colony twice daily (0800 and 1600). Animals born during the day (8-hour resolution) are used to record responses at 11 to 25 days and those born at night (16-hour resolution) are used at 26 days and thereafter.
18. I. Tasaki, H. Davis, J. P. Legouix, *J. Acoust. Soc. Am.* **5**, 502 (1952).
19. R. A. Schmiedt and J. J. Zwislocki, *ibid.* **61**, 133 (1977).
20. A. Finck, C. D. Schneck, A. F. Hartman, *J. Comp. Physiol. Psychol.* **78**, 375 (1972).
21. N. K. Woolf and A. F. Ryan, *Hearing Res.* **13**, 277 (1984).
22. The location of our first-turn recording electrode in the adult gerbil can be estimated from published data: ST_1 = 10 mm from apex (*19*). The difference between the estimated cut-off frequency for their first-turn recordings (10.5 kHz) and ours may be the result of a slight but consistent discrepancy between the two laboratories in the placement of the recording electrode.
23. D. A. N. Hoyte, *Am. J. Anat.* **108**, 1 (1961).
24. G. Ehret, *J. Am. Audiol. Soc.* **1**, 179 (1976).
25. We thank J. Santos-Sacchi for his assistance. Supported in part by NIH grant NS08635 to P.D. This material was presented at the 6th Midwinter Research Meeting of the Association for Research in Otolaryngology, St. Petersburg Beach, January 1983.

23 January 1984; accepted 17 May 1984

Leukotriene B_4 Produces Hyperalgesia That Is Dependent on Polymorphonuclear Leukocytes

Abstract. *Leukotriene B_4, at the same intracutaneous doses as bradykinin, reduced the nociceptive threshold in the rat paw. The mechanism of leukotriene B_4–induced hyperalgesia was distinguished from that of the hyperalgesia elicited by prostaglandin E_2 and bradykinin by its dependence on polymorphonuclear leukocytes and independence of the cyclooxygenation of arachidonic acid.*

The most characteristic sensory abnormality associated with inflammation is tenderness or hyperalgesia. Hyperalgesia develops when nerve fiber terminals of polymodal nociceptors are sensitized by mediators of inflammation (*1*), such as prostaglandin products of the cyclooxygenation of arachidonic acid. Prostaglandins produce hyperalgesia at low concentrations (*2*) without evoking pain (*3*). Nonsteroidal anti-inflammatory drugs (NSAID's) reverse hyperalgesia, in part by inhibiting the biosynthesis of prostaglandins (*4*). However, the failure of NSAID's to reverse consistently the hyperalgesia of inflammation at concentrations that suppress prostaglandin generation (*5*) and the finding that many noninjurious stimuli can elicit the generation of prostaglandins without producing hyperalgesia (*6*) both suggest that inflammatory mediators other than prostaglandins contribute to the sensitization of nociceptors.

Leukotriene products of the NSAID-resistant 5-lipoxygenation of arachidonic acid are potent stimuli of many components of inflammation. Such lipoxygenase products mediate hyperalgesia in animals (*7*) and humans (*8*). We now report that leukotriene B_4 (LTB_4) produces hyperalgesia, in rat paws, that is not dependent on the integrity of the cyclooxygenase pathway but does require polymorphonuclear leukocytes that recognize specifically and respond functionally to LTB_4.

We defined hyperalgesia as a decrease in nociceptive threshold in male Sprague-Dawley rats (200 to 250 g) and quantified it by determining the decrease in local pressure (measured in grams per square centimeter) required to evoke withdrawal of the paw (*9*). We increased the pressure applied to the dorsum of the paw linearly with time and recorded the pressure when the rat withdrew its paw. This reflex correlates well with hyperalgesia evoked in humans in response to intradermal injections of noxious agents and with responses to analgesic compounds (*10*). After measurement of the baseline nociceptive threshold, a test or control substance (10 μl) was injected intradermally in the dorsum of one hind paw. The intensity of the hyperalgesia, at the site of the injection, produced by the substance at each time was expressed as the percentage decrease in the nociceptive threshold from the baseline value.

