*Biochemical Pharmacology*, Vol. 36, No. 19, pp. 3201–3204, 1987.
Printed in Great Britain.

0006–2952/87 $3.00+0.00
Pergamon Journals Ltd.

# PHARMACOLOGICAL EVIDENCE FOR A ROLE OF LIPOXYGENASE PRODUCTS IN PLATELET-ACTIVATING FACTOR (PAF)-INDUCED HYPERALGESIA

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(*Received* 5 *December* 1986; *accepted* 24 *February* 1987)

**Abstract**—Platelet-activating factor (PAF), a potent inflammatory mediator, decreases the nociceptive threshold in the rat hindpaw. Pain sensitivity, measured by the applied pressure necessary to induce vocalization, was increased maximally at 3 and 4 hr after injection of synthetic PAF. The hyperalgesic response to PAF was specifically inhibited by agents that interfere with the lipoxygenase pathway of arachidonic acid metabolism and was not affected by cyclooxygenase inhibitors. BW-755C (3–30 mg/kg, p.o.) and L-615,919 (0.01–0.3 mg/kg, p.o.) significantly reduced PAF-induced hyperalgesia, whereas indomethacin had no effect. The finding that L-615,919, a specific 5-lipoxygenase inhibitor, was a potent inhibitor of this model of hyperalgesia leads to speculation that leukotrienes are important mediators of inflammatory pain.

There is considerable evidence that platelet-activating factor (PAF‡; 1-*O*-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine), which is released from a variety of inflammatory cells in response to various stimuli [1–3], is an important mediator of the inflammatory response. When administered intratracheally or into the pulmonary artery, PAF produces pulmonary vasoconstriction and edema [4, 5]. Acute lung inflammation is also characterized by infiltration of polymorphonuclear leukocytes (PMNs) and macrophages [6, 7]. Intravenous administration of PAF into rats causes acute hypotension due to decreased vascular resistance and depletion of blood volume [8]. Intradermal administration of PAF in rabbit [9] and guinea pig [10] skin induces increased vascular permeability and leukocyte infiltration. In humans, intradermal PAF causes an early wheal and flare response and, subsequently, erythema and cellular infiltration at 3 hr [11].

PAF also causes aggregation and degranulation of neutrophils (PMNs) obtained from various species including humans [12, 13], and it has been shown to stimulate the synthesis and release of leukotrienes from these cells [14, 15]. PAF-induced degranulation of PMNs was found to be dependent on leukotrienes as evidenced by selective inhibition by agents that inhibit the lipoxygenase pathway [13, 14]. It is thought that some of the *in vivo* effects of PAF may be mediated by stimulation of leukotriene synthesis. For example, $LTC_4$ and $LTD_4$ were found in lung effluent after PAF challenge *in vivo*, suggesting that pulmonary vasoconstriction following PAF administration may be due to these potent mediators [4]. In addition, Voelkel *et al.* [5] reported that the pulmonary vasoconstriction and edema induced by PAF are inhibited by eicosatetraynoic acid, a lipoxygenase inhibitor, and not by indomethacin.

PAF has been shown to produce edema and hyperalgesia when injected subcutaneously into the rat hindpaw [16, 17]. The hyperalgesia response to PAF was found to be either unaffected, or partially inhibited, by indomethacin. The present study provides evidence that lipoxygenase products play a role in the hyperalgesia induced by PAF.

## METHODS

Female weanling Sprague–Dawley rats (Taconic Farms, Germantown, NY), 40–50 g, were fasted overnight prior to all experiments requiring oral administration of drug. Sensitivity to pain, either hyperalgesia or hypoalgesia, was measured by vocalization threshold (V.T.) in the hindpaw utilizing the method of Winter and Flataker [18], where V.T. is defined as the pressure necessary to elicit vocalization. Synthetic PAF (l-lecithin β-acetyl-*o*-alkyl, Calbiochem), at doses of 0.1 to 10 μg dissolved in 0.9% saline, was injected subcutaneously into the plantar surface of the right hindpaw in a volume of 0.1 ml, while control rats received saline alone. The pressure in mm Hg (V.T.) was measured in both the injected and non-injected paw 1, 2, 3 and 4 hr afterwards. In separate experiments, rats were injected with 1 μg lyso-PAF (1-*o*-hexadecyl-sn-glycero-3-phosphorylcholine, Bachem.), 1 μg PAF, or an equal volume of saline, and tested for pain sensitivity 1, 3, 4, 6 and 24 hr afterwards. Drugs were administered by various routes 30 min before PAF injection [19]. All drugs were suspended in 1% methylcellulose except for yohimbine, phentolamine and methysergide which were dissolved in saline. The mean V.T. was determined for each experimental con-

