Effects of Some Non-Steroidal Anti-Inflammatory Drugs and Other Agents on Cyclooxygenase and Lipoxygenase Activities in Some Enzyme Preparations

Katsuhiko MIYAZAWA, Yayoi IIMORI, Masao MAKINO, Takashi MIKAMI and Katsuhiko MIYASAKA
Pharmacological Research Department, Teikoku Hormone Mfg. Co., Ltd., Shimosakunobe 1604, Takatsu-ku, Kawasaki 213, Japan
Accepted March 4, 1985

Abstract—The inhibiting influences of some non-steroidal anti-inflammatory drugs (NSAIDs) and other agents on cyclooxygenase (CO) and lipoxygenase (LO) activities in some enzyme preparations were investigated. Washed rat platelets (CO and 12-LO), rat polymorphonuclear leukocytes (PMNs, CO and 5-LO), rat renal medulla homogenate (CO), and purified soybean LO (15-LO) were used as enzyme preparations. The IC50 values of drugs on the enzyme activities were determined in each preparation. In addition, the inhibitory activities of the drugs on the generation of chemiluminescence from PMNs stimulated by phorbol myristate acetate were tested. NSAIDs (indomethacin, ketoprofen and phenylbutazone) showed a selective inhibition of CO in each preparation, but benoxaprofen inhibited both enzymes, especially in PMNs. BW755C, 1-phenyl-3-pyrazolidone (phenidone), toluene-3,4-dithiol (dithiol), 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline (ethoxyquin) and acetone phenylhydrazone (APH) inhibited both enzyme activities. Nordihydroguaiaretic acid (NDGA) showed a relatively selective inhibition of LO in all the preparations used. APH inhibited soybean 15-LO markedly compared with the other enzymes tested. Ethoxyquin inhibited COs more markedly than LOs, and with regard to LO inhibition, it inhibited 5-LO in PMNs markedly. BW755C, phenidone, ethoxyquin, NDGA, APH and dithiol, which strongly inhibited 5-LO, showed an inhibitory activity on the generation of chemiluminescence from PMNs activated with phorbol ester.

Unsaturated fatty acids, especially arachidonic acid, are metabolized not only by cyclooxygenase (CO) but also by lipoxygenase (LO) in various cells and tissues. Since the inhibition of CO was proposed as the common mechanism for the anti-inflammatory action of some non-steroidal anti-inflammatory drugs (NSAIDs) by Vane (1), there have been many reports which support his hypothesis. However, the anti-inflammatory action of NSAIDs is not always explained solely by the inhibition of CO (2, 3).

Recently, leukotriene B4, one of the LO products of arachidonic acid, has been reported to increase vascular permeability in combination with polymorphonuclear leukocytes (PMNs) and PGE2 (4, 5), to show a potent chemokinetic (6) and chemotactic activity (7) for PMNs, and to stimulate lysosomal enzyme secretion for PMNs (8). Leukotrienes C4 and D4, other LO products of arachidonic acid, have been reported to increase vascular permeability (9). These findings suggest that LO products as well as CO products of arachidonic acid may be involved in the initiation and development of acute inflammatory reactions. In general, NSAIDs have no inhibitory activity on LO, although there are some exceptions like benoxaprofen which inhibits the LO of rabbit PMNs (10).
Recently, a variety of LO inhibitors have been reported, and most of them also have an inhibitory activity on CO. Though there are many reports about inhibitory effects of various drugs including NSAIDs, antioxidants and polyunsaturated fatty acids, etc. on the arachidonic acid cascade, it is difficult to compare the data obtained from different reports, because the enzyme preparations used in these reports differ from each other. In this study, to elucidate the characteristics of inhibitory effects of some typical anti-inflammatory drugs and antioxidants on the arachidonic acid cascade, we compared these drugs to each other with regard to their effects on arachidonic acid metabolism. For this purpose, we used 3 different CO-systems and 3 different LO-systems: washed rat platelets (CO and 12-LO) and rat PMNs (CO and 5-LO) as intact cell systems and rat renal medulla homogenate (CO) and purified soybean LO (15-LO) as cell free systems.

