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Eicosanoid production and cell accumulation induced by intrapleural injection of sodium arachidonate in the rat. Characterization of the model

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The proinflammatory effects of arachidonic acid (AA*) have been evaluated *in vivo*. Although AA lacks intrinsic chemotactic properties [1], high doses injected into rabbit skin significantly increase leukocyte infiltration [2]. Topically applied AA produces an edema in the ears of mice that can be modulated by mixed inhibitors of the 5-lipoxygenase (LO)/cyclooxygenase (CO) pathway including nor-

* Abbreviations: AA, arachidonic acid; NaAA, sodium arachidonate; CO, cyclooxygenase; HETE, hydroxyeicosatetraenoic acid; PG, prostaglandin; LT, leukotriene; LO, lipoxygenase; RIA, radioimmunoassay; PMNL, polymorphonuclear leukocytes; and i.p., intraperitoneal.

dihydroguaiaretic acid and phenidone [3]. The products of AA mediate inflammatory events including vasodilation, hyperemia, pain, edema and cell accumulation at the site of injection [4–6]. Thus, a model where release of biologically active LO products by cells at a locus where both leukocyte infiltration and LO product formation can be analyzed could prove useful for evaluation of 5-LO or mixed LO/CO enzyme inhibitors. A preliminary report of our results has been presented elsewhere [7].

Methods

Fasted adult male Sprague–Dawley rats (200–250 g, Charles River Breeding Laboratories, Wilmington, MA) were lightly anesthetized with carbon dioxide prior to intrapleural injection of either 1–40 mg/kg NaAA containing 0.08% filtered trypan blue (Gibco, 0.4% in normal saline) as an indicator or 0.9% saline plus 0.08% trypan blue (0.1 ml/100 g body weight). Rats were euthanized at various times (3 min–24 hr) after NaAA injection. Pleural cavities were washed with 6 ml of 0.9% saline containing heparin (Upjohn, 10 units/ml) Exudate fluids were centrifuged at 600 *g*, 4° for 10 min, and supernatant fractions were saved for HPLC analysis. The cell pellet was washed with 6 ml of Hanks' balanced salt solution (Gibco) and centrifuged as above, and the total cell and differential cell counts were determined using a Coulter counter (ZBI). Rats were treated i.p. with drug 1 hr prior to intrapleural injection of NaAA. Each drug treatment was administered to at least eight animals per group in two to three separate experiments, and these data were compared with those obtained after administration of the methylcellulose vehicle (0.25%, 1 ml/100 g body weight). Results are reported as the mean ± SE for each group. An analysis of variance followed by a Duncan's Multiple Range test was utilized with a value of $P \leq 0.05$ considered statistically significant.

NaAA was prepared for intrapleural injection by adding the AA (Nu-Chek Prep, Inc.) to a stoichiometric amount of base (NaOH and Na_2CO_3) in physiological saline under argon (final pH = 8). AA metabolites were extracted from cell-free supernatant fractions using a Baker 10 Extraction System (J. T. Baker, Phillipsburg, NJ) fitted with octadecyl (C18) extraction columns [8]. Columns were conditioned with methanol followed by water. The cell-free supernatant fractions were passed over the column, and the columns were washed with water. Samples were eluted from the columns with methanol, dried under nitrogen at room temperature, and resuspended in methanol for HPLC analysis. Prostaglandin B_2 was added to samples as an internal standard before extraction. Recoveries determined using labeled AA metabolites were greater than 80% using this extraction procedure. HPLC separation was achieved on a Zorbax C8 4.6 mm × 250 mm column (du Pont, Wilmington, DE) isocratically eluted with methanol/water/acetic acid (75:25:0.01) at a flow rate of 1 ml/min. Absorbance was monitored at a wavelength of 232 nm. Authentic 5-, 12- and 15-HETE standards were run with each set of samples, and elution times of sample peaks were compared with these standards. The presence of 5-HETE in cell free supernatant fractions from NaAA-injected rats was confirmed by RIA (Advanced Magnetics) of the appropriate HPLC peak after recovery using the C18 extraction column and elution of the 5-HETE with methanol:trifluoroacetic acid:triethylamine (99.8:0.12:0.06). Radioimmunoassays were conducted using commercial kits: LTB_4 (Wellcome Diagnostics, Dartford, U.K.), LTC_4 (NEN, Boston, MA) and PGE (Advanced Magnetics). The antibody for LTB_4 determination was described by Salmon *et al.* [9]. The LTC_4 antibody cross-reacted with LTD_4 (60%) and LTE_4 (9%) [10], and the PGE antibody had complete cross-reactivity with PGE_1 and PGE_2.

