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INHIBITION OF PROSTAGLANDIN SYNTHASE BY PIRPROFEN

STUDIES WITH SHEEP SEMINAL VESICLE ENZYME*

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Summary

1. Pirprofen, racemic 2-[3-chloro-4(3-pyrrolinyl)phenyl]propionic acid, was evaluated for its ability to inhibit the conversion of arachidonic acid into prostaglandin E_2 by sheep seminal vesicle prostaglandin synthase in vitro.

2. The compound proved to be a potent inhibitor with a K_i value of about 1.2 μM. Like indomethacin, aspirin and certain other non-steroidal anti-inflammatory drugs, pirprofen inhibited the enzyme competitively with respect to substrate. Unlike most non-steroidal anti-inflammatory drugs, however, pirprofen did not promote time-dependent inactivation of the enzyme. It behaved as a competitive, reversible inhibitor, whereas most of the other agents acted as competitive, irreversible inhibitors.

3. The results suggest that inhibition of prostaglandin synthesis accounts in large part for the pharmacological effects of pirprofen.

Introduction

Inhibition of prostaglandin synthase is well established as an important molecular mechanism underlying the biological effects of indomethacin, aspirin and most other non-steroidal anti-inflammatory drugs [1—8]. Pirprofen (Su-21524) is an investigational drug whose pharmacological and therapeutic effects are similar to those of indomethacin [9—12] and whose chemical structure (Fig. 1) resembles certain 2-arylpropionic acids that are among the most potent known inhibitors of prostaglandin synthesis [5]. It was of interest,

Abbreviations and trivial names: for further identification of the various prostaglandins, see refs 31 and 32.

* A preliminary account of this work was presented at the 57th Annual Meeting of the Federation of American Societies for Experimental Biology, April 15—20, 1973, Atlantic City, N.J. (ref. 26).

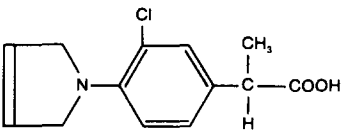

Fig. 1. Structure of pirprofen, racemic 2-[3-chloro-4(3-pyrrolinyl)phenyl] propionic acid.

therefore, to evaluate pirprofen for its ability to inhibit prostaglandin synthase. The results of studies with sheep seminal vesicle enzyme in vitro are presented in this report.

Materials and Methods

Enzyme, reagents and other materials

Lyophilized sheep seminal vesicle microsomes, isolated according to Takeguchi et al. [13], were utilized as the prostaglandin-synthesizing enzyme preparation.

Epinephrine (levorotatory form, free base), glutathione and Tris were purchased from Sigma Chemical Co., arachidonic acid and [1-^{14}C] arachidonic acid (55.5—58.0 Ci/mol) from Applied Science Labs, Inc., spectroquality ethyl acetate and acetone from Matheson, Coleman and Bell. Other solvents and chemicals used in the enzyme assay were reagent grade commercial preparations. Silica Gel 60 thin-layer plates (F-254) were obtained from Brinkmann Instruments. 2,7-Naphthalenediol was purchased from Aldrich Chemical Co., Inc. and recrystallized before use. 5,8,11,14-Eicosatetraynoic acid was provided by Dr Herbert Sheppard, Hoffmann-LaRoche, Inc., Nutley, N.J. Aspirin (U.S.P. powder) was obtained commercially. Other anti-inflammatory drugs were gifts from the manufacturers or were synthesized in these laboratories.

Enzyme assay

Enzyme activity was assayed by a modification of the procedure described by Takeguchi et al. [13]. The incubation mixture (total volume usually 1 ml) contained 0.2—10 μM [1-^{14}C] arachidonic acid (specific activity 14—58 Ci/mol), 1 mM epinephrine, 1 mM glutathione, the appropriate concentration of the compound being tested and the enzyme preparation (10—30 μg/ml) in 0.1 M Tris · HCl buffer (pH 8.3). The reaction was started by addition of enzyme. The mixture was incubated at 25 °C, a temperature at which the rate of auto-inactivation of the enzyme was not excessive [14]. Arachidonic acid was first dissolved in ethanol; the final concentration of ethanol was kept constant in each experiment and did not exceed 5% (v/v). This level did not affect the enzyme assay. Compounds being tested were dissolved in ethanol or directly in buffer, in some cases with the aid of NaOH. Care was taken that the final pH of the incubation medium did not deviate from pH 8.3.

