Neutropenia Induced by Systemic Infusion of 5,12-Dihydroxy-6,8,10,14-eicosatetraenoic Acid

CORRELATION WITH ITS IN VITRO EFFECTS UPON NEUTROPHILS

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ABSTRACT 5(S), 12(S)-Dihydroxy-*cis*-14,*trans*-6,8,10-eicosatetraenoate (compound I), 5(S),12(R)-dihydroxy-*cis*-14,*trans*-6,8,10-eicosatetraenoate (compound II), and 5(S),12(R)-dihydroxy-*cis*-6,14,*trans*-8,10-eicosatetraenoate (compound III) were prepared from rabbit peritoneal neutrophils challenged with arachidonic acid plus ionophore A23187. Each arachidonate metabolite caused rabbit neutrophils to aggregate and, in cells treated with cytochalasin B, release granule-bound enzymes. Compound III was 10- to 100-fold more potent than compounds II and I. When intravenously infused into rabbits at doses of 100–1,000 ng/kg, compound III induced abrupt, profound, transient neutropenia associated with a rapidly reversing accumulation of neutrophils in the pulmonary circulation. This in vivo action correlated closely with the ability of the fatty acid to activate neutrophils in vitro: neutropenia, aggregation, and degranulation occurred at similar doses of stimulus and the rapid, reversing kinetics of the neutropenic response paralleled the equally rapid, reversing formation of aggregates. The fatty acid did not alter the circulating levels of lymphocytes or platelets and did not aggregate platelets in vitro. At comparable doses (i.e., 100–1,000 ng/kg), compounds I and II did not cause neutropenia. Thus, compound III possesses a high degree of structural and target-cell specificity in stimulating neutrophils in vitro and in vivo. Clinical and experimental syndromes associating neutropenia with increased levels of circulating arachidonate metabolites may involve compound III as a mediator of neutrophil sequestration in lung.

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INTRODUCTION

Polymorphonuclear neutrophils (PMN)[1] disappear from the peripheral circulation during septicemia, endotoxemia, and anaphylaxis (1, 2). Many of these cells are lodged in the microvasculature of lung, liver, or other vital organs (3, 4). Investigations in several laboratories suggest how this effect is mediated. C5a, a polypeptide generated during complement activation (complement is activated in septicemia/endotoxemia [5]); *N*-formylmethionyl-leucyl-phenylalanine (FMLP), an analogue of factors released by growing bacteria (6); and platelet activating factor (PAF), a phospholipid released into the blood of animals during anaphylaxis (7), aggregate and degranulate PMN in vitro and, when injected into rabbits, produce neutropenia (7–10). Neutropenia appears closely related to aggregation: both responses develop at comparable doses of stimulus, become maximal within 0.5–3 min, and reverse within 1–5 min. Furthermore, a fourth degranulating agent, phorbol myristate acetate, also aggregates PMN and causes neutropenia. In contrast to the other stimuli, however, phorbol myristate acetate causes irreversible aggregation and sustained (>3 h) neutropenia (11). These correlations suggest that the respective in vitro and in vivo actions of the stimuli have a similar basis; i.e., they induce surface membrane hyperadhesiveness and/or aggregation in circulating PMN. The hyperadherent or aggregated cells marginate to capillary endothelium or clump in capillary networks. In either event, the PMN sequester and are lost from the cir-

[1] *Abbreviations used in this paper*: 5,12-diHETE, 5,12-dihydroxy-6,8,10,14-eicosatetraenoate; FMLP, *N*-formylmethionyl-leucyl-phenylalanine; LPP, large particle percentage; PAF, platelet-activating factor; PMN, polymorphonuclear neutrophil.

culation (7–11). Endogenously formed C5a and PAF or FMLP-like factors released by bacteria may cause the neutropenia seen in various clinical syndromes such as endotoxemia and anaphylaxis.