Results and discussion

The number of resident pleural cavity cells was approximately $3 \pm 0.4 \times 10^6$, of which 85–90% were macrophages and <2% were neutrophils (PMNL). Injection of saline containing trypan blue did not alter significantly the number of cells in the pleural cavity. Within 3 min after injection of 5 mg/kg NaAA, leukopenia developed in the pleural cavity of these rats as compared to saline-injected rats. Including trypsin in the wash to remove adherent cells did not increase cell recovery from the pleural cavity, and cell aggregates were not observed. Four hours after injection of 5 mg/kg NaAA, the number of PMNL was elevated significantly ($P \leq 0.01$) as compared to the 30-min time period (Fig. 1A). Total cell influx was increasing at 24 hr in NaAA-injected but not in saline-injected rats; PMNL in NaAA-injected rats returned to control levels by 24 hr after NaAA injection. Over the 24-hr time course, no increase in recoverable volume could be found following NaAA or saline injection.

The initial leukopenia in the pleural cavity washes following NaAA injection was accompanied by dose-related

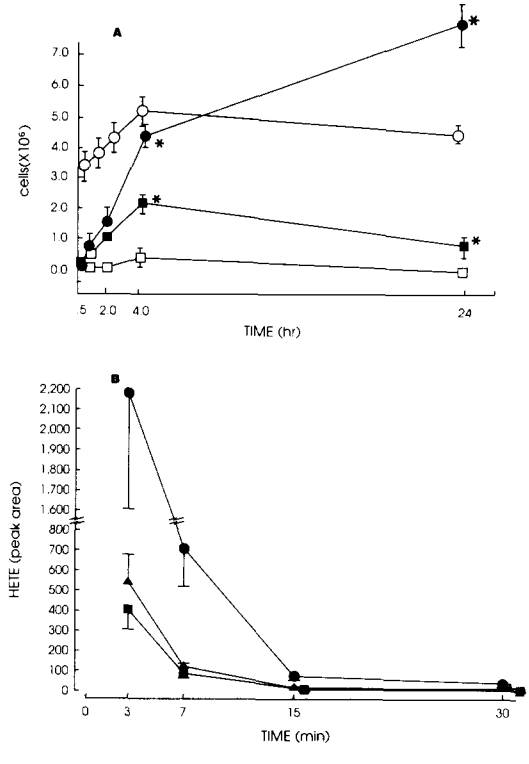

Fig. 1. (A) Time course of cell accumulation following intrapleural injection of NaAA. Animals were injected intrapleurally with NaAA (5 mg/kg) or saline and killed at various times after injection as described under Methods. Total cells following NaAA (●) or saline (○) and PMNL following NaAA (■) or saline (□) were plotted versus time. Approximately 3×10^6 cells were recovered from pleural cavities of noninjected rats. Values are means ± SE, N = 8 rats per group. Key: (*) $P \leq 0.01$ vs 30-min time point. (B) Time course of AA metabolite production in rat pleural cavity following intrapleural injection of NaAA (5 mg/kg). Production of 5-HETE (▲), 12-HETE (●) and 15-HETE (■) were measured in the pleural cavity wash following treatment with NaAA. HETE dropped below detectable levels (<10 ng) by 45 min after NaAA and could not be detected through 4 hr. Values are means ± SE, N = 6–14 rats per group at each time point.

increases in the activity of lactate dehydrogenase, a cytoplasmic enzyme, suggesting cell lysis. To distinguish whether the AA metabolites played a role in the cellular influx or whether the influx was due to cell lysis, the cytolytic agent Triton X-100 (not expected to induce AA metabolites) was injected intrapleurally. Triton X-100 (0.042, 0.21, 1.63 mg/rat) caused a dose-related cytopenia (51% reduction at the lowest dose) but produced neither an increase in PMNL at 4 hr after the injection nor HETE production even at the highest dose tested.