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‡Abbreviations: PAF, platelet-activating factor; $LTB_4$, leukotriene $B_4$; $LTC_4$, leukotriene $C_4$; and $LTD_4$, leukotriene $D_4$.

dition and, in some cases, data from different experiments were pooled. Results are also expressed as percent change from control. Significance was determined by the Dunnett's multiple range test for multiple dose testing or the unpaired, two-tailed Student's *t*-test, for single dose tests.

The materials used for this study were: indomethacin, sulindac sulfide, diflunisal, cyproheptadine (Merck & Co. Inc., Rahway, NJ); naproxen and ibuprofen (obtained from Dr. N. Jensen formerly of this institute); piroxicam (obtained from Dr. A. Tischler of this institute); BW-755C [3-amino-l-3-(trifluoromethyl)-phenyl-2-pyrazoline, synthesized by Dr. A. Tischler]; aspirin (acetylsalicylic acid, Aldrich Chemical Co., Milwaukee, WI); benoxaprofen (Lilly Research Laboratories, U.K.); verapamil hydrochloride (Knoll Pharmaceuticals Co., Whippany, NJ); theophylline (Sigma Chemical Co., St. Louis, MO); yohimbine (Hoffmann-LaRoche, Nutley, NJ); phentolamine (Ciba-Geigy Co., Summit, NJ); methysergide (Sandoz Pharmaceutical, Hanover, NJ); and L-615,919 (4-chlorophenothiazine-3-one, synthesized by Dr. Y. Guindon of Merck Frosst, Canada).