The substances used were synthetic bradykinin (Sigma Chemical Company, St. Louis, Missouri), prostaglandin E_2 (PGE_2) (Upjohn Company, Kalamazoo, Michigan), and LTB_4 and LTD_4 (Dr. J. Rokach, Merck-Frosst Co., Dorval, Canada). Native LTB_4, 5(*S*),12(*S*)-dihydroxyeicosa-6,8,10-*trans*-14-*cis*-tetraenoic acid [12(*S*)-6-*trans*-LTB_4], and 12(*R*)-6-*trans*-LTB_4 were extracted from supernatants of suspensions of human neutrophils, incubated with arachidonic acid (50 μg/ml) and 2 μ*M* ionophore A23187, and purified by high-performance liquid chromatography (HPLC). The incubation of 1 mg of arachidonic acid with 10,000 units of purified soybean 15-lipoxygenase (Sigma) in 2 ml of 0.2*M* sodium borate (*p*H 8.8) for 30 minutes at 37°C generated a mixture of 15-HETE and diHETE's, from which 8(*S*), 15(*S*) - dihydroxy - 5 , 11 - *cis* -9 , 13 -*trans*-eicosatetraenoic acid (8,15-diHETE) was extracted and isolated by HPLC (*11*). All compounds tested for their ability to induce hyperalgesia were dissolved in phosphate-buffered saline. Indomethacin (Sigma) was dissolved in sodium bicarbonate (2 g/100 ml) titrated to *p*H 7.2 with sodium phosphate and was administered intravenously (2 mg/kg) 20 to 30 minutes before intradermal injections.

Polymorphonuclear leukocytes were depleted by giving hydroxyurea (Squibb Company, Princeton, New Jersey) or methotrexate (Lederle Company, Puerto Rico) to rats intravenously for 3 days (*12*). The administration of hydroxyurea, 200 mg/kg on day 1 and 100 mg/kg on days 2 and 3, or methotrexate, 2 mg/kg on day 1 and 1½ mg/kg on days 2 and 3, eliminated polymorphonuclear leukocytes, other granulocytes, and mononuclear leukocytes from the circulation on days 4, 5, and 6 as assessed with Wright-stained slides of peripheral blood. Only rare polymorphonuclear leukocytes were observed on each of two slides of peripheral blood prepared from each rat treated with hydroxyurea or methotrexate. No mortality or morbidity was observed in rats receiving either drug. Since similar results were obtained with both agents, and all groups contained equal numbers of rats treated with either

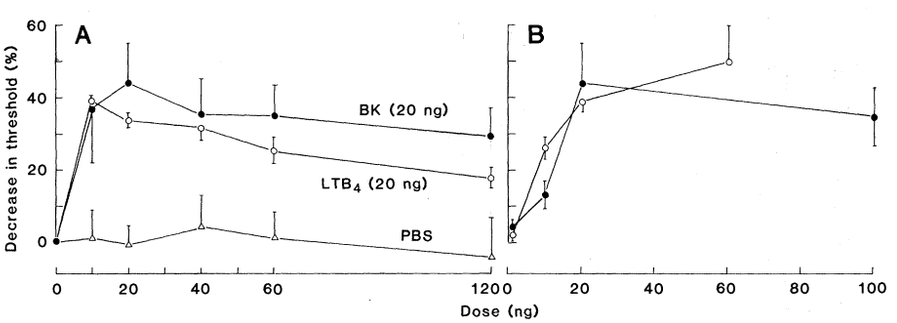

Fig. 1. (A) Time course of the effects of LTB_4 on nociceptive pressure thresholds in the rat paw. Leukotriene B_4, BK, and the phosphate-buffered saline (PBS) vehicle were injected intradermally in the dorsum of the paw. Each point and bracket represents the mean ± standard error of the results of studies of six rats. (B) Concentration dependence of the effect of BK and LTB_4 on nociceptive pressure thresholds. Measurements were made 20 minutes after the intradermal injection of BK (●) and LTB_4 (○). Each point represents an average of six experiments.