After an appropriate incubation period (10 min unless otherwise indicated), the enzyme reaction was stopped by the addition of one drop of concentrated HCl per ml of incubation medium. The acidified mixture was saturated with Na_2SO_4 and extracted with two 5-ml portions of ethyl acetate containing 7 μM arachidonic acid. The latter was added as a carrier and also to

facilitate the subsequent thin-layer chromatographic separation of labeled compounds. The combined extracts were evaporated to dryness. The residue was dissolved in acetone and chromatographed on thin-layer plates in the solvent system described by Tomlinson et al. [15]. The plates were scanned with a Varian Aerograph Radio Scanner; the radioactive zones corresponding to prostaglandin E_2 (the predominant product) were scraped off, transferred to liquid scintillation vials and counted.

Results

Inhibition studies based on a 10-min incubation period

Like indomethacin, pirprofen acted as a potent, competitive inhibitor of sheep seminal vesicle prostaglandin synthase (Fig. 2). The K_i of pirprofen was about 1.2 μM, one of the lowest values noted among several known inhibitors of the enzyme (Table I). The latter included non-steroidal anti-inflammatory drugs [4—8] and 5,8,11,14-eicosatetraynoic acid [19,20], all of which behaved as competitive inhibitors. Also included was 2,7-naphthalenediol [21], which blocked prostaglandin E_2 synthesis in a non-competitive manner.

Time-dependent inactivation studies

Certain inhibitors of sheep seminal vesicle prostaglandin synthase, such as indomethacin, aspirin and 5,8,11,14-eicosatetraynoic acid, promote destruction of the enzyme in a time-dependent manner [6,18—20,22—26]. This effect is considered to be distinct from the instantaneous interference with enzyme activity that is observed during brief incubation periods. The time-dependent destruction can be prevented [20,24], and it does not occur with all compounds that block activity of the enzyme [23,24]. Inhibitors can be classified as reversible or irreversible on the basis of their behavior upon extended incubation with sheep seminal vesicle prostaglandin synthase (for further discussion see refs 6, 18 and 23).

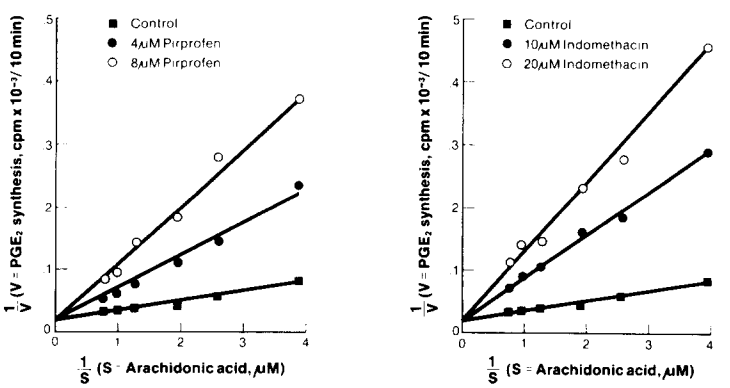

Fig. 2. Double-reciprocal plots [16] showing competitive inhibition of prostaglandin E_2 synthesis by pirprofen and indomethacin. Prostaglandin E_2 synthesis is expressed as cpm ($\times 10^{-3}$) per 0.5 ml of incubation medium during a 10-min incubation period (10 000 cpm equivalent to 0.06 nmol of prostaglandin E_2). The results of typical experiments are shown: each point represents a single measurement. The same pattern of inhibition was consistently noted in additional experiments.