PMN aggregation and degranulation responses appear mediated by arachidonate metabolism: C5a, PAF, and FMLP cause PMN to metabolize their stores of arachidonate (12–16); arachidonate antimetabolites block PMN responses to these stimuli (14, 16, 17); and certain arachidonate metabolites, i.e., 5,12-dihydroxy-6,8,10,14-eicosatetraenoates (5, 12-diHETE), formed by stimulated PMN are themselves powerful aggregating and degranulating agents (15, 18–22). Ultimately, then, a naturally derived arachidonate derivative may be involved in C5a-, PAF-, or FMLP-induced neutropenia as well as the neutropenia occurring in endotoxemia, anaphylaxis, and other syndromes that associate PMN sequestration with increased blood levels of arachidonate metabolites (23, 24). While this manuscript was in preparation, a report showed that 1 μg/kg of a 5,12-diHETE caused neutropenia in rabbits (25). Here we find a similar result. Furthermore, we show that this bioaction is dose-related, target-cell specific, stereospecific for one 5,12-diHETE isomer, closely related to the in vitro bioactions of the fatty acid, and predominately due to an accumulation of PMN in pulmonary vasculature.

METHODS

Lipid preparations. The diHETE (i.e., compounds I, II, and III) were purified by a modification of previously described procedures (18–22). Briefly, 2×10^9 rabbit peritoneal PMN in 100 ml of 37°C modified Hanks' buffer (10) containing 1.4 mM calcium chloride and no magnesium were incubated with 10 μM A23187 and 110 μM arachidonic acid (which we purified to >99% purity from commercial supplies [Sigma Chemical Co., St. Louis, MO]) for 4 min. The cells were extracted and the residue of this extract applied to a heat-activated (130°C for 3 h) silicic acid column. The column was sequentially eluted as described (22). The partially purified residue from the ethyl acetate eluent of this column (22) was applied to a 30 × 0.78-cm μ-Bondapak C_{18} Water Associates (Milford, MA) column and eluted with methanol:water:acetic acid (75:25:0.01, vol/vol/vol) at 4 ml/min. Monitored at 280 nm, three poorly resolved major peaks, I, II, and III, eluted at ~6.4, 6.8, and 7.3 min, respectively. To improve resolution, the peaks were pooled, reapplied to the column, and eluted with a more polar solvent system (methanol/water/acetic acid; 69:31:0.01; vol/vol/vol). Under these conditions, each peak resolved from one another (elution times 21, 23, and 25 min, respectively) and appeared >95% pure as judged by the absence of contaminants when rechromatographed. Three ultraviolet maxima (performed at 25°C on samples dissolved in 100% ethanol with a Cary 219 UV-vis spectrometer [Varian Associates, Inc., Palo Alto, CA]) were obtained for each compound. These maxima were at 259, 269, and 281 nm for compound I; 260, 270, and 281 nm for compound II; and 261, 271, and 282 nm for compound III. After treatment with diazomethane, platinum oxide/hydrogen, and *bis*-trimethylsilylimidazole/triethylamine, compound I gave characteristic ion fragments at m/e 299, 215, and 203 when subjected to mass spectroscopy (generously performed by Dr. M. M. Bursey, University of North Carolina, Chapel Hill, NC on a DuPont model 21-492B mass spectrometer) [DuPont Instruments, Wilmington, DE]. These data indicate that compound I is a 5,12-dihydroxyeicosanoate (21). Quantification of each 5,12-diHETE was performed by measuring absorbancy at 280 nm with a molecular extinction coefficient of 37,400, as described (21, 26).