In addition, we tested the influences of the drugs on the generation of chemiluminescence (CL) from activated PMNs to investigate the relationship between their effects on the arachidonic acid cascade and those on the generation of CL.

Materials and Methods

The main chemicals used in the experiments were as follows: [1-14C]-arachidonic acid (55 mCi/mmol, 98–99% purity) (New England Nuclear, Boston, MA, U.S.A.), arachidonic acid (grade 1, approx. 99% purity), 4,3-phorbol 1 2'3-myristate 1 3a acetate (PMA), I-epinephrine bitartrate, lambda carrageenan (Sigma, St. Louis, MO, U.S.A.), indomethacin (Sumitomo Chemical, Osaka, Japan), phenylbutazone (Leisinger, Corona/Ti, Switzerland), 1 -phenyl-3-pyrrozolidone (phenidone), glutathione (Wako, Osaka, Japan), 6-ethoxy-1,2-dihydro-2,2,4-trimethyl-quinoline (ethoxyquin) (Tokyo Kasei, Tokyo, Japan), nordihydroguaiaretic acid (NDGA), toluene-3,4-dithiol (dithiol), gelatin fine powder (Nakarai, Kyoto, Japan), and calcium ionophore A23187 (Calbiochem-Behring, La Jolla, CA, U.S.A.). BW755C, ketoprofen, benoxaprofen and acetone phenylhydrazone (APH) were synthesized in our laboratory. Soybean lipoxygenase [EC 1.13.11.12] type I was purchased from Sigma, St. Louis, MO, U.S.A.. Precoated silica gel 60 plastic sheets for thin layer chromatography were products of E. Merck, Darmstadt, FRG. In this study, propylene glycol (less than 2% at the final concentrations) was used to dissolve NDGA and dithiol and had no effect on enzyme activities. All other drugs were dissolved with equimolar NaOH or HCl in the buffer used in each assay.

CO and 12-LO activities in platelets: Rat blood containing 0.1 volume of 100 mM EDTA-saline, pH 7.4, was centrifuged at 250xg for 10 min. Platelet-rich plasma was collected by pipetting and then centrifuged at 2,000xg for 10 min. Cells were washed with buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.4) and suspended in the same buffer to a concentration of 1×10^9 cells/ml (washed platelet suspension). One hundred /tl of 0.5 μCi/ml [1-14C]-arachidonic acid (55 mCi/mmole), 50 /ul of drug solution and 50 /ul of washed platelet suspension were mixed and incubated at 37°C for 2 min with shaking. This enzymatic reaction was stopped by the addition of 0.9 ml of 4 mM citric acid. Arachidonic acid and its metabolites were extracted with 2.4 ml of chloroform/methanol (1:1, v/v) and fractionated by thin layer chromatography (TLC) using the following solvent system: chloroform/methanol/acetic acid/water (90:8:1:0.8, v/v/v/v) (11). The radioactive areas on the TLC plates detected by autoradiography were scraped into toluene scintillator fluid containing 10% methanol, and their radioactivities were counted in an Aloka LSC-651 liquid scintillation counter. Both the fractions containing 12-hydroxyxicosatetraenoic acid (HETE) and its intermediary metabolite, 12-hydroperoxycicosatetraenoic acid, (HPETE) were regarded as 12-LO products. Thromboxane B2 and 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) fractions were regarded as CO products.

CO and 5-LO activities in PMNs: Male Wistar rats weighing 250–350 g were lightly anesthetized with ether and 0.1 ml of 2% lambda carrageenan in sterile saline was injected into the right pleural cavity using a procedure similar to that described by Hurley.
et al. (12) except that a 26-gauge needle was used instead of a 22-gauge lumbar puncture needle. After 4 hr, pleural exudate was collected and centrifuged at 250×g for 10 min. Cells were suspended in 50 mM Tris-HCl (pH 7.4) containing 100 mM NaCl to a concentration of 1.6×10⁸ cells/ml. Fifty μl of 2.0 μCi/ml [1-14C]-arachidonic acid, 50 μl of 2 μM calcium ionophore A23187 and 50 μl of drug solution were mixed. These three solutions were prepared with 50 mM Tris-HCl (pH 7.4) containing 100 mM NaCl and 1.33 mM CaCl₂. Then 50 μl of PMN suspension was added to the mixture and incubated at 37°C for 2 min. The subsequent procedures were the same as in the assay for platelet enzymes except for the solvent system: diethylether/hexane/acetic acid (30:20:1, v/v/v). Leukotriene B₄ and 5-HETE fractions were regarded as 5-LO products, and HHT was regarded as a CO product.