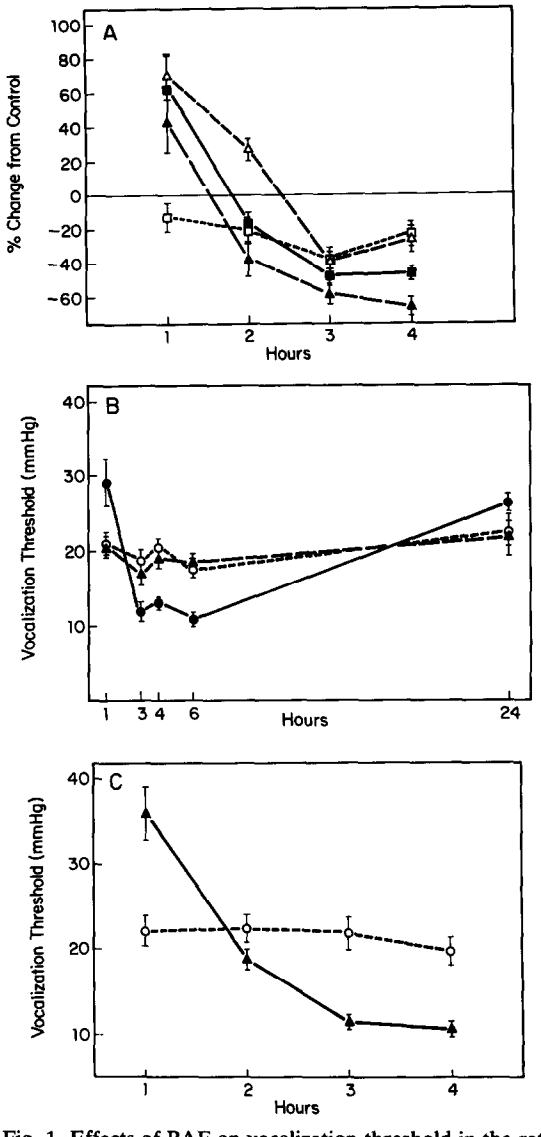


Fig. 1. Effects of PAF on vocalization threshold in the rat hindpaw. (A) PAF [(□) 0.1 μg; (△) 0.5 μg; (■) 1 μg; (▲) 5 μg], dissolved in saline was injected into the hindpaw, and the V.T. was measured 1–4 hr afterwards. Data are expressed as percent change in the V.T. as compared to saline-injected control paws (mean ± SEM, N = 20; see panel C for absolute values). (B) PAF 1 μg (●), lyso-PAF 1 μg (▲), or saline (○) was injected into the rat paw, and V.T. was measured 1–24 hr afterwards (mean ± SEM, N = 20). (C) V.T. 1–4 hr after injection of PAF 1 μg (▲) or saline (●) (mean ± SEM, N = 20).

## RESULTS

Figure 1A illustrates the time course of change in vocalization thresholds after injection of PAF (0.1 to 5 μg) into the hindpaw. One hour after PAF, significant hypoalgesia was observed at 0.5 and 1 μg doses. Hyperalgesia, a significant increase in sensitivity to pain, was seen at 3 and 4 hr after PAF at all doses. Low concentrations (0.1, 0.5 or 1 μg/paw) had little or no effect on the V.T. in the non-injected paw, whereas 5 μg of PAF caused significant hyperalgesia in the non-injected paw, suggesting a systemic effect. The hyperalgesic response to 1 μg PAF lasted up to 6 hr, but disappeared by 24 hr (Fig. 1B). Lyso-PAF, 1 or 10 μg/paw, had no significant effect on pain sensitivity at any time point. In contrast, a dose of 10 μg PAF caused 60% lethality and no consistent changes in V.T. in the survivors.

After numerous pilot experiments, a dose of 1 μg/paw of PAF was found suitable for the purpose of evaluating pharmacological agents. This concentration produces maximum local hyperalgesia without significant systemic activity. The 3-hr time point was chosen since it was the time of onset of hyperalgesia, and pain sensitivity was not significantly different between 3 and 4 hr (Fig. 1C). Results are expressed as the percent change from the PAF injected paw in vehicle-treated control groups. Note that inhibition of hyperalgesia is evidenced by an increase in V.T. and percent change.

Table 1 illustrates that cyclooxygenase inhibitors, such as indomethacin, had no significant effect on V.T., whereas BW-755C, a mixed cyclooxygenase and lipoxygenase inhibitor, significantly inhibited the hyperalgesic response to PAF. Benoxaprofen, a weak lipoxygenase/cyclooxygenase inhibitor [19], caused a small but significant rise in vocalization threshold. Yohimbine, an $\alpha_2$-receptor antagonist, significantly potentiated the hyperalgesia induced by PAF, whereas another $\alpha_2$-antagonist, phentolamine, had no effect, Verapamil, a calcium channel blocker, and theophylline, a phosphodiesterase inhibitor, had no significant effects. Serotonin receptor antagonists, methysergide and cyproheptadine (which is also antihistamine), had no effect.

Table 2 demonstrates the dose-related inhibition of PAF-induced hyperalgesia by BW-755C. Significant increases in vocalization threshold were observed after pretreatment with 3, 10 and 30 mg/kg perorally. Indomethacin, at doses of 1, 3 and 10 mg/kg, p.o., had no significant effect on hyperalgesia.

L-615,919 (4-chlorophenothiazine-3-one) is one of

Table 1. The effects of various drugs on the hyperalgesia induced by 1 μg PAF

| Treatment | Dose (mg/kg) | N | Vocalization threshold (mm Hg) | % Change |
|---|---|---|---|---|
| BW-755C | 30 p.o. | 20 | 27.6 ± 3.4* | +184.5 |
| Indomethacin | 10 p.o. | 10 | 11.8 ± 1.6 | + 34.1 |
| Sulindac sulfide | 30 p.o. | 10 | 15.6 ± 4.6 | + 69.6 |
| Piroxicam | 10 p.o. | 20 | 10.9 ± 1.3 | + 16.0 |
| Naproxen | 30 p.o. | 10 | 14.2 ± 3.0 | + 54.3 |
| Aspirin | 100 p.o. | 10 | 11.0 ± 1.6 | + 19.6 |
| Benoxaprofen | 30 p.o. | 20 | 15.9 ± 2.1† | + 69.1 |
| Diflunisal | 30 p.o. | 30 | 13.5 ± 1.8 | + 37.8 |
| Ibuprofen | 30 p.o. | 10 | 10.6 ± 1.2 | + 10.4 |
| Verapamil | 50 p.o. | 10 | 11.2 ± 1.5 | + 1.8 |
| Cyproheptadine | 10 p.o. | 10 | 14.0 ± 2.1 | + 27.3 |
| Theophylline | 50 p.o. | 10 | 10.4 ± 1.9 | − 5.5 |
| Yohimbine | 5 s.c. | 20 | 6.8 ± 0.6* | − 36.4 |
| Phentolamine | 5 s.c. | 10 | 10.4 ± 1.0 | − 5.5 |
| Methysergide | 5 s.c. | 10 | 11.0 ± 1.4 | + 5.8 |