TABLE I

INHIBITION OF PROSTAGLANDIN E_2 SYNTHESIS BY PIRPROFEN AND VARIOUS OTHER COMPOUNDS

Prostaglandin E_2 synthesis was measured as described in the text. K_i values were determined at substrate concentrations ranging from 0.2 to 2 μM and a constant inhibitor concentration [17]. Reversibility was assessed by determining the net synthesis of prostaglandin E_2 during a 16-h incubation in the presence of excess arachidonic acid (initial concentration of 2—6 μM) and varying inhibitor concentrations (multiples of the K_i) [18]. Each K_i value represents the mean of two to four separate determinations. Values from experiment to experiment varied somewhat, but in no case by a factor greater than 3. The mode of inhibition was shown to be consistent from experiment to experiment.

Compound	K_i (μM)	Mode of inhibition
Pirprofen	1.2	Competitive, reversible
Meclofenamic acid	2.6	Competitive, irreversible
Mefenamic acid	3.2	Competitive, irreversible
2,7-Naphthalenediol	3.4	Non-competitive, —
5,8,11,14-Eicosatetraynoic acid	4.9	Competitive, irreversible
Indomethacin	6.5	Competitive, irreversible
Oxyphenbutazone	730	Competitive, irreversible*
Phenylbutazone	98	Competitive, irreversible*
Aspirin	5500	Competitive, irreversible

* Weakly irreversible in comparison to the other irreversible inhibitors listed [18].

Irreversible inhibition by indomethacin is evident upon inspection of the curves in Fig. 3. The rate of prostaglandin E_2 synthesis tapered off after 15—30 min of incubation, and the total amount synthesized during 60 min was markedly depressed. Pirprofen, however, did not behave in this manner. As shown in

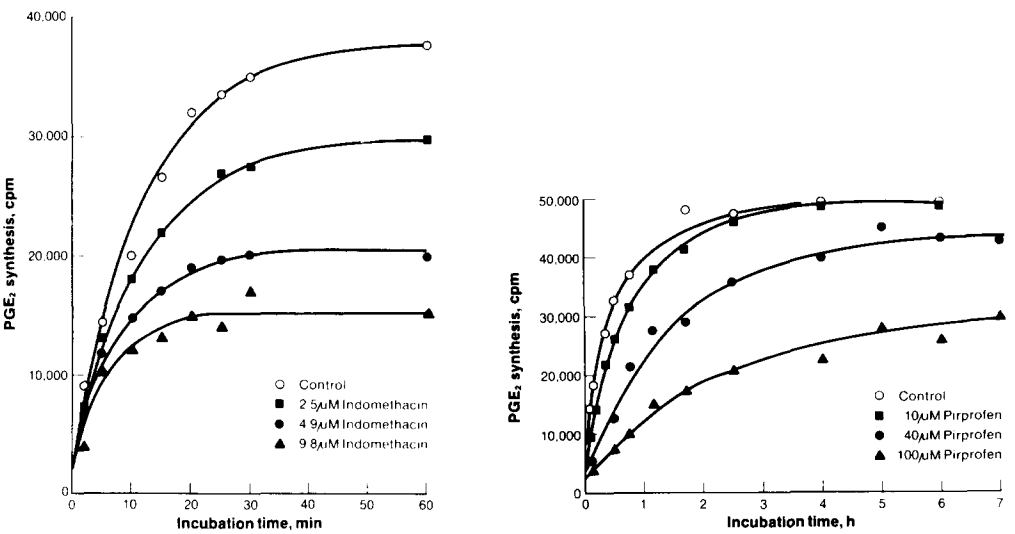

Fig. 3. Time course of inhibition of prostaglandin E_2 synthesis by indomethacin. The initial arachidonic acid concentration was 6 μM. Prostaglandin E_2 synthesis is expressed as cpm per 0.5 ml of incubation medium (10 000 cpm equivalent to 0.35 nmol of prostaglandin E_2). The results of a typical experiment are shown; each point represents a single measurement. The same pattern of inhibition was consistently noted in other experiments.

