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PHARMACOLOGICAL MODULATION OF EICOSANOID LEVELS AND HYPERALGESIA IN YEAST-INDUCED INFLAMMATION

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Abstract—Injection of brewer's yeast into the rat paw results in edema and a subsequent hyperalgesia. The edema was accompanied by an increase in 5-lipoxygenase products, and the hyperalgesia coincided with the formation of both cyclooxygenase and 5-lipoxygenase products. When administered perorally, indomethacin inhibited cyclooxygenase product formation, phenidone inhibited 5-lipoxygenase product formation, and 3-amino-1-(*m*-[trifluoromethyl]-phenyl)-2-pyrazoline (BW 755C) inhibited formation of products of both pathways. These compounds were also effective analgesic agents. The correlation of these effects with the suppression of hyperalgesia suggests the participation of products from both cyclooxygenase and 5-lipoxygenase pathways in the mediation of hyperalgesia.

Brewer's yeast, when injected into the rat paw, causes edema and hyperalgesia [1–3], and pharmacological studies with cyclooxygenase inhibitors suggest that these responses are mediated by products derived from the arachidonic acid (AA) cascade [4]. Both leukotrienes (LT) and prostaglandins (PG) increase sensitivity to pain when injected into rat paws [3–8]. Recently, Carey and Haworth [9] demonstrated that yeast-injected rat paws generate products from both cyclooxygenase (CO†) and 5-lipoxygenase (5-LO) pathways in temporal association with the development of hyperalgesia. The present paper demonstrates the association of arachidonic acid oxygenation products with both edema and hyperalgesia of rat paw inflammation induced by yeast-injection. We also report the modulation of arachidonic acid metabolites at the peak of hyperalgesia after peroral dosing with various pharmacological agents.

MATERIALS AND METHODS

Reagents. Indomethacin and 3-amino-1-(*m*-[trifluoromethyl]-phenyl)-2-pyrazoline (BW 755C) were prepared at Merck Sharp & Dohme Research Laboratories (Rahway, NJ). 1-Phenyl-3-pyrazolidone (phenidone) was purchased from the Sigma Chemical Co. (St. Louis, MO). Brewer's yeast was obtained from the Philadelphia Dry Yeast Co. (Philadelphia, PA). PGE_2 was purchased from the Ono Pharmaceutical Co., Ltd. (Osaka, Japan). Leukotrienes were provided by J. Rokach, Merck-Frosst (Montreal, Canada). PGE_2-antisera were purchased from Miles Research Products (Elkhart, IN) and leukotriene-antisera were from our laboratories [10]. Radioimmunoassay (RIA) reagents for thromboxane B_2 and 5-HETE were purchased from New England Nuclear (Boston, MA) and Seragen, Inc. (Boston, MA) respectively.

Animal studies. Groups of four to ten female weanling Sprague–Dawley rats weighing 40–60 g (Taconic Farms, Germantown, NY) were fasted overnight prior to all experiments requiring oral dosing. A subplantar injection of 0.1 ml of a 5% (w/v) suspension of brewer's yeast in saline was made in the rat hindpaw. Drugs were suspended in either Aqueous Vehicle (NaCl, 0.9%; carboxymethylcellulose, 0.5%; Tween 80, 0.4%; benzyl alcohol, 0.9%) or 1% methylcellulose and administered perorally in a volume of 0.1 ml 2 hr after yeast injection. Control groups received vehicle alone.

Edema. Paw volume was measured by mercury displacement utilizing a Buxco plethysmograph. Baseline measurements of each animal were taken prior to injection, and these values were subtracted from all subsequent readings. Paws were injected with 0.9% sterile saline or brewer's yeast, and foot volume was again measured 5, 15, 30 min, 1, 2, 3, 4 and 6 hr afterwards.