Bioassays. In vitro assays were performed on PMN suspended in a modified Hanks' balanced salt solution or on platelets suspended in a Tyrode's solution (10). PMN aggregation was performed with a Coulter Counter (Coulter Electronics Inc., Hialeah, FL) as described (10). Results are reported as either the large particle percentage (LPP) (i.e., percentage of all enumerated particles that are aggregated) or the maximal change in the LPP (i.e., the greatest LPP found 1/4, 1/2, 1, 2, 4, or 8 min after stimulation minus the LPP found just before stimulation). Degranulation of cytochalasin B-treated PMN was performed as described (10), and is reported as percentage of total cellular enzyme released. Rabbit platelets were isolated from blood and aggregated as described (10) using a Chrono-Log Aggregometer (Chrono-Log Corp., Havertown, PA). Cytopenia was studied in albino rabbits weighing 2–3 kg by injecting 0.5 ml of a test substance into catheterized femoral veins over a 0.25-min period and analyzing catheterized femoral artery blood (0.5 ml) for cell counts as described (9). Stasis of PMN in pulmonary vessels was measured by placing catheters in the aorta and vena cava via the femoral vessels and analyzing the PMN counts obtained from blood withdrawn from the two catheters simultaneously. Results are presented as the pulmonary clearance of PMN(C): $C = (V - A/V) \times 100$, where V and A are the respective vena caval and aortic PMN concentrations. This previously used formulation indexes pulmonary accumulation ($C > 0$) or release ($C < 0$) of PMN (27).

RESULTS

Each 5,12-diHETE, i.e., compounds I, II, and III, degranulated cytochalasin B-treated PMN. 5,12-diHETE are known (15, 20) to degranulate human and guinea pig PMN, inducing net release of 20% lysozyme and β-glucuronidase at optimally active dosages (~1–10 μM). We found, however, that compound III was much more potent than this in degranulating rabbit PMN: at optimal doses (~40–100 nM) the compound caused release of 70% lysozyme and 60% β-glucuronidase (Fig. 1). Compounds I and II, isomers of III, were 10- to 100-fold less potent degranulating agents (Fig. 1). Two earlier reports (18, 19) found that 5,12-diHETE, in concentrations as low as 1 nM, caused rat and human PMN to aggregate. We found that they also aggregated rabbit PMN and were active in doses between 3 and 100 nM; moreover, compounds I and II were 10- to 100-fold less active than compound III (Fig. 2, inset). The response to each compound developed rapidly and began to reverse within 0.5 min (Fig. 2).

Based on the consistent association between the aggregating and neutropenia-inducing activities of other

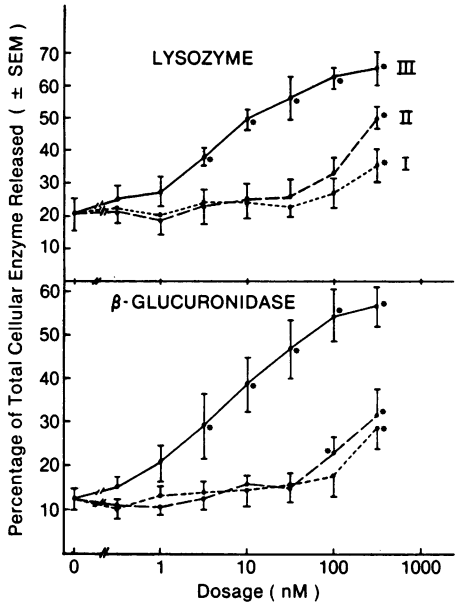

FIGURE 1 Release of lysozyme and β-glucuronidase by rabbit peritoneal neutrophils exposed to varying dosages of compounds I, II, or III. Cells were incubated in modified Hanks' buffer containing 1.4 mM calcium and 0.7 mM magnesium. They were challenged with 5 μg/ml cytochalasin B plus a stimulus for 5 min. Asterisks indicate values significantly higher than background release.

stimuli (8–10), the above data suggest that the compounds may cause transient neutropenia in vivo. Indeed, when infused into rabbits at 100–1,000 ng/kg, compound III significantly depressed PMN counts; at the 1,000-ng/kg dosage it caused virtually all PMN to disappear briefly from the circulation (Fig. 3). At all dosages its actions were transient and began to reverse within 1–3 min. However, the compound caused identical responses when repeatedly injected at 15-min intervals into one rabbit, which indicates that circulating PMN respond fully to the stimulus shortly after a previous neutropenic response reverses (data not shown). The 5,12-diHETE did not influence lymphocyte or platelet levels (Table I). Correlating with this lack of effect on circulating platelets, compound III did not aggregate rabbit platelets in vitro in doses as high as 1 μM (data not shown). Animals infused with any dosage of this diHETE did not show alterations in respiratory rate, patterns of respiration, or heart rate: they appeared well and had no overt signs of cardiorespiratory distress.

