophylline			With theophylline		
	cyclic AMP, pmol per 10^7 PMN	LTB_4, pmol per 10^7 PMN	O_2^-, nmol per 0.5 × 10^7 PMN	cyclic AMP, pmol per 10^7 PMN	LTB_4, pmol per 10^7 PMN	O_2^-, nmol per 0.5 × 10^7 PMN
Unstimulated	4.6	0	0	7.6	0	0
10 μM fMet-Leu-Phe	5.6	351	36.2	9.6	181	18.0
+ 2.8×10^{-9} M PGE_1	6.6	399 (0)	35.1 (3)	11.5	88 (51)	11.4 (37)
+ 2.8×10^{-8} M PGE_1	6.0	134 (62)	17.9 (51)	12.0	35 (81)	5.5 (69)
+ 2.8×10^{-7} M PGE_1	9.0	73 (79)	9.3 (74)	15.5	0 (100)	2.7 (85)
+ 2.8×10^{-6} M PGE_1	10.8	26 (93)	8.1 (78)	18.3	4 (98)	2.3 (87)
2.8×10^{-6} M PGE_1	7.8	0	0	15.5	0	0

Rat peritoneal PMN were preincubated for 5 min at 37°C with and without 0.5 mM theophylline. Subsequent incubation conditions are as described in the legend to Table 1. O_2^- measurements were obtained as follows in a separate experiment. These incubation mixtures, which contained 5×10^6 PMN per 0.5 ml, were prepared in the described buffer made with 15 mM Hepes (pH 7.4) instead of Tris·HCl. A preincubation with 2.5 μg of cytochalasin B for 5 min was followed by a 2-min incubation with various PG concentrations with and without 0.5 mM theophylline and the incubations were continued for 2 min with 10 μM fMet-Leu-Phe. O_2^- production was determined by spectrophotometric assay of O_2^- dismutase-inhibitable reduction of cytochrome *c* (23). These data are representative of five experiments. The % inhibition is indicated in parenthesis.

inflammation is the recruitment of the PMN and their adhesion to endothelial cells, the latter may well be the source of such PGs. The endothelial cell has a large capacity for producing PGE_2 and PGI_2. Boxer *et al.* have shown that fMet-Leu-Phe promotes the adherence of human peripheral PMN to nylon fibers (26). In a more recent report, these workers extended these studies by determining that adherence of activated human PMN to endothelial cells is suppressed by nanogram levels of PGI_2 (27). The known action of LTB_4 to promote adherence of PMN to endothelial cells (7), our finding that PGs depress LTB_4 production by PMN, and the observation by Boxer *et al.* (27) that PGI_2 suppresses PMN adherence to the endothelial cell argue for the concept that PGs produced in the vascular endothelium serve to suppress adhesion between the two cell types by inhibiting LTB_4 production by the PMN. The purpose of such an intercellular interaction could be either to serve as a negative feedback system or to facilitate the passage of the PMN through the blood vessel wall into the site of inflammation, or both, as shown schematically in Fig. 2. These findings with respect to LTB_4 and O_2^- also pose the possibility that PGs may have antiinflammatory actions in addition to their established pro-inflammatory properties, as suggested in earlier studies by others on the basis of the suppressive effects of PGE_1 on adjuvant arthritis (28–30).

There are a number of reports that nonsteroidal antiinflammatory agents (NSAIDs), acting by inhibiting PG synthesis, enhance or induce leukotriene-like responses (31, 32). In fact, there is a group of people who respond to aspirin and other such drugs with symptoms of asthma (33). Because AA is a substrate common to both pathways, this enhancement of leukotriene synthesis has been attributed to the shunting of substrate from the PG to the leukotriene pathway (31, 32). Nevertheless, in the

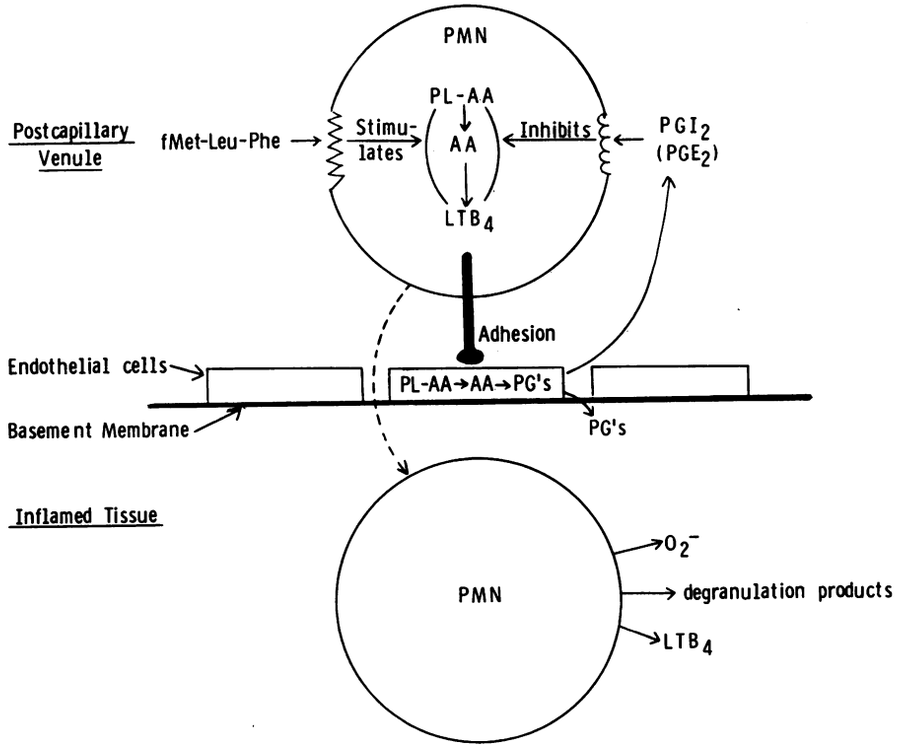

FIG. 2. Proposed interactions between PMN and endothelial cells. PL, phospholipid.

mouse peritoneal macrophage, a phagocytic cell in which both pathways prevail, no increased leukotriene accrues after NSAID action (34). Furthermore, we are not aware that added AA alone fosters a significant increase in synthesis of leukotrienes in the absence of a stimulator such as A23187. The findings in this report offer a viable alternative to the shunting concept. The action of NSAIDs in inhibiting cyclooxygenase would necessarily prevent the repressive action of PGs on LTB_4 production. As a consequence, LTB_4 synthesis would be able to proceed unimpaired. Because the action of PGs is at the rate-limiting step of leukotriene formation, other products, including LTC_4 and LTD_4, which have been implicated in asthma, would be expected to rise after NSAID treatment. The report that indomethacin causes a 5-fold increase in the production of slow-reacting substance of anaphylaxis (LTC_4 and LTD_4) by sensitized guinea pig lungs, whereas PGI_2 suppresses its release, suggests that the cells present in the lung responsible for the production of slow-reacting substance of anaphylaxis are similarly regulated by PGs (35). The possibility that other circulating leukocytes, which have the capacity for yielding lipoxygenase-derived products, are also controlled by PGs is implied by studies showing that adhesion of eosinophils and basophils as well as PMN to glass capillaries is inhibited by PGs (36).

We thank M. S. Springer for helpful suggestions and J. M. Liesch for gas chromatographic/mass spectral analyses. We also thank E. H. Cordes, A. S. Rosenthal, and A. W. Ford-Hutchinson for their interest and helpful discussions.

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