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# PHARMACOLOGICAL EFFECTS OF NON-STEROIDAL ANTIINFLAMMATORY AGENTS ON PROSTAGLANDIN AND LEUKOTRIENE SYNTHESIS IN MOUSE PERITONEAL MACROPHAGES

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**Abstract**—Resident mouse peritoneal macrophages, exposed to zymosan, synthesized and released products of both the cyclooxygenase and lipoxygenase pathways. The effects of various non-steroidal antiinflammatory agents were evaluated for their abilities to inhibit zymosan-stimulated prostaglandin $E_2$ ($PGE_2$) and leukotriene $C_4$ ($LTC_4$) synthesis. The order of potencies to inhibit $PGE_2$ synthesis and release was: indomethacin ≥ sulindac sulfide > ibuprofen ≥ aspirin > 3-amino-1-[3-(trifluoromethyl)-phenyl]-2-pryazoline (BW755C) > benoxaprofen ≥ nordihydroguaiaretic acid (NDGA) > 5,8,11-eicosatriynoic acid (ETYA). BW755C and ETYA also inhibited zymosan-stimulated $LTC_4$ production. None of the compounds tested showed selective inhibition of lipoxygenase products.

Two distinct arachidonic acid oxygenation pathways are found in mouse peritoneal macrophages [1, 2]. One pathway utilizes a cyclooxygenase enzyme to give rise to the prostaglandin (PG) intermediates $PGG_2$ and $PGH_2$, which are rapidly converted to $PGE_2$ and $PGI_2$ [1]. The other pathway found in the macrophage involves a 5-lipoxygenase, which leads to the production of leukotriene (LT)$B_4$ and $LTC_4$ [2, 3]. The products of both pathways can be increased dramatically by the addition of zymosan [3], whereas only $PGE_2$ and $PGI_2$ synthesis is increased by macrophages exposed to lipopolysaccharide or phorbol myristate acetate [3]. Thus, it is clear that these two pathways can be stimulated independently by inflammatory stimuli and presumably by the addition of inhibitors specific for either the cyclooxygenase or lipoxygenase enzymes.

Many of the non-steroidal antiinflammatory drugs (NSAIDS) are potent inhibitors of the fatty acid cyclooxygenase [4, 5]. Recently, one of the new antiinflammatory agents, benoxaprofen, has been reported to have low cyclooxygenase inhibitory activity but to have potent inhibitory capacity for the lipoxygenase pathway of the rabbit PMN [6]. We have studied several of the NSAIDS including indomethacin, benoxaprofen, sulindac sulfide and aspirin for their abilities to inhibit zymosan-stimulated PG and LT synthesis and secretion by macrophages. From these studies it is clear that leukotriene synthesis by mouse peritoneal macrophages, unlike that by rabbit PMN, was not inhibited by benoxaprofen. In addition, high levels of sulindac sulfide inhibited both LT and PG synthesis and secretion.

## MATERIALS AND METHODS

Female CFW-1 mice (15–25 g) and male Hartley guinea pigs were purchased from the Charles River Laboratories, Wilmington, MA. The mice and guinea pigs were maintained on standard pellet diets and water *ad lib.*

M-199 tissue culture medium, other tissue culture reagents, and porcine serum were purchased from the Grand Island Biological Co., Grand Island, NY. The porcine serum was heat inactivated (HIPS) by heating at 56° for 30 min. Twelve well tissue culture cluster plates (2.4 × 1.7 cm wells) were from the Linbro Division of Flow Laboratories, McLean, VA. Zymosan was from ICN, K&K Laboratories, Plainview, NY, and was prepared as previously described [7]. Indomethacin, sulindac sulfide (5-fluoro-2-methyl[4-methylthiobenzylidene]-inden-3-yl acetic acid), ibuprofen (2-methyl-4-(2-methylpropyl)-benzene-acetic acid), BW755C (3-amino-1-[3-(trifluoromethyl)-phenyl]-2-pyrazoline), NDGA (nordihydroguaiaretic acid), ETYA (5,8,11-eicosatriynoic acid) and aspirin were obtained from Dr. N. Jensen of this institute. Benoxaprofen (2-[4-chlorophenyl]-$\alpha$-methyl-5-benzoxazole-acetic acid) was a gift from Dr. W. Dawson, Lilly Research Laboratories, U.K. [5,6,8,9,11,12,14,-15-$^3$H]Arachidonic acid ([$^3$H]AA, 64 Ci/mmole) was obtained from the New England Nuclear Corp., Boston, MA.