To determine where (e.g., pulmonary vs. systemic circulation) PMN are during the neutropenia, vena caval and aortic blood were simultaneously assayed for PMN counts after infusing compound III (750 ng/kg). Fig. 4 shows these data. For 1 min after infusion, ve-

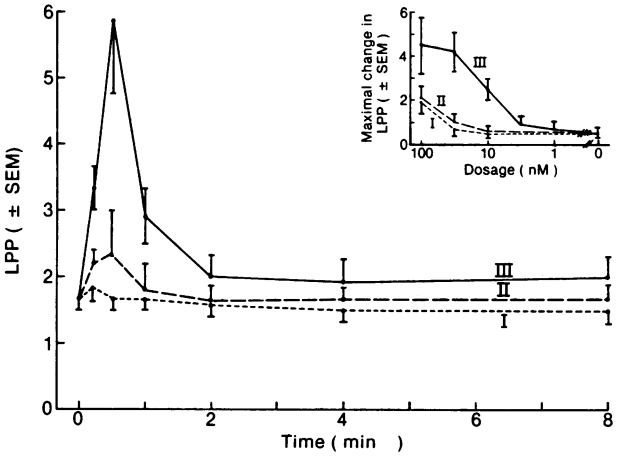

FIGURE 2 Aggregation response (LPP) of rabbit peritoneal neutrophils at varying times after challenge with 100 nM compounds I, II, or III. The inset shows the maximal change in the LPP induced by varying dosages of these compounds. Cells were incubated in modified Hanks' buffer containing 1.4 mM calcium and 0.7 mM magnesium for 5 min before challenge.

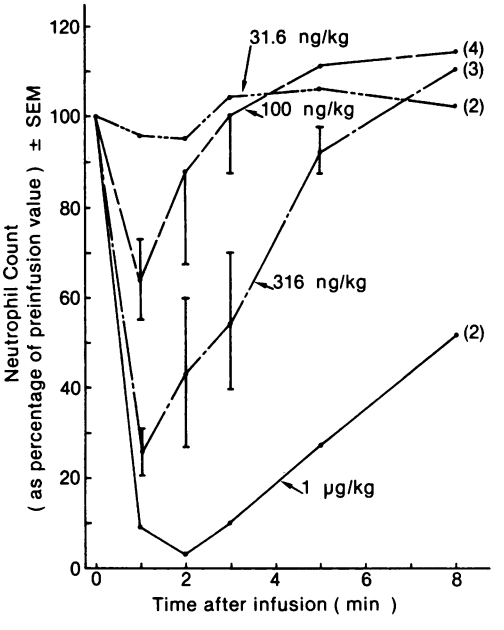

FIGURE 3 Neutrophil counts (as percentage of preinfusion value) in femoral arteries of rabbits after intravenous injection of varying amounts of compound III. Numbers in parentheses are the number of animals injected. The abcissa indicates time after the onset of a 15-s injection. The 1-, 2-, and 3-min postinjection values for the 316-ng/kg dose and the 1-min postinjection value for the 100-ng/kg dose are significantly ($P < 0.05$, Students' t test) lower than preinjection values or similarly timed values for animals injected with buffer. Statistical analyses were not performed on the values for the 1-μg/kg dose.

TABLE I

*Neutrophil, Lymphocyte, and Platelet Counts 2 min after Beginning a 15-s Injection of Compounds I, II, and III**

Compound injected‡	Cell type		
	Neutrophil	Lymphocyte	Platelet
I (3)	97.8±4.9§	106±5.1	96.9±10.5
II (3)	100.2±6.1	111±6.8	94.1±10.9
III (2)	3	107	104

* Albino rabbits were injected intravenously with 1 μg/kg of an indicated substance and arterial blood was sampled at 1, 2, 3, 5, 8, and 15 min thereafter. Compounds I and II did not produce significant cytopenia and compound III did not depress lymphocyte or platelet counts at any time after infusion: these recorded 2-min postinjection values are typical of the values obtained at all sampled times. Compound III produced neutropenia as shown in Fig. 2.