Rat pleural washes were analyzed for the presence of CO and LO products 7 min after injection of 5 mg/kg NaAA. LTB_4 (35 ± 6 ng/rat), LTC_4 (21 ± 1 ng/rat) and PGE (26 ± 3 ng/rat) (mean ± SE, N = 5 rats per group) were detected by RIA 7 min after intrapleural injection of NaAA and declined to near background levels by 30 min. Similarly, production of HETEs (12-HETE > 5-HETE > 15-HETE), detected by HPLÇ, was maximal at the earliest time periods tested, 3 and 7 min after intrapleural NaAA, and declined rapidly but was still significantly above background levels by 30 min (Fig. 1B). No HETEs were detected after injection of the saline–trypan blue vehicle at any time tested (3, 7, 15 and 30 min). Dose-related increases in 5-HETE at 7 min (5–40 mg/kg NaAA) and cell accumulation at 4 hr (5–20 mg/kg NaAA) were observed; a plateau was not reached in either response. The toxicity of NaAA precluded its use at higher doses. Seven minutes after NaAA, 27 ± 5 ng/rat (mean ± SE, N = 6 rats) of 5-HETE was detected when HPLC fractions were analyzed by RIA. Others [11] have found approximately 20 ng LTB_4/100 g body weight by RIA alone when 5 mg/kg NaAA was administered, which reached background by 30 min after injection.

No direct temporal correlation was observed between HETE production and cell influx. These results were similar to those of Smith *et al.* [12] who reported an influx of cells 6 hr after injection of LTB_4 in guinea pigs, a time when LTB_4 could not be detected. Simmons *et al.* [13] showed that maximal LTB_4 concentrations in carrageenan-soaked sponges correlated with the fastest rate of leukocyte infiltration rather than the largest cell number. Both investigators concluded, as one must in the present study, that the presence of LO products in the exudates did not prove a cause–effect relationship between detection of HETEs and/or LTB_4 and cell accumulation. The generation of LO products may represent a local control mechanism for the accumulation of leukocytes at the inflammatory site.

NaAA injection caused acute damage in the pleural cavity but did not lead to chronic inflammation (≥24 hr) which is seen with other inflammagens such as carrageenan. In this model, 6–8 × 10^6 cells accumulated at the peak time of PMNL influx (4 hr), whereas in carrageenan pleurisy 60–90 × 10^6 PMNL are mobilized at 3–5 hr after 500 μg carrageenan [14]. This difference in cell influx may be related to the particulate nature of the carrageenan which provides a sustained inflammatory stimulus. In contrast, NaAA is soluble and can be rapidly metabolized by multiple pathways. Intrapleural NaAA injection caused cell influx which was not associated with edema, giving it a very different character than that of non-specific inflammagens including carrageenan.

To assess the relative importance of LO and CO metabolites on cell accumulation, the effects of the CO inhibitor indomethacin and the dual CO/LO inhibitors BW755C and phenidone were evaluated for their abilities to influence HETE production and cell accumulation 7 min and 4 hr, respectively, after NaAA injection. Dosing was i.p. to minimize absorption effects. Results are shown in Table 1 and Fig. 2. Under the conditions described, indomethacin did not decrease ($P > 0.05$) HETE production or PMNL accumulation. Cell influx was inhibited significantly ($P \leq 0.01$) by BW755C at 20, 50 and 100 mg/kg and by phenidone at 100 mg/kg. 5-HETE production was reduced by BW755C, but the reduction was not statistically significant due to the variability in the 5-HETE values. Phenidone significantly inhibited 5-HETE production at 75 and 100 mg/kg ($P \leq 0.05$).