Resident peritoneal macrophages were obtained by lavage from the peritoneal cavity of mice and placed into cell culture as previously described [3, 7]. The macrophages ($1 \times 10^6$) were incubated with 1.5 $\mu$Ci of [$^3$H]AA in 1 ml of M-199 containing 1% HIPS. After 16–20 hr, the medium was removed, the radiolabeled cells were washed twice with 2 ml of M-199 containing 1% HIPS, and additions of the antiinflammatory compounds were made in 1 ml of this tissue culture medium. The test compounds were added by diluting a 1000-fold concentrated DMSO stock solution 1:1000 into culture medium. The com-

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pounds were incubated 2 hr prior to the addition of zymosan. Three hours after zymosan addition, the media were removed, acidified to pH 4.5, extracted and chromatographed for both [$^3$H]$LTC_4$ and [$^3$H]$PGE_2$ as previously described [3].

One millilitre of 0.1% Triton X-100 in phosphate buffered saline was added to the cells, and the lysed cells were removed from the plate. Lactic dehydrogenase activity was determined in both the media and cell lysates by copper-neocuprione colorimetry coupled to the reduction of NAD [3]. *N*-acetyl-β-glucosaminidase activity in both the media and cell lysates was similarly determined by the method of Woolen *et al.* [8] adapted to Titertek automated colorimetry.*

For the determinations of $LTC_4$ activity by muscle contraction assays, six identical cultures per observation were incubated. After 3 hr of zymosan exposure, the media were removed, acidified to pH 4.5, and extracted with ethyl ether as previously described. The extracted aqueous phases were combined (total volume = 6 ml) and concentrated to approximately 1 ml with a Savant Speed-Vac concentrator. The volumes of the aqueous phases were measured precisely, and the amount of $LTC_4$ in the total sample was determined by bioassay on segments of guinea pig ileum as previously described [3].

## RESULTS

Resident mouse peritoneal macrophages synthesized and secreted both $LTC_4$ and $PGE_2$ when exposed to zymosan (Fig. 1A). Concomitant with the synthesis of these arachidonic acid oxygenation products, zymosan also promoted the selective secretion of the lysosomal acid hydrolase *N*-acetyl-β-glucosaminidase (NAG). The cytoplasmic enzyme lactic dehydrogenase (LDH) was retained by the cells (Fig. 1B).

The effects of various non-steroidal antiinflammatory drugs on zymosan-stimulated $LTC_4$ and $PGE_2$ synthesis were evaluated (Table 1). The cyclooxygenase inhibitors (aspirin, ibuprofen and indomethacin) selectively inhibited zymosan-stimulated $PGE_2$ synthesis without affecting the synthesis of $LTC_4$. In contrast, BW755C inhibited the zymosan-stimulated formation of both $LTC_4$ and $PGE_2$ in a dose-dependent manner. None of the compounds affected the secretion of NAG or the cellular retention of LDH.

Nordihydroguaiaretic acid (NDGA), reported to be an inhibitor of 5-lipoxygenase, inhibited zymosan-stimulated $LTC_4$ synthesis in a dose-dependent manner with an approximate $EC_{50}$ of 3 μM (Table 2). However, at 10 μM, NDGA also inhibited zymosan-stimulated $PGE_2$ synthesis. Similarly, 5,8,11-eicosatriynoic acid, which has also been reported as a specific inhibitor of 5-lipoxygenase, was a more effective inhibitor of zymosan-stimulated $LTC_4$ synthesis than zymosan-stimulated $PGE_2$ synthesis (Table 2). However, at concentrations greater than 10 μM, cytotoxicity was noted, as evidenced by LDH release (data not shown).

Benoxaprofen at concentrations from 3 to 30 μM

* P. Cameron, personal communications.

