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

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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: [$1\text{-}^{14}\text{C}$]-arachidonic acid (55 mCi/mmol, 98–99% purity) (New England Nuclear, Boston, MA, U.S.A.), arachidonic acid (grade 1, approx. 99% purity), 4β -phorbol 12 β -myristate 13 α -acetate (PMA), 1-epinephrine bitartrate, lambda carrageenan (Sigma, St. Louis, MO, U.S.A.), indomethacin (Sumitomo Chemical, Osaka, Japan), phenylbutazone (Leisinger, Corona/Ti, Switzerland), 1-phenyl-3-pyrazolidone (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 phenylhydrazine (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 $250\times g$ for 10 min. Platelet-rich plasma was collected by pipetting and then centrifuged at $2,000\times g$ 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 μl of 0.5 $\mu\text{Ci/ml}$ [$1\text{-}^{14}\text{C}$]-arachidonic acid (55 mCi/mmol), 50 μl of drug solution and 50 μl 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-hydroxyeicosatetraenoic acid (HETE) and its intermediary metabolite, 12-hydroperoxyeicosatetraenoic acid, (HPETE) were regarded as 12-LO products. Thromboxane B_2 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 $250\times g$ for 10 min. Cells were suspended in 17 mM Tris-HCl (pH 7.2) containing 0.75% NH_4Cl to lyse red cells and then centrifuged at $250\times g$ for 10 min. Cells were resuspended in 50 mM Tris-HCl (pH 7.4) containing 100 mM NaCl to a concentration of 1.6×10^6 cells/ml. Fifty μl of 2.0 $\mu\text{Ci/ml}$ [$1\text{-}^{14}\text{C}$]-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 CaCl_2 . 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 B_4 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 lipoxygenase (150 U/ μg protein) was dissolved in 0.15 M potassium phosphate buffer (pH 8.0) to a concentration of 50 $\mu\text{g/ml}$. 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 $\mu\text{Ci/ml}$ [$1\text{-}^{14}\text{C}$]-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 extracted into the organic phase. The organic and the aqueous layers were separated by freezing the latter on dry ice-acetone, and the radioactivity in each layer was measured in a liquid scintillation counter. Unreacted arachidonic acid was present in the organic phase, and LO products were in the aqueous phase. The conversion rate of arachidonic acid to LO products was calculated.

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 $\mu\text{Ci/ml}$ [$1\text{-}^{14}\text{C}$]-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 PGE_2 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 1×10^8 cells/ml. Hanks' balanced salt solution (pH 7.4) (9.7 ml) containing 1% gelatin, 0.1 ml of 200 $\mu\text{g/ml}$ 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

IC_{50} 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

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

Drugs	Enzymes					
	PL CO	PMNs CO	R. Med. CO	PL 12-LO	PMNs 5-LO	Soybean 15-LO
Indomethacin	5.90 $\pm$ 0.25	4.78 $\pm$ 0.94	5.07 $\pm$ 0.57	916 $\pm$ 130	332 $\pm$ 10	N.E. (1000)
Ketoprofen	1.45 $\pm$ 0.25	0.58 $\pm$ 0.17	2.20 $\pm$ 0.70	N.E. (5000)	N.E. (1500)	N.E. (3000)
Phenylbutazone	79.3 $\pm$ 27.2	15.7 $\pm$ 2.1	20.9 $\pm$ 2.7	N.E. (1500)	N.D.	N.E. (1000)
Benoxaprofen	2360 $\pm$ 440	19.3 $\pm$ 0.8	767 $\pm$ 137	4440 $\pm$ 690	171 $\pm$ 26	N.E. (3000)
BW755C	21.6 $\pm$ 1.0	11.6 $\pm$ 6.6	12.1 $\pm$ 1.6	66.2 $\pm$ 2.3	55.7 $\pm$ 2.6	38.5 $\pm$ 3.9
Phenidone	89.3 $\pm$ 11.2	125 $\pm$ 19	147 $\pm$ 18	66.8 $\pm$ 4.9	24.4 $\pm$ 9.7	7.19 $\pm$ 2.06
Ethoxyquin	17.3 $\pm$ 1.0	5.93 $\pm$ 2.20	8.90 $\pm$ 2.80	N.E. (1500)	25.3 $\pm$ 4.7	1333 $\pm$ 300
NDGA	38.1 $\pm$ 10.2	40.6 $\pm$ 3.8	86.3 $\pm$ 12.8	3.72 $\pm$ 1.15	2.40 $\pm$ 0.75	1.08 $\pm$ 0.34
APH	4.03 $\pm$ 0.50	9.60 $\pm$ 4.37	7.20 $\pm$ 0.40	8.07 $\pm$ 0.47	4.84 $\pm$ 0.91	0.12 $\pm$ 0.02
Dithiol	6.62 $\pm$ 1.04	26.3 $\pm$ 0.8	43.3 $\pm$ 10.3	20.1 $\pm$ 3.9	24.4 $\pm$ 1.5	219 $\pm$ 99

The results represent the mean $\pm$ 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 (μ M). N.D.: not determined, because the maximal inhibition was less than 50%.

preparation, but benoxaprofen inhibited both enzyme activities, especially in PMNs.

Phenylbutazone caused the maximal inhibition (60%) of CO in PMNs at 50 μ M, and no further increase in inhibition was seen at concentrations more than 50 μ M. 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 $\pm$ 0.25 $\times$ 10⁵ (mean $\pm$ 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

Fig. 1. Effect of phenylbutazone on arachidonic acid (AA) metabolism in PMNs. Values are means $\pm$ S.E.M. obtained from 5 experiments. —●—, AA fraction; —○—, HHT fraction; —■—, LTB₄+5-HETE fractions; —□—, residual fractions.

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 μ M. Whereas CO was

Table 2. The inhibitory activities of various drugs on the generation of chemiluminescence from stimulated PMNs

Drugs	Concentrations (μ M)				
	50	5	0.5	0.05	0.005
Indomethacin	-16				
Ketoprofen	0				
Phenylbutazone	3				
Benoxaprofen	-8				
BW755C			72	44	12
Phenidone		82	12		
Ethoxyquin		79	20	1	
NDGA	97	94	73	13	
APH	93	68	14		
Dithiol	98	83	22		

The values represent % inhibition.

inhibited, arachidonic acid metabolism was accelerated significantly and dose-dependently up to 1,500 μ M. 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 drugs 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 phorbol ester, 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.

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