‡ Numbers in parenthesis are the number of rabbits infused.

§ Cell count, as percentage of preinfusion value, ±SEM.

nous counts outnumbered arterial (upper panel), lung clearance (see Methods) of PMN rose to high positive values (lower panel), and, therefore, more PMN entered than exited the lung. Conversely, at 2 and 3 min postinfusion arterial PMN outnumbered venous, lung clearance fell to low negative values, and, therefore, more cells exited than entered the lung. Thereafter, all parameters normalized (Fig. 4). These data show that lung first accumulates and then releases PMN in rapid succession. The timing of these events strongly

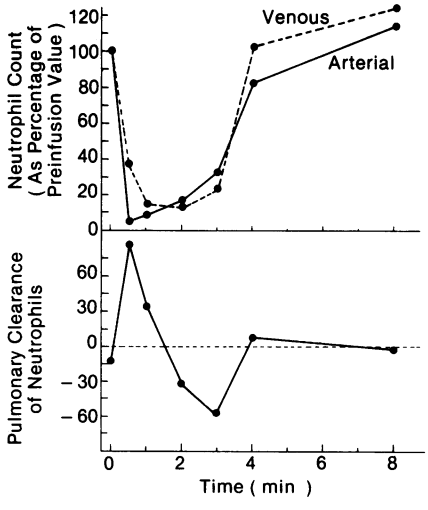

FIGURE 4 Upper panel: Neutrophil counts (as percentage of preinfusion values) in the vena cava and aorta of a rabbit injected with 750 ng/kg of compound III. Lower panel: Clearance of neutrophils by lung as determined with the data from the upper panel (see Methods). The abcissa gives time after injection.

indicate that the fall and rise in circulating PMN levels are due to transient stasis of these cells in pulmonary vasculature. Similar results and conclusions occur using C5a (27).

In contrast to compound III, compound I and II, in dosages as high as 1 μg/kg, did not influence the levels of circulating blood cells (Table I). However, these two compounds were much less effective in activating PMN in vitro (Figs. 1 and 2). On the basis of extrapolation from their relative potencies in vitro, >3 μg/kg of compounds I or II would have been necessary to cause appreciable depression of the PMN counts in vivo. These quantities of the two compounds were unavailable.

DISCUSSION

We prepared the 5,12-diHETE from rabbit PMN by established procedures (21, 22). With these procedures the first two 5,12-diHETE eluting from the C-18 μ-Bondapak reverse-phase column are a pair of diasteriomeric diols, 5(S), 12(S)-dihydroxy-*cis*-14,*trans*-6,8,10-eicosatetraenoic acid and 5(S),12(R)-dihydroxy-*cis*-14,*trans*-6,8,10-eicosatetraenoic acid. The third and major component eluting from the column is a 5,12-diHETE containing two *cis* and two *trans* double bonds (21, 22). Further studies have identified this compound as 5(S),12(R)-dihydroxy-*cis*-6,14,*trans*-8,10-eicosatetraenoic acid (25). The following considerations imply that compounds I, II, and III are these three respective isomers. First, their retention volumes and quantitative yield off of the C-18 μ-Bondapak column were very similar to those reported (21). Second, their ultraviolet absorbancy spectra are virtually identical to previous reports (19, 21, 25). And, finally, mass spectroscopy of compound I confirmed that it was a 5,12-dihydroxyeicosanoate. There is little doubt, therefore, that the three compounds are the isomers reported by others (19, 21, 25).