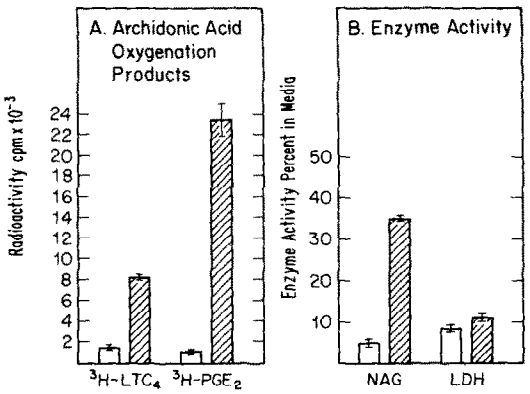


Fig. 1. Zymosan stimulation of $LTC_4$ and $PGE_2$ synthesis and the selective secretion of NAG. Resident mouse peritoneal macrophages were prelabeled with [$^3$H]arachidonic acid and subsequently exposed to zymosan (50 μg/ml) for 3 hr as described in Materials and Methods. The amounts of [$^3$H]$LTC_4$ and [$^3$H]$PGE_2$ in the media and the amounts of *N*-acetyl-β-glucosaminidase (NAG) and lactic dehydrogenase (LDH) in both the media and cells were determined as described in Materials and Methods. Key: (□) no addition; and (▨) zymosan (50 μg/ml). Results are mean ± S.D. (N = 3).

Table 1. Effects of non-steroidal antiinflammatory agents on zymosan-stimulated $LTC_4$ and $PGE_2$ synthesis*

| Compound | Conc (μM) | Percent inhibition of zymosan-stimulated synthesis $LTC_4$ | $PGE_2$ |
|---|---|---|---|
| Indomethacin | 30 | 8 | 90 |
| | 0.1 | 14 | 86 |
| | 0.03 | 10 | 70 |
| | 0.01 | 8 | 50 |
| BW755C | 30 | 97 | 99 |
| | 10 | 69 | 90 |
| | 3 | 36 | 56 |
| | 1 | 28 | 29 |
| Benoxaprofen | 30 | 0 | 77 |
| | 10 | 0 | 52 |
| | 3 | 10 | 28 |
| | 1 | 5 | 7 |
| Ibuprofen | 30 | 0 | 95 |
| | 10 | 0 | 90 |
| | 3 | 13 | 84 |
| | 1 | 6 | 53 |
| | 0.3 | 15 | 32 |
| Aspirin | 30 | 0 | 96 |
| | 10 | 1 | 91 |
| | 3 | 0 | 60 |
| | 1 | 0 | 36 |

* The compounds were preincubated with the [$^3$H]arachidonic acid-labeled cells for 2 hr. Zymosan (50 μg/ml) was then added. After 3 hr the [$^3$H]$PGE_2$ and [$^3$H]$LTC_4$ in the culture media were determined as described in Materials and Methods. The percent inhibition of the zymosan-stimulated product synthesis is the mean of three separate experiments. Zymosan stimulated [$^3$H]$PGE_2$ synthesis from 1,766 ± 219 to 39,244 ± 7,563 cpm and [$^3$H]$LTC_4$ synthesis from 4,786 ± 2,117 to 18,470 ± 3,069 cpm (the mean ± S.D. from three separate experiments).

Table 2. Effects of nordihydroguaiaretic acid (NDGA) and 5,8,11-eicosatriynoic acid (ETYA) on zymosan-stimulated $LTC_4$ and $PGE_2$ synthesis*

| Compound | Conc ($\mu$M) | Percent inhibition of zymosan-stimulated synthesis $LTC_4$ | $PGE_2$ |
|---|---|---|---|
| NDGA | 10 | 100 | 96 |
| NDGA | 3 | 48 | 9 |
| NDGA | 1 | 0 | 0 |
| ETYA | 10 | 63 | 43 |
| ETYA | 3 | 64 | 31 |
| ETYA | 1 | 46 | 12 |

* The compounds were incubated with the radiolabeled cells and exposed to zymosan as described in Table 1. Zymosan stimulated [$^3$H]$PGE_2$ synthesis from 2,016 ± 6.6 to 31,320 ± 238 cpm and [$^3$H]$LTC_4$ from 3,280 to 18,960 ± 504 cpm.

showed dose-dependent inhibition of zymosan-stimulated $PGE_2$ synthesis. However, in this macrophage cell culture system, this compound did not prevent the increased synthesis of $LTC_4$.

Sulindac sulfide, the reduced metabolite of sulindac is a potent inhibitor of zymosan-stimulated $PGE_2$ synthesis in this system with an $EC_{50}$ of approximately 0.02 $\mu$M. In addition to its established role as an inhibitor of cyclooxygenase, sulindac sulfide at high concentrations also inhibited zymosan-stimulated $LTC_4$ synthesis (Table 3). This dose-dependent inhibition was established by both the radio-release assay and independently by bioassay of the macrophage culture media on guinea pig ileum.

A summary of the pharmacological effects of these agents on zymosan-stimulated $LTC_4$ and $PGE_2$ synthesis is presented in Table 4.