Hyperalgesia. Sensitivity to pain was assessed utilizing the method of Winter and Flataker [2]. Increasing pressure was applied to the plantar surface of the hindpaw by means of a compressed-air driven piston with a 2 mm tip, and the vocalization threshold (mm Hg) was determined. For the time course of hyperalgesia, thresholds were obtained before and 1, 3, 4 and 6 hr after injection of brewer's yeast. For pharmacological studies, measurements were obtained 4 hr after paw injections. The analgesic index, computed for each drug, represents the dose

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† Abbreviations: CO, cyclooxygenase; 5-HETE, 5-hydroxyeicosatetraenoic acid; 5-LO, 5-lipoxygenase; LT, leukotriene; PGE_2, prostaglandin E_2; RP-HPLC, reverse phase–high performance liquid chromatography; and TXB_2, thromboxane B_2.

(mg/kg) required to raise the mean vocalization threshold in the inflamed paw to that of the non-injected paw of the control group.

Biochemical measurements. Animals were killed by CO_2 asphyxiation, and their test paws were amputated and placed immediately into liquid nitrogen for several minutes. Frozen paws were then stored at −80° until needed for biochemical analysis of AA metabolites.

Frozen paws were pulverized in a Freezer-Mill (Spex, Metuchen, NJ) and transferred to a glass high-speed centrifuge tube. Extraction buffer (4.0 ml) was added (75% methanol, 25% 0.1 M sodium acetate, adjusted to pH 3.0 with HCl) and vortexed several times. The extracted tissue was centrifuged at 12,000 *g* for 10 min at 4°. The supernatant fluid was filtered through glass wool and diluted with water to a final concentration of 15% methanol. This solution was loaded onto a C18 Sep-Pak cartridge (Waters Associates, Milford, MA) that had been prewashed with 10 ml methanol, 10 ml H_2O and 10 ml 15% methanol. After loading the Sep-Pak, an additional 10 ml of 15% methanol was passed through the cartridge, followed by 20 ml of H_2O. AA metabolites were eluted with 5 ml methanol, evaporated under nitrogen, taken up in 0.5 ml methanol, and stored at −20° until analysis by RIA. The efficiencies of recovery as determined by injection of radiolabeled eicosanoids into amputed paws were as follows (mean percent ± SD, N = 4): LTB_4, 48 ± 7%; LTC_4, 28 ± 5%; PGE_2, 63 ± 6%; TXB_2, 76 ± 4%; and 5-HETE, 38 ± 12%.

Analysis of eicosanoids by RIA utilized the dextran-coated charcoal binding method as described previously [11]. For analysis of 5-HETE, a portion of the methanolic extract was fractionated by RP-HPLC (Supelco 5 μm C-18 column, methanol–water–acetic acid, 74:26:0.1, by vol., adjusted to pH 6.2 with NH_4OH, flow rate 1 ml/min, 34°), and RIA was performed on appropriate fractions corresponding to the retention time of authentic standard. The limits of detection by RIA were 10 pg for PGE_2 and $LTB_4/C_4/D_4$, 5 pg for TXB_2, and 8 pg for 5-HETE.

RESULTS

Brewer's yeast caused an immediate edema, with significant increases in foot volume evident at 5 min. The edema was maximal at 15–30 min and remained elevated for as long as 6 hr (Fig. 1a). The effect of yeast injection on vocalization threshold is shown in Fig. 1b. At 1 hr, a marked hypoalgesia was seen. Hyperalgesia was evident 3–6 hr after yeast injection, whereas saline had no significant effect on the vocalization threshold at any time point. Similar time courses for these responses have been reported [2, 3].

Injection of brewer's yeast into the rat paw stimulated the formation of arachidonic acid oxygenation products by both the CO and LO pathways; two peaks of product formation were found. The first increase in metabolites measured in extracted tissue occurred at 5 min after yeast injection and coincided with the onset of edema. The metabolites consisted of only the 5-LO products LTC_4, LTD_4 and 5-HETE