15-LO activity of purified soybean lipoxygenase: Soybean 15-LO activity was measured by the procedure described by Knippel et al. (13), with some modifications. Soybean lipoxydase (150 U/μg protein) was dissolved in 0.15 M potassium phosphate buffer (pH 8.0) to a concentration of 50 μg/ml. One hundred μl of drug solution and 100 μl of enzyme solution were mixed and pre-incubated at room temperature for 10 min. Then, 100 μl of 0.4 μCi/ml [1-14C]-arachidonic acid was added and incubated at room temperature for 10 min. One ml of n-hexane/diethylether (49:1, v/v) was added to stop the reaction, and unreacted arachidonic acid was removed by extracting twice with 0.6 ml of n-hexane/ethyl acetate (2:1, v/v). The radioactivity of whole metabolites in the aqueous phase which consisted mainly of PGE₂ was counted in a liquid scintillation counter.

CO activity in renal medulla homogenate: CO activity in renal medulla homogenate was measured using a procedure similar to that described by Baumann et al. (14). Male Wistar rats weighing 250–300 g were decapitated, and the kidneys were immediately removed. Then, the inner medulla was excised, frozen under liquid nitrogen and stored at −70°C. The medulla was homogenized in the cold in 0.1 M potassium phosphate buffer (pH 8.0) containing 1.6 mg/ml glutathione, at a protein concentration of 15 mg/ml. Twenty-five μl of 1.46 mg/ml L-epinephrine bitartrate, 25 μl of 0.4 mM unlabeled arachidonic acid, 50 μl of 0.5 μCi/ml [1-14C]-arachidonic acid, 50 μl of drug solution and 50 μl of medulla homogenate were mixed and incubated at 37°C for 30 min with shaking. The reaction was stopped by the addition of 0.1 ml of ethanol and unreacted arachidonic acid was removed by extracting twice with 0.6 ml of n-hexane/ethyl acetate (2:1, v/v). The radioactivity of whole metabolites in the aqueous phase which consisted mainly of PGE₂ was counted in a liquid scintillation counter.

Measurement of chemiluminescence (CL) generated from PMNs: The procedure for preparing the PMN suspension was the same as described above, except that the final concentration of PMNs was 1×10⁸ cells/ml. Hanks’ balanced salt solution (pH 7.4) (9.7 ml) containing 1% gelatin, 0.1 ml of 200 μg/ml PMA in 50% dimethylsulfoxide and 0.1 ml of drug solution in 50 mM Tris-HCl (pH 7.4) containing 100 mM NaCl were mixed in a vial. Immediately after the addition of 0.1 ml of PMN suspension, the CL generation from activated PMNs was counted in a liquid scintillation counter (Aloka LSC-651) for 9 min. In this measurement, the counter was set at the following conditions: tritium channel, coincidence mode was changed to the summation mode, 0.1 min repeat. Total counts in 9 min (it took 9 min for 30 cycles of 0.1 min count and data output) was assumed to be the amount of CL generated in the reaction.

Results

IC50 values for the drug inhibition of CO and LO activities are shown in Table 1. NSAIDs except benoxaprofen, i.e. indomethacin, ketoprofen and phenylbutazone showed a selective inhibition of CO in each
preparation, but benoxaprofen inhibited both enzyme activities, especially in PMNs.

Phenylbutazone caused the maximal inhibition (60%) of CO in PMNs at 50 μM, and no further increase in inhibition was seen at concentrations more than 50 μM. Arachidonic acid metabolism was accelerated at these concentrations (Fig. 1). Phenylbutazone also showed a weak inhibition of 5-LO in PMNs. It was less than 50%, even at the maximal inhibition.