Fig. 4. Time course of inhibition of prostaglandin E_2 synthesis by pirprofen. Experimental conditions were the same as in Fig. 3 except for a prolonged incubation period. Data from a single, representative experiment are expressed as in Fig. 3 (10 000 cpm equivalent to 0.28 nmol of prostaglandin E_2).

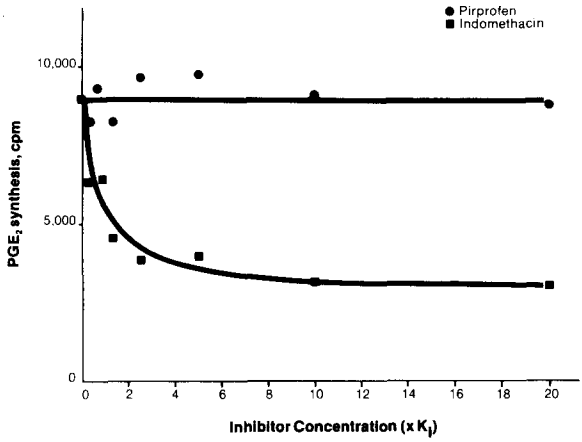

Fig. 5. Plots showing the difference in behavior of pirprofen and indomethacin with regard to time-dependent destruction of sheep seminal vesicle prostaglandin synthase during a 16-h incubation period. Experimental conditions were the same as in Fig. 3 except for the longer incubation. Data from a single, representative experiment are expressed as in Fig. 3 (10 000 cpm equivalent to 0.45 nmol of prostaglandin E_2).

Fig. 4, the final yield of prostaglandin E_2 approached the control value except when the inhibitor was present in great excess. Whereas the indomethacin time curves tapered off at inhibitor concentrations near the K_i (Fig. 3), this was not the case with pirprofen at concentrations up to at least 20 times the K_i (Fig. 3). Thus, pirprofen behaved as a competitive, reversible inhibitor.

All of the compounds listed in Table I were evaluated over a wide concentration range for their ability to promote inactivation of sheep seminal vesicle prostaglandin synthase during a 16-h incubation period (for further details see ref. 18). With the exception of pirprofen, all of the competitive inhibitors could be classified as irreversible. Under the specified experimental conditions indomethacin (Fig. 5) and most of the other compounds prevented prostaglandin E_2 synthesis to the extent of about 50% at concentrations 1—2 times their K_i value. In the case of phenylbutazone and oxyphenbutazone, concentrations 7—10 times the respective K_i values were required to produce 50% inhibition [18]. The net production of prostaglandin E_2 was not affected by pirprofen at concentrations up to 20 times the K_i (Fig. 5).

Competitiveness between pirprofen and indomethacin in their interaction with sheep seminal vesicle prostaglandin synthase

Since both pirprofen and indomethacin acted as competitive inhibitors, it was reasoned that they should compete with each other for the substrate-binding site or for a common binding site nearby. Thus, under appropriate conditions pirprofen would be expected to interfere with the time-dependent destruction of the enzyme in the presence of indomethacin. The curves in Fig. 6 show that this indeed occurred. Inactivation of the enzyme by 10 μM indomethacin was antagonized by 10 or 30 μM pirprofen. As expected, inhibition of prostaglandin E_2 synthesis during early stages of incubation was greater in the presence of both agents than in the presence of indomethacin alone.