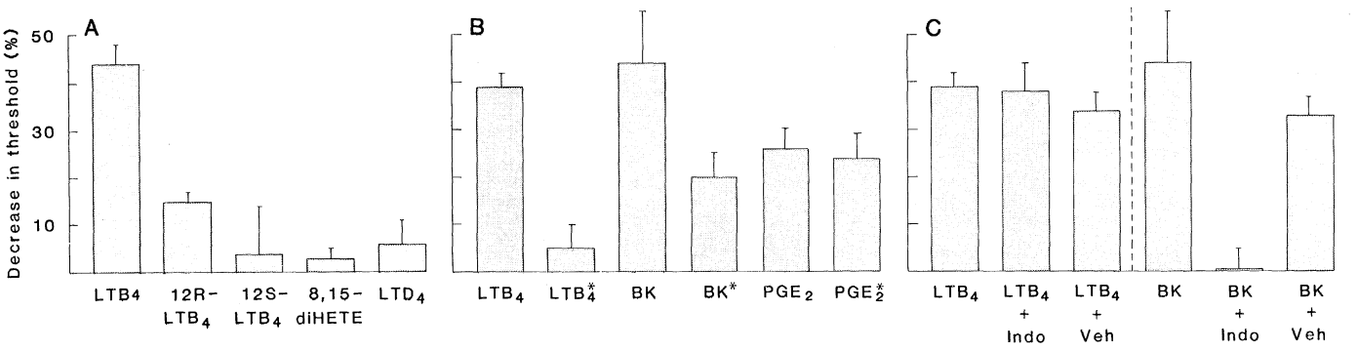

Fig. 2. (A) Effects of different products of lipoxygenase pathways on the nociceptive threshold. Measurements were made 20 minutes after intradermal injection of 20 ng of LTB_4, 12(*R*)-6-*trans*-LTB_4 (12R-LTB_4), 12(*S*)-6-*trans* LTB_4 (12S-LTB_4), 8,15-diHETE, and LTD_4. Each bar represents an average of six experiments. (B) Effect of polymorphonuclear leukocyte depletion on the responses of the nociceptive pressure threshold. Measurements were made 20 minutes after intradermal injection of 20 ng of LTB_4, BK, and PGE_2 into control rats and rats that had been depleted of neutrophils (indicated by an asterisk). Significant blunting of the effect of LTB_4 ($P < 0.025$) and BK ($P < 0.025$), but not PGE_2, on the pressure threshold was produced by neutrophil depletion. Each bar represents an average of six experiments. (C) Lack of effect of indomethacin on the change in nociceptive pressure threshold induced by LB_4. Leukotriene B_4 (20 ng) or BK (20 ng) were injected intradermally after administration of indomethacin (Indo) or vehicle alone (Veh). Each bar depicts the mean ($n = 6$) of each set of data.

drug, the data from animals treated with either of the drugs have been combined for presentation. The statistical comparison of means was done by Student's *t*-test.

The hyperalgesia produced by intradermal injection of 20 ng of LTB_4 was similar in magnitude and duration to that elicited by 20 ng of bradykinin (BK) and achieved a maximum level at 10 to 20 minutes (Fig. 1A). The saline vehicle was without statistically significant effect. The concentration-dependence relationships for the induction of hyperalgesia by LTB_4 and BK were similar, and 20 ng of each agonist evoked the maximum hyperalgesia at 20 minutes (Fig. 1B). Hence the molar potency of LTB_4 is approximately three times that of BK. The hyperalgesic effects of 20 ng of LTD_4, 8,15-diHETE, and the 12(*S*) and 12(*R*) isomers of 6-*trans*-LTB_4 were compared to the hyperalgesic effects of LTB_4 to examine the structural determinants of hyperalgesic activity (Fig. 2A). The 12(*R*) isomer of 6-*trans*-LTB_4 produced significant hyperalgesia ($P < 0.05$), whereas LTD_4, 8,15-diHETE, and the 12(*S*) isomer of 6-*trans*-LTB_4 were not significantly active at the same concentration.

Treatment of rats with the NSAID indomethacin (2 mg/kg) attenuated the hyperalgesic effect of a subsequent intradermal injection of BK (Fig. 2B) but had no significant effect on the hyperalgesia produced by 20 ng of LTB_4. The vehicle for indomethacin had no significant effect on the hyperalgesia induced by BK or LTB_4.