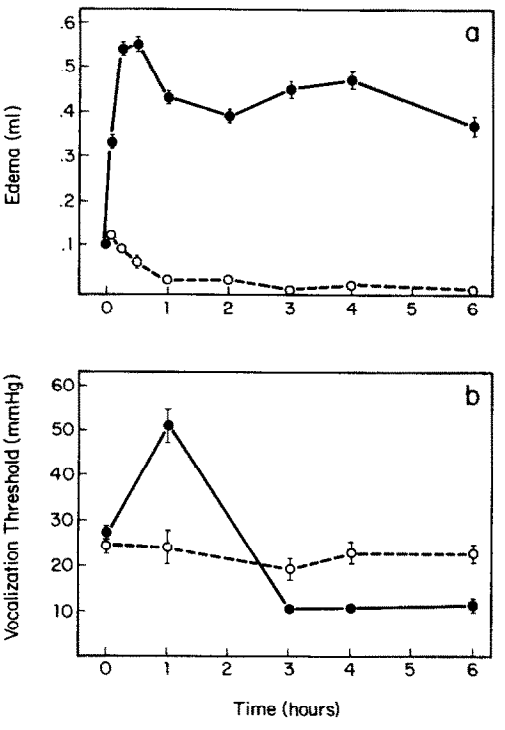

Fig. 1. Time course of edema and hyperalgesia in the rat paw. (a) Edema. Paw volumes were measured before and at various times after injection of saline (○) or brewer's yeast (●), and pre-injection volume was subtracted from these values. Points represent the mean ± SE; N = 18. (b) Hyperalgesia. Vocalization threshold (mm Hg) was measured before and at various times after injection of saline (○) or brewer's yeast (●). Points represent the mean ± SE; N = 10.

(Fig. 2, a and b). Both the amounts of these products and the edema were maximal at 15 min. The leukotrienes C_4 and D_4 existed in approximately equal amounts, and their identities were confirmed by immunoreactivity of RP-HPLC fractions corresponding to authentic standards (data not shown). Our antisera to LTC_4 cross-reacted 50% with LTD_4 and the measurements as expressed in LTC_4 equivalents were 116 ± 11 ng/paw at the 15-min peak. The amount of 5-HETE measured in the same 15-min extracts was 48 ± 7 ng/paw. Increased amounts of both 5-LO products and CO products were found at 4 hr after yeast injection, a time which is associated with maximum hyperalgesia within the inflamed paw [3, 9]. 5-Hydroxyeicosatetraenoic acid and LTB_4 were the 5-LO products (Fig. 2, b and c) present in amounts of 42 ± 3 and 35 ± 4 ng/paw respectively. The identities of the 5-LO products were confirmed by RIA of fractions from RP-HPLC (data not shown). The CO products consisted primarily of TXB_2 and PGE_2 (Fig. 3, a and b), in amounts of 8 ± 0 and 14 ± 2 ng/paw respectively.

The abilities of various compounds that are standard in the literature to modulate eicosanoids associated temporally with hyperalgesia were investigated. Drugs were administered perorally 2 hr after yeast injection, and animals were killed 2 hr later. Indomethacin inhibited both PGE_2 and TXB_2 formation