Agents were administered 30 min prior to subplantar injection of PAF either perorally (p.o.) or subcutaneously (s.c.). The V.T. was measured 3 hr after PAF injection (mean ± SEM). The percent change in V.T. was calculated as compared to V.T. in the inflamed paws of the vehicle-treated control group (range from 9 to 11 mm Hg). The V.T. in the non-injected paws ranged from 19 to 25 mm Hg.

*,†Significance was determined by the unpaired Student's *t*-test: *$P < 0.002$, and †$P < 0.01$ (two-tailed test).

a class of potent and specific inhibitors of 5-lipoxygenase *in vitro* with an $IC_{50}$ <80 nM [20]. L-615,919, administered orally at 0.001 to 0.3 mg/kg, significantly inhibited PAF-induced hyperalgesia in a dose-dependent manner between 0.01 and 0.3 mg/kg (Table 2).

Table 2. Effects of BW-755C, indomethacin, and L-615,919 (4-chlorophenothiazine-3-one) on the hyperalgesia induced by 1 μg PAF

| Treatment | Dose (mg/kg, p.o.) | N | Vocalization threshold (mm Hg) | % Change |
|---|---|---|---|---|
| Vehicle | | 30 | 9.7 ± 0.7 | |
| BW-755C | 0.3 | 10 | 10.8 ± 1.6 | + 11.3 |
| | 1.0 | 20 | 15.1 ± 1.8 | + 55.7 |
| | 3.0 | 30 | 19.5 ± 1.7* | +101.0 |
| | 10.0 | 30 | 20.9 ± 2.0* | +115.5 |
| | 30.0 | 20 | 27.6 ± 3.4* | +184.5 |
| Vehicle | | 10 | 8.8 ± 0.6 | |
| Indomethacin | 1 | 10 | 11.2 ± 1.0 | + 27.3 |
| | 3 | 10 | 10.4 ± 1.1 | + 18.2 |
| | 10 | 10 | 11.8 ± 1.6 | + 34.1 |
| Vehicle | | 40 | 11.1 ± 0.8 | |
| L-615,919 | 0.001 | 20 | 11.4 ± 1.1 | + 2.7 |
| | 0.003 | 20 | 16.6 ± 2.1 | + 49.5 |
| | 0.01 | 30 | 21.5 ± 2.5* | + 93.7 |
| | 0.03 | 20 | 33.1 ± 4.1* | +198.2 |
| | 0.1 | 39 | 30.6 ± 2.4* | +175.7 |
| | 0.3 | 29 | 33.9 ± 2.8* | +205.4 |

Agents were administered perorally 30 min prior to subplantar injection of PAF. The V.T. was measured 3 hr after PAF injection (mean ± SEM), and the percent change was calculated as compared to the vehicle-treated control group.

*Significance was determined by the Dunnett's multiple range test ($P < 0.01$, two-tailed test).

## DISCUSSION

Platelet-activating factor (PAF) causes edema and hyperalgesia when injected into the rat hindpaw. The specificity of the hyperalgesic response to PAF is evidenced by the lack of response to lyso-PAF, a structurally-related, biologically-inactive form. It was shown previously in our laboratories [21] that the edematous response is maximal 1 hr after injection of PAF and declines rapidly thereafter. In contrast, hyperalgesia is a delayed response, first occurring at 3 hr. The cause of the hypoalgesia seen 1 hr after PAF injection is not known and may be due to the edematous state and the release of other mediators. A similar response occurs 1 hr after injection of Brewer's yeast [22]. The time course of response to PAF was similar to that reported by Bonnet *et al.* [16]. Vargaftig and Ferreira [17], using a similar method, found PAF-induced hyperalgesia to peak at 2 hr and decline thereafter. The reason for this difference is unknown. Archer *et al.* [11] also noted a dual response to PAF after injection into human skin. An acute wheal and flare response was followed by a delayed erythema and hyperalgesia.