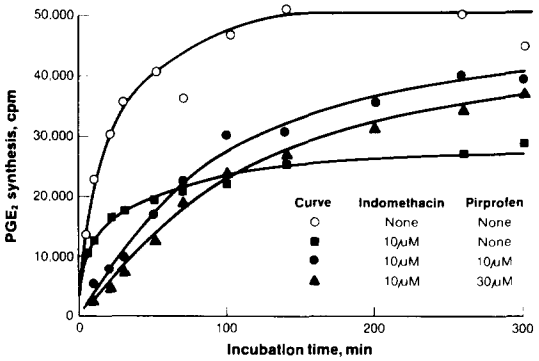

Fig. 6. Protective effect of pirprofen against the time-dependent destruction of sheep seminal vesicle prostaglandin synthase by indomethacin. Experimental conditions were the same as in Fig. 3. Data from a single, representative experiment are expressed as in Fig. 3 (10 000 cpm equivalent to 0.28 nmol of prostaglandin E_2).

Concentrations of pirprofen higher than 30 μM caused persistent inhibition of the enzyme, which made it impossible to discern protection against inactivation.

The competitiveness between pirprofen and indomethacin is also illustrated in the multiple-inhibition experiment shown in Fig. 7. The increase in the apparent K_i of pirprofen in the presence of indomethacin is indicative of competition between the two compounds as they interact with the enzyme [27].

Inhibitory potencies of stereoisomers of pirprofen and naproxen

It has previously been noted in studies with several enantiomeric pairs of 2-arylpropionic acids that the dextrorotatory isomers are much more effective inhibitors of prostaglandin synthesis than their enantiomers [5,15,21,28]. The data presented in Table II show that dextrorotatory pirprofen was also more potent than its levorotatory isomer as an inhibitor of sheep seminal vesicle

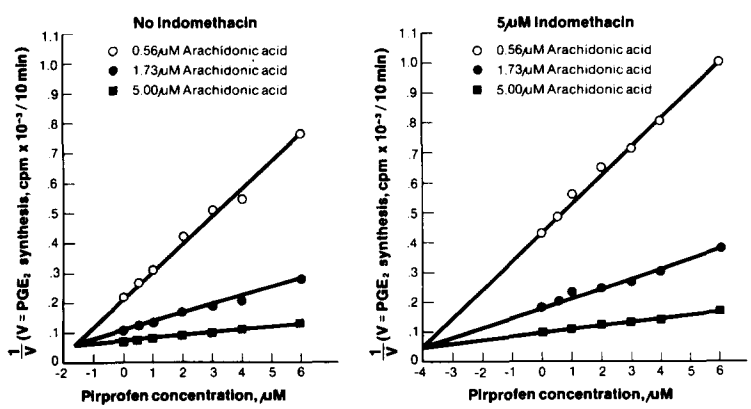

Fig. 7. Plots of multiple-inhibition data [27] showing competitiveness between pirprofen and indomethacin as inhibitors of sheep seminal vesicle prostaglandin synthase. Data from representative experiments are expressed as in Fig. 2 (10 000 cpm equivalent to 0.07 nmol of prostaglandin E_2).

TABLE II

INHIBITION OF PROSTAGLANDIN E_2 SYNTHESIS BY STEREOISOMERS OF PIRPROFEN AND NAPROXEN

K_i values were determined as described in Table I. Each value represents the mean of two or more separate determinations. Values from experiment to experiment did not vary beyond the range specified in Table I.

Compound	K_i (μM)
Dextrorotatory pirprofen	1.3
Levorotatory pirprofen	8.3
Naproxen	1.8
Levorotatory naproxen	240

prostaglandin synthase. However, the difference in potency between the two isomers was far less than that between naproxen and its enantiomer. The dextrorotatory and levorotatory isomers of both compounds behaved as competitive inhibitors in 10-min incubation experiments. Of the isomers listed, only naproxen was evaluated in extended incubation experiments. Inhibition was weakly irreversible as in the case of phenylbutazone and oxyphenbutazone.

Discussion

The data in this report show that pirprofen is a potent inhibitor of sheep seminal vesicle prostaglandin synthase in vitro, that it acts competitively with respect to substrate, and that unlike certain other competitive inhibitors, it does not cause time-dependent inactivation of the enzyme. The compound is also a potent inhibitor of prostaglandin synthesis by bovine cerebral cortex microsomes [29], microsomal fractions of tissues from the rabbit eye [30] and 0—9000 × *g* sediments of human platelet homogenates (Ku, E.C. and Signor, C., unpublished).