The depletion of circulating polymorphonuclear leukocytes just before the intradermal injections attenuated the hyperalgesia elicited by 20 ng of LTB_4 without affecting that induced by PGE_2 (Fig. 2C). The hyperalgesic effect of BK was prevented only partially by the polymorphonuclear leukocytopenia.

That the hyperalgesic effect of LTB_4 appears to be a function of an influx of polymorphonuclear leukocytes is consistent with the previous findings that LTB_4 is the most potent polymorphonuclear leukocyte chemotactic and activating factor of the lipoxygenase pathways (*13*). The hyperalgesic effect of LTB_4 (Fig. 2A) is dependent on the position of the hydroxyl groups and the double bond geometry of the triene portion of the molecule. Leukotriene D_4, which is the most potent vasoactive and contractile leukotriene of the slow-reacting substances of anaphylaxis (*14*), did not show any hyperalgesic activity (Fig. 2A). The similarity of the LTB_4 structural requirements for hyperalgesia and polymorphonuclear leukocyte activation suggests that the principal effect of LTB_4 is the mobilization and localization of polymorphonuclear leukocytes at the site of the challenge. However, our data do not exclude the possibility of a polymorphonuclear leukocyte–derived mediator or of a separate and necessary effect of LTB_4 on nociceptive afferent neurons in the skin or deeper tissue. The hypothesis that LTB_4-induced hyperalgesia is mediated by release, from polymorphonuclear leukocytes, of a compound that in turn sensitizes polymodal nociceptors was supported by the finding that depletion of leukocytes prevented LTB_4-induced hyperalgesia. The leukopenia-inducing agents we used depleted both polymorphonuclear and mononuclear leukocytes, but the leukocytes that respond to LTB_4 in tissue are predominantly of the polymorphonuclear class (*15*).

Some of the actions of the lipoxygenase products in other systems are indirectly mediated by their ability to enhance activity in the cyclooxygenase pathways of endogenous arachidonic acid metabolism (*16*). Nevertheless, the failure of the cyclooxygenase inhibitor indomethacin to significantly antagonize LTB_4-induced hyperalgesia, while completely suppressing BK-induced hyperalgesia, suggests that the pathway by which LTB_4 induces hyperalgesia is independent of cyclooxygenase activity. Thus the mechanism of LTB_4-induced hyperalgesia is different from that produced by PGE_2 and other products of the cyclooxygenase pathway.

The complete antagonism of BK-induced hyperalgesia by indomethacin suggests that both the polymorphonuclear leukocyte–dependent and –independent components of BK-induced hyperalgesia involve cyclooxygenase products of arachidonic acid and differ from LTB_4-induced hyperalgesia.

Thus, in inflammatory lesions, LTB_4 may play a role in the attendant hyperalgesia. Pharmacological agents that block lipoxygenase pathways or antagonize actions of its metabolic products may constitute an important new class of analgesic compounds that are independent in effect from inhibitors of the cyclooxygenase pathways.

JON D. LEVINE*
WAI LAU
GEOFFREY KWIAT
Section of Rheumatology and Clinical Immunology and *Division of Oral and Maxillofacial Surgery, Department of Medicine, University of California, San Francisco 94143*
EDWARD J. GOETZL
Howard Hughes Medical Institute, and *Division of Allergy and Immunology, Department of Medicine, University of California*