In other studies [1, 15–20], CO inhibitors either inhibited or stimulated leukocyte numbers at inflammatory sites, while BW755C consistently reduced PMNL infiltration. Boctor *et al.* [11] confirmed our preliminary data [7] using a variation of the NaAA pleural injection and showed that BW755C and phenidone inhibited 5-HETE production. Using NaAA as the inflammagen, Griswold *et al.* [21] reported that the mixed LO/CO inhibitors phenidone and SK&F86002 decreased NaAA-induced PMNL influx to the peritoneal cavity of mice but that the CO inhibitor, naproxen, was inactive.

The pharmacological specificity of the intrapleural response to NaAA was evaluated using the corticosteroid, dexamethasone, and the histamine/serotonin antagonist,

Table 1. Effect of standard anti-inflammatory drugs on 5-HETE levels 7 min after intrapleural injection of NaAA

Drug	Dose* (mg/kg, i.p.)	5-HETE† (% of control)	N‡
Indomethacin	10	101 ± 26	2
Phenidone	50	51 ± 17	5
	75	23 ± 12§	2
	100	5§	1
BW755C	20	54 ± 5	2
	50	63 ± 27	2
	100	37 ± 38	2
Dexamethasone	1	99 ± 42	2
	10	104 ± 1	2

* Drugs were administered i.p. 1 hr prior to challenge with NaAA.

† Based on HPLC peak area (mean ± SD). Vehicle control peak area: 1.42 ± 0.16 × 10^6 area units (mean ± SE).

‡ Numbers of separate experiments each containing 6–10 rats per group.

§ Significantly different vs control at $P \leq 0.05$ (Duncan's Multiple Range test).

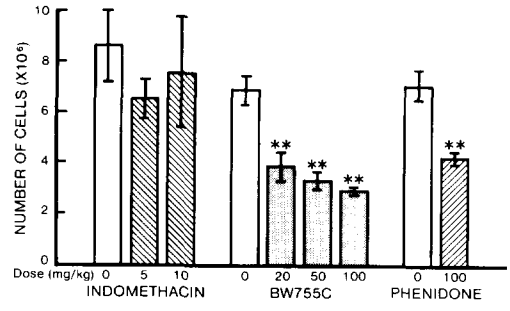

Fig. 2. Effects of CO and mixed LO/CO inhibitors on cell influx into the pleural cavity 4 hr after intrapleural injection of 5 mg/kg NaAA. Drugs were given i.p. 1 hr prior to the NaAA injection. Values are means ± SE of 4–5 experiments, 6–12 rats per group per experiment. (**) $P \leq 0.01$ (Duncan's Multiple Range test).

cyproheptadine. Animals treated with dexamethasone (1 and 10 mg/kg) demonstrated a statistically significant reduction in cell accumulation ($10.6 \pm 4.2 \times 10^6$ total cells reduced to 1.5 ± 0.2 and $1.1 \pm 0.1 \times 10^6$ cells using 1 and 10 mg/kg, respectively) with no alteration in AA metabolite production. These data suggested that the glucocorticoid effect in this model was on the PMN and their ability to respond to a chemotactic stimulus rather than on the generation of NaAA metabolites. Animals treated with cyproheptadine, 1 mg/kg, had no significant change in cell accumulation or 5-HETE production, suggesting that neither histamine nor serotonin served as mediators in rats injected intrapleurally with NaAA.

In summary, intrapleural injection of NaAA produced both a cellular response and the generation of hydroxy fatty acid metabolites of AA without edema. The cellular influx was inhibited by mixed LO/CO inhibitors and by a glucocorticoid but not by CO inhibitors. These results suggest that a model using intrapleural injection of NaAA may have utility for detecting systemically active 5-LO inhibitors. Those compounds active at reducing the cellular influx could be tested further for their effect on the generation of lipoxygenase products to distinguish between 5-LO inhibitors and compounds with glucocorticoid-like activity.

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