BW755C, phenidone, dithiol, ethoxyquin and APH inhibited both enzyme activities. NDGA caused a relatively selective inhibition of LO in all the preparations used. Ethoxyquin inhibited CO activities more markedly than LO activities, and with regard to LO inhibition, it inhibited 5-LO in PMNs markedly. APH inhibited CO and LO activities to approximately the same extent except for 15-LO which was markedly inhibited. Benoxaprofen inhibited CO and 5-LO activities in PMNs markedly.

The inhibitory activities of the drugs tested on the CL generation from activated PMNs are shown in Table 2. The mean cpm of reference controls in 9 min was 3.57±0.25×10⁵ (mean±S.E., n=4). In this experiment, BW755C, phenidone, ethoxyquin, NDGA, APH and dithiol, which strongly inhibited 5-LO, showed an inhibitory activity on the generation of CL. On the other hand, NSAIDs had no effect on the CL generation or slightly enhanced it.

### Discussion

Though indomethacin and ketoprofen caused a selective and dose-dependent inhibition of CO as many reports have shown, the pattern of inhibitory action of phenylbutazone on CO was somewhat different. The inhibitory effect of phenylbutazone on CO in PMNs reached a plateau (60% inhibition) at 50 μM. Whereas CO was

---

**Table 1.** The IC₅₀ (μM) for drug inhibition of cyclooxygenase (CO) and lipoxygenase (LO) activities

| Drugs      | PL CO | PMNs CO | R. Med. CO | PL 12-LO | PMNs 5-LO | Soybean 15-LO |
|------------|-------|---------|------------|----------|-----------|---------------|
| Indomethacin | 5.9±0.25 | 4.7±0.94 | 5.07±0.57 | 916±130 | 332±10 | N.E. (1000) |
| Ketoprofen  | 1.45±0.25 | 0.68±0.17 | 2.20±0.70 | N.E. (5000) | N.E. (1500) | N.E. (3000) |
| Phenylbutazone | 79.3±27.2 | 15.7±2.1 | 20.9±2.7 | N.E. (1500) | N.D. | N.E. (1000) |
| Benoxaprofen | 2360±440 | 19.3±0.8 | 767±137 | 4440±680 | 171±26 | N.E. (3000) |
| BW755C     | 21.6±1.0 | 11.6±6.6 | 12.1±1.6 | 66.2±2.3 | 55.7±2.6 | 38.5±3.9 |
| Phenidone   | 89.3±11.2 | 125±19 | 147±18 | 66.8±4.9 | 24.4±9.7 | 7.19±2.06 |
| Ethoxyquin  | 17.3±1.0 | 5.9±2.20 | 8.9±2.80 | N.E. (1500) | 25.3±4.7 | 133±300 |
| NDGA       | 38.1±10.2 | 40.6±3.8 | 86.3±12.8 | 3.7±1.15 | 2.40±0.75 | 1.08±0.34 |
| APH        | 4.03±0.50 | 9.60±4.37 | 7.20±0.40 | 8.07±0.47 | 4.84±0.91 | 0.12±0.02 |
| Dithiol    | 6.62±1.04 | 26.3±0.8 | 43.3±10.3 | 20.1±3.9 | 24.4±1.5 | 219±99 |

The results represent the mean±S.E.M. of values obtained from at least 3 experiments. PL.: platelets, PMNs: polymorphonuclear leukocytes. R. Med.: renal medulla homogenate. N.E.: no inhibitory effect at or below the concentration given in parentheses (μM). N.D.: not determined, because the maximal inhibition was less than 50%.