References and Notes

1. P. Bessou and E. R. Perl, *J. Neurophysiol.* **32**, 1025 (1969); R. E. Beitel and R. Dubner, *ibid.* **39**, 1160 (1976).
2. H. Juan and F. Lembeck, *Naunyn-Schmiedeberg's Arch. Pharmacol.* **283**, 151 (1974).
3. P. Crunkhorn and A. L. Willis, *Br. J. Pharmacol.* **41**, 49 (1971); E. W. Horton, *Nature (London)* **200**, 892 (1963).
4. R. J. Flower, S. Moncada, J. R. Vane, in *The Pharmacological Basis of Therapeutics*, A. G. Gilman, L. S. Goodman, A. Gilman, Eds. (Macmillan, New York, 1980), pp. 682–728.
5. E. C. Huskinsson, *Semin. Arthritis Rheum.* **7**, 1 (1977).
6. S. Moncada, S. H. Ferreira, J. R. Vane, in *Handbook of Experimental Pharmacology*, J. R. Vane and S. H. Ferreira, Eds. (Springer-Verlag, Berlin, 1979), pp. 588–616.
7. A. Rackham and A. W. Ford-Hutchinson, *Prostaglandins* **25**, 193 (1983).
8. R. A. Lewis, N. A. Soter, E. J. Corey, K. F. Austen, *Clin. Res.* **29**, 492A (1981).
9. L. O. Randall and J. J. Selitto, *Arch. Int. Pharmacodyn.* **3**, 409 (1957).
10. S. H. Ferreira, in *Advances in Pain Research and Therapy* (Raven, New York, 1983), pp. 627–634.
11. M. Phillips, W. M. Gold, E. J. Goetzl, *J. Immunol.* **131**, 906 (1983); D. W. Goldman, W. C. Pickett, E. J. Goetzl, *Biochem. Biophys. Res. Commun.* **117**, 282 (1983).
12. P. M. Calabresi and R. R. Parks, Jr., in *Pharmacological Basis of Therapeutics*, A. G. Gilman, L. S. Goodman, A. Gilman, Eds. (Macmillan, New York, 1980), pp. 1256–1313; A. Johnson and A. B. Malik, *Am. Rev. Respir. Dis.* **122**, 561 (1980).
13. D. W. Goldman and E. J. Goetzl, *J. Immunol.* **129**, 1600 (1982).
14. R. A. Lewis, K. F. Austen, J. M. Drazen, D. A. Clark, A. M. Marafat, E. J. Corey, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3710 (1979).
15. B. Samuelsson, *Trends Pharmacol. Sci.* **1**, 227 (1980).
16. R. A. Lewis and K. F. Austen, *Nature (London)* **293**, 103 (1981).

17. We thank A. Basbaum for discussions of the manuscript. This work was supported in part by grants AM 32634, DE 05369, HL 31809, and AI 19784 from the National Institutes of Health, and by grants from the KROC Foundation, the Northern California Arthritis Foundation, and the Academic Senate of the University of California, San Francisco. J.D.L. is a fellow of the Hartford Foundation.

* To whom correspondence should be addressed at 473 HSW, Section of Rheumatology, University of California, San Francisco 94143.

5 March 1984; accepted 14 June 1984

Methylation Increases Sodium Transport into A6 Apical Membrane Vesicles: Possible Mode of Aldosterone Action

Abstract. *When isolated apical membrane vesicles prepared from cultured A6 epithelia were incubated in vitro with the methyl donor* S-*adenosylmethionine, the control rate of amiloride-inhibitable sodium transport was doubled. The methylation inhibitors 3-deazaadenosine and* S-*adenosyl homocysteine returned the* S-*adenosylmethionine–stimulated sodium transport to control levels. Neither these agents nor adenosine affected sodium transport into control vesicles. In vesicles incubated with* S-*adenosyl-[*^{3}*H-methyl]methionine, both membrane phospholipids and proteins were labeled, and this labeling was inhibited by deazaadenosine. In vesicles prepared from A6 cells treated with aldosterone, sodium transport was twice the control value and* S-*adenosylmethionine did not cause any further stimulation of transport. In those vesicles, both lipid and protein methylation were increased. These results suggest that methylation, which increases the rate of amiloride-sensitive sodium transport, is involved in the action of aldosterone at the apical membrane level in epithelia.*