Table 4. Approximate $EC_{50}$ values of selected antiinflammatory compounds to inhibit zymosan-stimulated $PGE_2$ and $LTC_4$ synthesis

| | $EC_{50}$ for the inhibition of zymosan-stimulated synthesis ($\mu$M) $LTC_4$ | $PGE_2$ |
|---|---|---|
| Indomethacin | | 0.01 |
| Sulindac sulfide | 40 | 0.02 |
| Aspirin | | 2 |
| Ibuprofen | | 1 |
| Benoxaprofen | | 8 |
| BW755C | 5 | 3 |
| 5,8,11-ETYA | 2 | >10 |
| NDGA | 3 | 7 |

## DISCUSSION

Two previous independent studies by Bray and Gordon [9] and Glatt *et al.* [10] have demonstrated that the assay of PG production from mouse peritoneal macrophages is a suitable *in vitro* system for the evaluation of antiinflammatory drugs. It is now established that, in addition to cyclooxygenase products, these cells synthesize and secrete large amounts of lipoxygenase products. Thus, this cell culture system allows the study of the regulations of the inductive steps leading to increased production of both of these products as well as of putative inhibitors of either or both of these pathways. These inhibitors can be studied with regard to potency and selectivity. We have shown previously that indomethacin and aspirin inhibit zymosan- and immune complex-induced $PGE_2$ production by cultured macrophages [11]. These classic antiinflammatory drugs, along with other compounds that have antiinflammatory activities, were compared for their effects on macrophage production of $PGE_2$ and $LTC_4$ in the same cultures. As expected, aspirin, ibuprofen and indomethacin blocked $PGE_2$ production without affecting $LTC_4$ synthesis and secretion. ETYA and BW755C were nearly equipotent in blocking zymosan-induced synthesis of these two products. Benoxaprofen, reported to inhibit the lipoxygenase pathway of rabbit PMN, had no effect on $LTC_4$ production by macrophages up to near-toxic levels but did show a dose-dependent inhibition of $PGE_2$ synthesis. Benoxaprofen has been shown to be an inhibitor of prostaglandin synthetase in microsomal preparation of ram and bovine seminal vesicles and bovine lung with a potency much less than that of indomethacin but approximately equipotent to aspirin [6, 12]. Finally, sulindac sulfide, the active metabolite of

Table 3. Sulindac sulfide inhibition of $LTC_4$ synthesis evaluated by [$^3$H]$LTC_4$ synthesis or by guinea pig ileum bioassay*

| | Conc ($\mu$M) | Percent inhibition of $LTC_4$ synthesis Radio-release assay | Muscle contraction bioassay |
|---|---|---|---|
| Sulindac sulfide | 100 | 65 | 92 |
| | 50 | 62 | 92 |
| | 25 | 32 | 49 |
| | 10 | 6 | +13 |

* Sulindac sulfide was incubated for 2 hr with macrophages or macrophages prelabeled with [$^3$H]arachidonic acid. Zymosan (50 $\mu$g/ml) was added. After 3 hr the media were collected. The [$^3$H]$LTC_4$ media from the [$^3$H]arachidonic acid-labeled cells were measured as described in Materials and Methods. The amount of $LTC_4$ activity in the media from unlabeled cells was quantified by bioassay on segments of guinea pig ileum as described in Materials and Methods. (+) Denotes a stimulation of contraction.

sulindac, blocked $PGE_2$ synthesis by macrophages as expected but, at a concentration of 25–30 $\mu$M, also inhibited $LTC_4$ production as determined either by the radio-release measurement or by muscle contractile activity. A summary of the $EC_{50}$ values for these compounds to inhibit zymosan-stimulated $PGE_2$ and $LTC_4$ synthesis is presented in Table 4.

All of these compounds tested (aspirin, ibuprofen, indomethacin, BW755C, ETYA, sulindac sulfide and benoxaprofen) have been shown to inhibit ram seminal vesicle cyclooxygenase, albeit with widely different potencies [5, 6, 13]. In the macrophage culture system, the order of potencies of these compounds for inhibition of zymosan-induced $PGE_2$ synthesis and secretion is as follows: indomethacin $\geq$ sulindac sulfide > ibuprofen $\geq$ aspirin > BW755C > benoxaprofen $\geq$ NDGA > ETYA. Two compounds, BW755C and ETYA, have been reported to inhibit lipoxygenase activity, and these compounds also blocked this synthesis of $LTC_4$ in cultured macrophages. None of the compounds tested showed selectivity for the lipoxygenase pathway in these cells.

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