---

**Fig. 1.** Effect of phenylbutazone on arachidonic acid (AA) metabolism in PMNs. Values are means ±S.E.M. obtained from 5 experiments. —○—, AA fraction; —■—, HHT fraction; —□—, LTB₄+5-HETE fractions; —□—, residual fractions.
inhibited, arachidonic acid metabolism was accelerated significantly and dose-dependently up to 1,500 μM. It is unknown what sort of metabolites were produced in this acceleration. Benoxaprofen did not affect soybean 15-LO activity (15) and 12-LO activity in the microsomal preparation of human platelets (16), but it showed an inhibitory effect on 5-LO in rabbit PMNs (10), guinea pig peritoneal cells, human promyelocytic leukemic cell line HL 60 (16) and rat basophil leukemia cells (RBL-1) (17). In accordance with these reports, in this study, benoxaprofen showed a marked inhibition of 5-LO in PMNs, a very weak inhibition of 12-LO in platelets and no inhibition of soybean 15-LO. Furthermore, it inhibited CO in PMNs markedly compared with other CO enzyme preparations. Benoxaprofen has a remarkable feature as an anti-inflammatory drug because it showed a relatively selective inhibition on the arachidonic acid cascade in PMNs.

All the drugs tested except the NSAIDs mentioned above showed inhibitory effects on both CO and LO activities. Iron is a cofactor of CO and also probably an essential component of LO, and agents which form a chelate complex with ferric ions inhibit LO activity (18, 19). In this study, dithiol, a ferric ion chelating agent, showed inhibitory effects on both enzyme activities which were observed at almost the same range of concentrations. These results suggest that its inhibitory effects on both enzyme activities are probably based on its chelating action against ferric ions. NDGA showed a relatively selective inhibition of LOs in this study. These results are consistent with those of Harvey and Osborn (20) that NDGA had no effect on CO, but selectively inhibited LO in guinea pig PMNs. APH inhibited soybean 15-LO markedly compared with the other enzymes tested. Ethoxyquin inhibited COs more markedly than LOs, and with regard to LO inhibition, it inhibited 5-LO in PMNs markedly.

Allen et al. (21) showed that human PMNs emitted CL during the process of phagocytosis and proposed that CL generated from PMNs reflected the generation of singlet oxygen. Oyanagui (22) demonstrated the participation of superoxide anions in the prostaglandin phase of rat carrageenan foot oedema, and it was reported that superoxide anion production in isolated guinea pig macrophages (23) and in guinea pig peritoneal exudate cells (24) was inhibited by NSAIDs. However in our study, NSAIDs failed to inhibit the generation of CL from PMNs. Oyanagui (23, 24) recorded the decrease of NADH absorption at 340 nm which indicates the superoxide anion production and suggested that NSAIDs blocked superoxide radical production, but did not work as radical scavengers.

In our experiment, PMNs were activated by phorbolester, and direct light emission from PMNs was monitored by a liquid scintillation counter. So, the different results may reflect
the differences in the experimental conditions, cell sources used and/or species of radicals concerned in each experimental system. In this study, only drugs which strongly inhibited 5-LO in PMNs showed an inhibitory activity on the generation of CL. It should be noted, however, that the number of drugs investigated is small, and therefore, one has to await further investigations before this statement can be generalized. It is uncertain whether their inhibitory activities on CL generation were ascribed to radical scavenger effects or other inhibitory effects on any processes in the pathways of superoxide radical generation and subsequent CL generation.