The findings of Voelkel *et al.* [5], showing leukotriene release in the lung after PAF challenge, prompted us to study the effects of lipoxygenase inhibitors on the hyperalgesic response to PAF. Our initial studies showed that BW-755C, a mixed lipoxygenase/cyclooxygenase inhibitor, caused significant dose-related inhibition of PAF-induced hyperalgesia. In contrast, cyclooxygenase inhibitors such as indomethacin had no significant effect on the response to PAF. More definitively, the discovery of 4-chlorophenothiazone-3-one, as an extremely potent 5-lipoxygenase inhibitor, allowed a more precise evaluation of the role of leukotrienes in hyperalgesia. The potency of 4-chlorophenothiazine-3-one in inhibiting 5-lipoxygenase activity *in vitro* was paralleled *in vivo* by its inhibition of PAF-induced hyperalgesia at 120 μg/kg, with maximal inhibition at 30 μg/kg perorally. These findings provide pharmacological evidence that lipoxygenase products are essential for the hyperalgesic response induced by PAF, with no significant role for cyclooxygenase products.

The hyperalgesic response to PAF is not mediated through histamine, serotonin, or α- or β-adrenergic receptors, as indicated by the lack of inhibition by their respective antagonists. Yohimbine has CNS effects including excitation, increased motor activity and irritability, which may be responsible for the potentiation of hyperalgesia observed [23]. While calcium channel blockers partially inhibit PAF-induced edema [16, 21], verapamil had no effect on the development of hyperalgesia in the present study. Theophylline, a phosphodiesterase inhibitor, also partially reduces the edematous response [16, 21], while having no effect on PAF-induced hyperalgesia. Clearly, PAF produces edema and hyperalgesia by different mechanisms.

There is some evidence that lipoxygenase products may play a role in inflammatory pain. Rackham and

Ford-Hutchinson [22] showed that, while $LTB_4$ itself causes only a small hyperalgesic effect, when it is injected into the hindpaw with Brewer's yeast, $LTB_4$ reverses the early hypoalgesic response and decreases the latency of onset of hyperalgesia. Levine *et al.* [24] found that $LTB_4$, but not $LTD_4$ induces hyperalgesia in the rat hindpaw. In our laboratory, we found that $LTB_4$ also causes significant increases in pain sensitivity in the rat. Increases in $LTB_4$ levels have been found in rat paws injected with Brewer's yeast and appear to play a role in the hyperalgesic response to this inflammatory agent [25]. In addition, in human skin, Lewis *et al.* [26] reported $LTB_4$ to cause tenderness and neutrophil infiltration; similar effects were seen when PAF was injected intradermally in humans [27].

Little is known about the effects of other lipoxygenase products on pain sensitivity. It is likely that a number of mediators interact with each other to produce the hyperalgesic response. O'Flaherty *et al.* [28] found that PAF, leukotrienes and 5-hydroxyeicosatetraenoic acid can interact to produce potent degranulation responses in PMNs. These relationships found *in vitro* may relate to responses seen *in vivo*.

The mechanisms by which PAF induces leukotriene synthesis and hyperalgesia are not known at this time. The observation by Levine *et al.* [24] that $LTB_4$-induced hyperalgesia is dependent upon PMN recruitment suggests a central role for this cell. PAF is both chemotactic for, and a stimulus of, leukotriene synthesis by these cells. A careful study of the role of PAF in recruitment of PMNs and stimulation of leukotriene synthesis in the model of hyperalgesia that we have described here will clarify these issues. It is already clear, however, that specific lipoxygenase products may be important mediators of pain, independent of prostaglandins, in some situations. This raises the prospect of inhibitors of leukotriene synthesis or leukotriene antagonists emerging as unique analgesic agents.

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