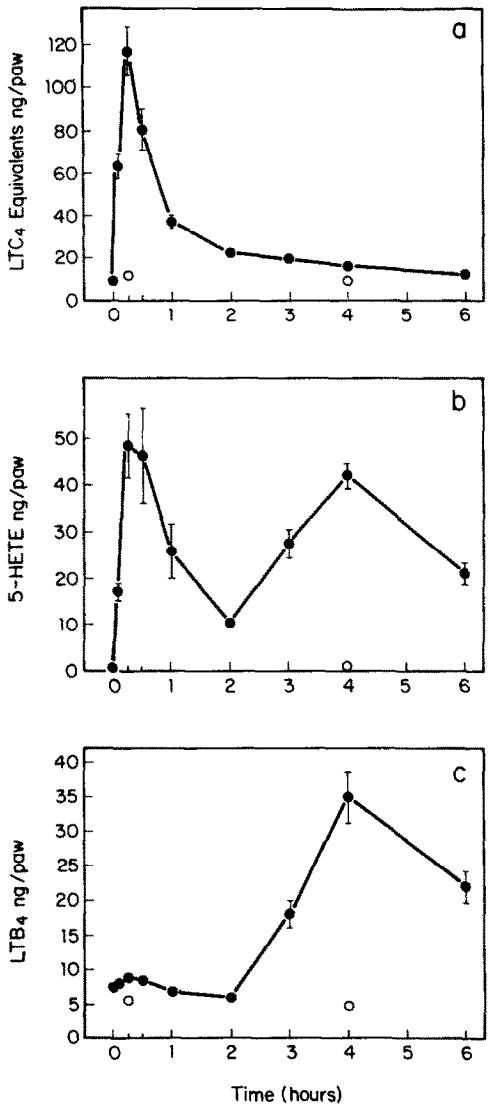

Fig. 2. Time course of 5-lipoxygenase product formation in rat paws after yeast-injection. Key: (a) LTC_4 equivalents, (b) 5-HETE, and (c) LTB_4. Animals were killed at various times, and 5-LO products were extracted and analyzed by RIA either directly (a and c) or after RP-HPLC (b). Filled circles represent yeast-injected paws; open circles represent saline-injected paws. Values are corrected for recovery efficiency and expressed as ng/paw ± SE; N = 8 for (a) and (c), and N = 4 for (b).

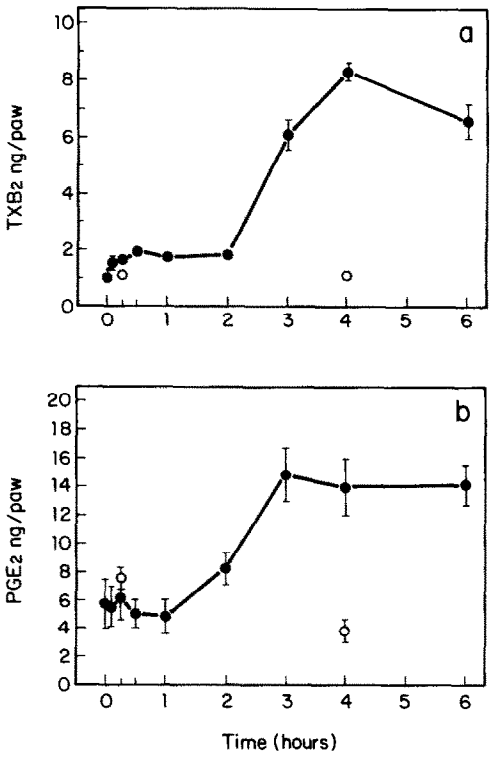

Fig. 3. Time course of cyclooxygenase product formation in rat paws after yeast-injection. Key: (a) TXB_2, and (b) PGE_2. Animals were killed at various times, and CO products were extracted and analyzed by RIA. Filled circles represent yeast-injected paws; open circles represent saline-injected paws. Values were corrected for recovery efficiency and expressed as ng/paw ± SE; N = 8.

with an ED_{50} of 0.2 mg/kg and was ineffective in inhibiting LTB_4 formation (Table 1 and Fig. 4, a and b). Dual inhibition of CO/5-LO was demonstrated by BW755C, which yielded ED_{50} values of 25.6, 7.4 and 2.4 mg/kg to inhibit LTB_4, TXB_2 and PGE_2 formation respectively (Table 1 and Fig. 4, c and d). Phenidone inhibited the production of LTB_4 with an ED_{50} of 13.9 mg/kg. No dose-related inhibition of TXB_2 or PGE_2 formation was observed (Table 1 and Fig. 4, e and f).

These pharmacological agents were also effective at suppressing the hyperalgesia in the inflamed rat paw. The quantitation of the analgesic response is presented in Table 2, designated as the "analgesic index" which represents the dose (mg/kg) required to raise the mean vocalization threshold in the inflamed paw to that of the noninjected paw of the control group. The relative participation of CO and/or 5-LO products in effecting the pain response is borne from the comparison of the drug analgesic indices with their effects on eicosanoid levels at that dose (Table 2). By this method of comparison, indo-

Table 1. Inhibition *in vivo* of eicosanoid formation by three agents p.o.