References
1 Vane, J.R.: Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nature New Biol. 231, 232–235 (1971)
2 Higgs, G.A., Eakins, K.E., Mugridge, K.G., Moncada, S. and Vane, J.R.: The effects of non-steroid anti-inflammatory drugs on leukocyte migration in carrageenin-induced inflammation. Eur. J. Pharmacol. 66, 81–86 (1980)
3 Mikami, T. and Miyasaka, K.: Effects of several anti-inflammatory drugs on the various parameters involved in the inflammatory response in rat carrageenin-induced pleurisy. Eur. J. Pharmacol. 95, 1–12 (1983)
4 Bray, M.A., Cunningham, F.M., Ford-Hutchinson A.W. and Smith, M.J.H.: Leukotriene B: a mediator of vascular permeability. Br. J. Pharmacol. 72, 483–486 (1981)
5 Wedmore, C.V. and Williams, T.J.: Control of vascular permeability by polymorphonuclear leukocytes in inflammation. Nature 289, 646–650 (1981)
6 Palmer, R.M.J., Stepney, R.J., Higgs, G.A. and Eakins, K.E.: Chemokinetic activity of arachidonic acid lipoygenase products on leukocytes of different species. Prostaglandins 20, 411–418 (1980)
7 Palmer, R.M.J. and Yeats, D.A.: Leukotriene B: A potent chemotactic agent and stimulus for lysosomal enzyme secretion for human neutrophils (PMN). Br. J. Pharmacol. 73, 260P–261P (1981)
8 Bokoch, G.M. and Reed, P.W.: Effect of various lipoygenase metabolites of arachidonic acid on degranulation of polymorphonuclear leukocytes. J. Biol. Chem. 256, 5317–5320 (1981)
9 Williams, T.J. and Piper, P.J.: The action of chemically pure SRS-A on the microcirculation in vivo. Prostaglandins 19, 779–789 (1980)
10 Walker, J.R. and Dawson, W.: Inhibition of rabbit PMN lipoygenase activity by benoxaprofen. J. Pharm. Pharmacol. 31, 778–780 (1979)
11 Vanderhoek, J.Y., Bryant, R.W. and Bailey, J.M.: 15-Hydroxy-5,8,11,13-eicosatetraenoic acid. A potent and selective inhibitor of platelet lipoygenase. J. Biol. Chem. 255, 5996–5998 (1980)
12 Hurley, J.V., Ryan, G.B. and Friedman, A.: The mononuclear response to intrapleural injection in the rat. J. Pathol. Bacteriol. 91, 575–587 (1966)
13 Knippel, I., Baumann, J., von Buchhause, F. and Wurm, G.: Interactions of sulfhydryl agents and soybean lipoygenase inhibitors. Biochem. Pharmacol. 30, 1677–1684 (1981)
14 Baumann, J., von Bruchhausen, F. and Wurm, G.: A structure-activity study on the influence of phenolic compounds and bioflavonoids on rat renal prostaglandin synthetase. Naunyn Schmiedebergs Arch. Pharmacol. 307, 73–78 (1979)
15 Kingston, W.P.: 15-Lipoxygenase: a rapid sensitive assay for lipoygenase inhibitor. Br. J. Pharmacol. 74, 919P–920P (1981)
16 Harvey, J., Parish, H., Ho, P.P.K., Boot, J.R. and Dawson, W.: The preferential inhibition of 5-lipoxygenase product formation by benoxaprofen. J. Pharm. Pharmacol. 35, 44–45 (1983)
17 Levine, L.: Inhibition of the A-23187-stimulated leukotriene and prostaglandin biosynthesis of rat basophil leukemia (RBL-1) cells by nonsteroidal anti-inflammatory drugs, anti-oxidants, and calcium channel blockers. Biochem. Pharmacol. 32, 3023–3028 (1983)
18 Pistorus, E.K. and Axelrod, B.: Iron, an essential component of lipoygenase. J. Biol. Chem. 249, 3183–3186 (1974)
19 Aharony, D., Smith, J.B. and Silver, M.J.: Inhibition of platelet lipoygenase by tolune-3,4-dithiol and other feric iron chelators. Prostaglandins Med. 6, 237–242 (1981)
20 Harvey, J. and Osborne, D.J.: A rapid method for detecting inhibitors of both cyclooxygenase and lipoygenase metabolites of arachidonic acid. J. Pharmcol. Methods 9, 147–155 (1981)
21 Allen, R.C., Sjostrom, R.L. and Steele, R.H.: Evidence for the generation of an electronic excitation state(s) in human polymorphonuclear leukocytes and its participation in bactericidal activity. Biochem. Biophys. Res. Commun. 47, 679–684 (1972)
22 Oyanagui, Y.: Participation of superoxide anions at the prostaglandin phase of carrageenan foot-
23 Oyanagui, Y.: Inhibition of superoxide anion production in macrophages by anti-inflammatory drugs. Biochem. Pharmacol. 25, 1465–1472 (1976)

24 Oyanagui, Y.: Inhibition of superoxide anion production in non-stimulated guinea pig peritoneal exudate cells by anti-inflammatory drugs. Biochem. Pharmacol. 27, 777–782 (1978)