Aldosterone increases sodium transport across responsive epithelia, including the epithelia formed in tissue culture by the amphibian kidney cell line A6 (*1*). Part of this action is an increase in sodium flux into the cells across their apical membranes (*2*), possibly caused by activation of previously quiescent sodium channels in those membranes (*3–5*). We found earlier that apical membrane vesicles prepared from cultured A6 epithelia contain amiloride-sensitive sodium channels (*6, 7*). Amiloride-sensitive sodium transport into these vesicles was twice as rapid when the A6 epithelia were incubated with aldosterone before vesicle preparation (*7*). This result was the starting point for the present studies. Wiesmann *et al.* (*8*) observed that aldosterone increased methylation in intact cultured TB-6c epithelia (a continuous toad bladder cell line) and that inhibition of the methylation prevented the increase in sodium transport caused by aldosterone. To test whether aldosterone acts by increasing methylation of the phospholipids surrounding sodium channels or by methylation of some proteins—perhaps the channels themselves—thereby increasing sodium flux through the channels, we methylated isolated apical membrane vesicles from A6 cells in vitro. Amiloride-sensitive sodium transport was measured and compared to the rate of transport observed in vesicles prepared from aldosterone-treated A6 cells (*9*).

Incubation of vesicles from control cells with *S*-adenosylmethionine ($10^{-4}M$) increased the amiloride-sensitive transport of sodium into the vesicles from 0.20 ± 0.02 to 0.37 ± 0.02 nmol/min per milligram of protein ($n = 9$; $P < 0.05$). In contrast, in vesicles prepared from cells incubated with aldosterone ($10^{-7}M$ for 4 hours) amiloride-sensitive sodium transport [0.42 ± 0.03 nmol/(min · mg)] was not further increased by *S*-adenosylmethionine [0.39 ± 0.02 nmol/(min · mg); $n = 5$]. In other words, the effects of aldosterone and *S*-adenosylmethionine were not additive. From the lack of effect of *S*-adenosylmethionine in vesicles made from aldosterone-treated cells, we infer that the methylating system was already fully activated in those cells and that this activation was not lost during the membrane isolation procedure.

Since methylating enzymes are associated with plasma membranes (*10*) and methyl groups can be incorporated into plasma membrane phospholipids (*11*), it seemed likely that addition of the methyl donor *S*-adenosylmethionine would increase methylation in the vesicles. As shown in Fig. 1, addition of *S*-adenosyl-[^{3}H-methyl]methionine resulted in labeling of phospholipids, and this labeling was inhibited by 3-deazaadenosine, an inhibitor of methylation. Protein carboxymethylation (*12*) was also observed (502 ± 25 dpm per milligram of protein) and was reduced (156 ± 12 dpm/mg) by the addition of $3 \times 10^{-4}M$ deazaadenosine. To test whether aldosterone indeed increased methylation at the apical membrane level, we incubated intact A6 epi-

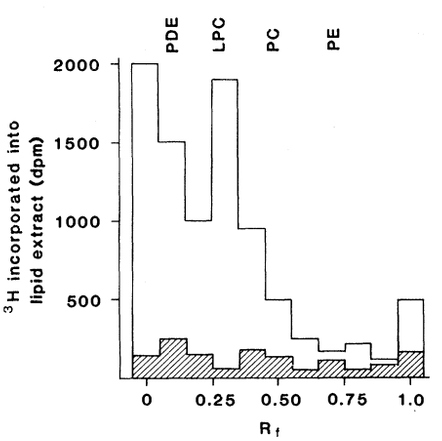

Fig. 1. Methylation by $10^{-4}M$ *S*-adenosylmethionine in vitro. Chromatographically pure *S*-adenosyl-[^{3}H-methyl]methionine (5 μCi/ml) was added to vesicles suspended in either diluent (open bars) or deazaadenosine (300 μ*M*) (hatched bars) and incubated for 35 minutes at room temperature. The suspension was then centrifuged at 17,000*g* for 10 minutes at 4°C, and the pellet was rinsed twice with ice-cold 145 m*M* NaCl. Lipids were extracted as described earlier (*17*). The lipid extract was chromatographed on silica gel plates (Baker) in a mixture of chloroform, propanol, propionic acid, and water (2:3:2:1). One-centimeter sections were scraped from the thin-layer plates directly into vials for liquid scintillation counting. Phospholipid standards run on the same plate were made visual with I_2 vapor. They had the following relative mobilities (R_f): phosphatidylcholine (PC), 0.52; phosphatidylethanolamine (PE), 0.73; lysophosphatidylcholine (LPC), 0.27; and phosphatidyldimethylethanolamine (PDE), 0.13.