Compound	ED_{50} (mg/kg) LTB_4*	TXB_2*	PGE_2†
Indomethacin	Not inhibited	0.2	0.2
BW755C	25.6	7.4	2.4
Phenidone	13.9	>60	ND‡

Compounds were administered 2 hr after yeast injection, and measurements were determined 2 hr later.

* The ED_{50} values were derived from the curves illustrated in Fig. 4.

† The ED_{50} values for PGE_2 were derived as for other eicosanoids.

‡ No dose response observed.

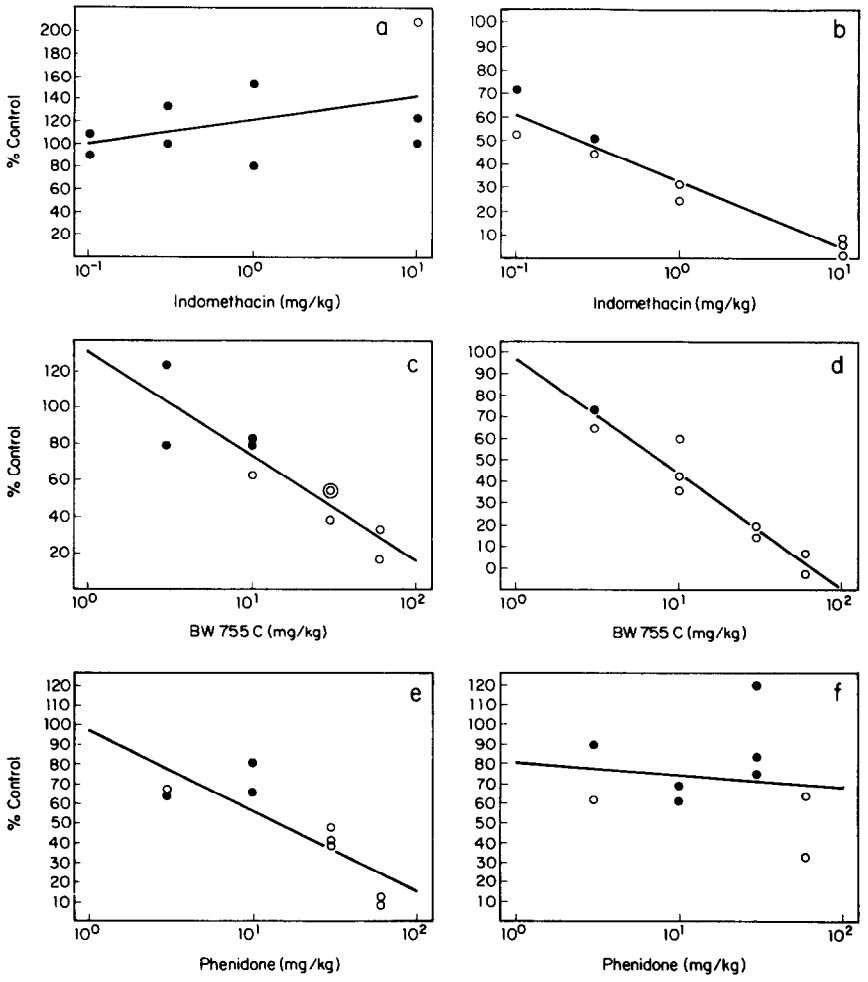

Fig. 4. Effect of perorally administered compounds to modulate eicosanoid formation in yeast-injected rat paws. Key: indomethacin (a) LTB_4 and (b) TXB_2; BW755C (c) LTB_4 and (d) TXB_2; and phenidone (e) LTB_4 and (f) TXB_2. Data are expressed as percent changes from the vehicle-dosed control group, and a combination of four experiments is shown. Each point represents the mean of four animals. The percent control was calculated after subtracting from all values the amounts of LTB_4 or TXB_2 measured in saline-injected paws. Open circles represent values significantly different from control, $P < 0.05$ by Student's *t*-test; filled circles represent values not significantly different from control, $P \geq 0.05$.