Compound III is a strikingly potent inducer of neutropenia (Fig. 4). This in vivo action of the agent correlated closely with its ability to stimulate PMN in vitro. Thus, if one assumes a rabbit plasma volume of 65 ml/kg and no extravasation of injected material, compound III equilibrates at plasma levels of 4, 14, and 40 nM after infusion of 100, 316, and 1,000 ng/kg, respectively, and, by extrapolation, depresses PMN counts by 50% at a plasma level of 10 nM. In vitro, the fatty acid caused increasing effects between 4 and 40 nM (Figs. 2 and 3) and induced half-maximal responses at 10 nM. Furthermore, compound III caused aggregation (Fig. 3) and neutropenic (Fig. 3) responses that developed rapidly and reversed within 0.5–3 min. Thus, as suggested for other PMN stimuli that induce

virtually identical aggregation and neutropenic responses (e.g., C5a, FMLP, and PAF) (7–11), compound III may cause neutropenia by aggregating and/or augmenting endothelial adhesion of circulating PMN, although other possibilities could explain the in vivo response. For instance, in hamsters, compound III causes circulating PMN to attach (individually) to capillary endothelium (28) and (in in vitro studies exclusively) C5a induces cultured endothelial cells to attach PMN (29). Conceivably, then, the neutropenic effects of compound III, as well as other PMN stimuli, could be due to primary actions on endothelium. However, the in vitro endothelial studies were performed only after 0.5 h of PMN-endothelial cell incubation: the kinetics of this response appear quite different that those for the in vivo response (29). Furthermore, PMN harvested from C5a-induced neutropenic animals are hyperadherent to foreign surfaces (27, 30). Finally, as outlined in the Introduction, this theory ignores the strikingly close association between in vitro PMN aggregation and in vivo neutropenia with respect to the wide range of stimuli effecting both responses, their dose–response relations, and their similar kinetics. It seems much more likely, therefore, that PMN stimuli such as compound III directly cause circulating PMN to adhere to each other, other blood cells, or endothelium. Further studies are needed to establish this point.

Neutropenia induced by compound III developed concomitantly with an influx of PMN into lung (Fig. 4). This influx was selective for PMN (data not shown) and was probably the dominant contributor to the ensuing neutropenia, i.e., the previously circulating PMN accumulated in pulmonary vasculature selectively. However, these data do not exclude a similar but quantitatively less significant accumulation of cells in the systemic circulation. Reversal of the neutropenia occurred concomitantly with PMN efflux from lung (Fig. 4), which suggests that the reversal of neutropenia occurred because lung released the accumulated cells. Thus, these data are fully compatible with the notion that compound III causes circulating PMN to sequester transiently in lung. Similar events occur with other PMN stimuli (8, 11, 27, 30).

Compound III had highly selective bioactions. In vitro it did not aggregate platelets and in vivo it did not induce thrombocytopenia or lymphocytopenia (Table I). Furthermore, compounds I and II were >10-fold less active than compound III (Table I and Figs. 1 and 2). Thus, a *cis* double bound at carbon-6 (compounds I and II possess a *trans* double bound at this location) is required for optimum potency. Indeed, the bioactions of the two steroisomers, compounds I and II, could be due to low level (e.g., <1–10%) contamination with compound III. In any event, the bioactivities of these fatty acids are target-cell specific and highly dependent upon small differences in lipid structure.

The bioactions of these compounds are of particular interest because they are naturally derived arachidonate metabolites formed by stimulated PMN and, perhaps, by other cell types from various species (13–16, 18–22). Furthermore, these compounds aggregate and degranulate PMN from humans, rabbits, and guinea pigs (although there appear to be species differences in potency; see Results). Thus, 5,12-diHETE could form in the blood of animals or humans exposed to PMN stimuli. This formation may be appreciable: 7×10^7 human PMN release >1.3 μg compound III when challenged in vitro (21, 22). Neutropenia induced by PMN stimuli or occurring in syndromes associated with elevated blood levels of arachidonate metabolites such as endotoxemia or anaphylaxis (23, 24) may therefore be mediated by 5,12-diHETE or structurally related compounds. Further attention should be focused on the role of these compounds in controlling PMN levels during a variety of pathological and clinical conditions.

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