Table 2. Comparison of eicosanoid inhibition with suppression of hyperalgesia in response to pharmacological agents

Compound	Analgesic index* (mg/kg)	% Inhibition at analgesic index†		
		LTB_4	TXB_2	PGE_2
Indomethacin	0.31 ± 0.09	No inhibition	54	55
BW755C	0.70 ± 0.55	No inhibition	No inhibition	22
Phenidone	21.9 ± 5.3	58	NR‡	NR

* Mean ± SEM of three experiments. The analgesic index was computed as explained in Materials and Methods, utilizing at least four doses of each compound and ten rats per dose.

† Derived from curves illustrated in Fig. 4, except PGE_2 (curves not shown).

‡ No dose response observed.

methacin's specific inhibition of TXB_2 and PGE_2 formation at the analgesic index indicates a role for CO products in the induction of pain. A role for 5-lipoxygenase products is also indicated. Phenidone specifically inhibited LTB_4 formation at the analgesic index, suggesting a role for this 5-LO product in mediating the pain response. However, BW755C inhibited hyperalgesia more potently than would have been expected based on its ability to inhibit 5-LO and CO products; the analgesic index for BW755C was a dose at which neither CO nor 5-LO was inhibited.

DISCUSSION

The initial edema induced within the rat paw after subplantar injection of brewer's yeast was associated with increased amounts of 5-LO products. Although other mediators of edema were probably generated concurrently (e.g. histamine, serotonin), the peptidoleukotrienes produced may also have contributed to the edematous response. The work of other investigators has shown that exogenous LTC_4 and LTD_4 increase vascular permeability [12, 13], while previous work from our laboratories indicates their association with the edema of mouse ear inflammation induced with topical application of arachidonic acid [14].

A second major increase of eicosanoids coincided with the onset of hyperalgesia. Carey and Haworth [9] have reported similar findings. There appeared to be a relationship between CO product inhibition and suppression of hyperalgesia, as demonstrated by the specific CO inhibitor, indomethacin. This is in good agreement with the reported analgesic activities of CO inhibitors [4]. Leukotriene B_4 is hyperalgesic when injected into the raw paw [3, 8]. In addition, our laboratories have found 5-HETE to cause hyperalgesia in the same model (unpublished results). We have demonstrated that both of these 5-LO products are formed in response to subplantar injection of brewer's yeast into the rat paw. The ability of phenidone to specifically inhibit 5-LO product formation at similar concentrations which inhibit hyperalgesia is supportive of the hypothesis that 5-LO products are associated with the increased sensitivity to pain. The effects of indomethacin and phenidone thus suggest that inhibition of CO or 5-LO product formation may be related to their analgesic responses.

BW755C inhibited LTB_4, TXB_2 and PGE_2 formation with ED_{50} values of 25.6, 7.4 and 2.4 mg/kg respectively (Table 1). However, hyperalgesia was suppressed by BW755C at concentrations lower than required to inhibit CO or 5-LO product formation. This discrepancy may have been due to the fact that the eicosanoid measurements were performed on extracts of the whole paws. Metabolites derived from the entire tissue may mask the modulation of product formation in the microenvironment of a specific target cell or nociceptor within the paws. Within this microenvironment mediators may act in concert to elicit a physiological response such as hyperalgesia. For example, the hypothesis of Williams and Peck [15, 16] suggests that edema is the net result of two mediators, namely complement components and prostaglandins. O'Flaherty *et al.* [17, 18] demonstrated that 5-HETE acts in synergy with other lipids to induce neutrophil degranulation. Therefore, the dual inhibition of 5-LO and CO product formation by BW755C within the microenvironment may have been responsible for its potent analgesic index.

These studies suggest a role for products of both the CO and the 5-LO oxygenation pathways of arachidonic acid in mediating hyperalgesia. Furthermore, these observations demonstrate that specific inhibition of either pathway may lead to a diminution of pain.

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