Although the experiments described herein were based upon measurement of prostaglandin E_2 synthesis, studies with bovine cerebral cortex enzyme in the presence of added Cu^{2+} have shown that pirprofen is equally effective as an inhibitor of prostaglandin $F_{2\alpha}$ synthesis [29]. This was expected, of course, in view of the fact that the compound acts competitively with respect to arachidonic acid which serves as the precursor of both prostaglandins through a common pathway [31,32]. Pirprofen might also be expected to interfere with the formation of prostaglandins G_2 and H_2, peroxide intermediates in the synthesis of prostaglandins E_2 and $F_{2\alpha}$ from arachidonic acid [31,32]. Pirprofen has not yet been evaluated for its ability to interfere with prostaglandin synthesis from poly-unsaturated fatty acids other than arachidonic acid.

Pirprofen apparently possesses some structural feature that prevents it from inactivating sheep seminal vesicle prostaglanding synthase, which distinguishes it from most of the other competitive inhibitors evaluated in this study. It seems likely that the competitive, irreversible inhibitors cause or promote inactivation of the enzyme subsequent to formation of the enzyme-inhibitor complex. The ability of pirprofen to protect against this effect, as described in the experiments with indomethacin and as also noted in studies with aspirin (Ku, E.C. and Wasvary, J.M., unpublished), may be attributable to competitive

occupancy of the binding site by the non-destructive inhibitor. The antioxidant ethoxyquin also inhibits sheep seminal vesicle prostaglandin synthase competitively without causing time-dependent inactivation, and it prevents the destructive action of the competitive, irreversible inhibitor 5,8,11,14-eicosatetraynoic acid [24]. However, competitive binding may not be the only factor underlying the protective effect of this compound, since α-naphthol, an antioxidant that inhibits the enzyme non-competitively, also prevents the destructive effect of 5,8,11,14-eicosatetraynoic acid [24]. The susceptibility of the pyrrolinyl moiety to oxidation [33] may be an important determinant of pirprofen's capacity to inhibit the enzyme competitively without causing time-dependent inactivation and also of its ability to protect against destruction by other inhibitors.

Preparations of prostaglandin synthase from different tissue sources are not equally susceptible to time-dependent inactivation. In experiments with bovine cerebral cortex microsomes [29], no distinction could readily be drawn between pirprofen and the competitive, irreversible inhibitors of the sheep seminal vesicle enzyme listed in Table I. None of the compounds exhibited a destructive effect in the absence of added Cu^{2+}. On the other hand, in studies with the 0—9000 × *g* sediment of human platelet homogenate (Ku, E.C. and Signor, C., unpublished), time-dependent inactivation was apparent in the presence of all of the compounds, including pirprofen. It remains to be determined whether intrinsic differences in the enzymes account for these variations in behavior. The crude enzyme preparations employed might have contained variable quantities of one or more substances that influenced the results.

It should be pointed out that the experimental conditions in our studies with sheep seminal vesicle prostaglandin synthase differed significantly from those in certain previously reported studies. When possible, we employed low concentrations of substrate, incubated briefly at 25°C and avoided preincubation of the enzyme with inhibitors. It has been our experience that these measures minimize the influence of enzyme inactivation by substrate and inhibitors. We also avoided the use of hydroquinone as a cofactor because of its ability to inhibit sheep seminal vesicle prostaglandin synthase [24]. These points should be taken into consideration when our data are compared with those from other studies.

In view of the present findings and of the mounting evidence that inhibition of prostaglandin formation is the principal mechanism of action of most non-steroidal anti-inflammatory agents [1—8], we consider it likely that the pharmacological effects of pirprofen are attributable in large part to inhibition of prostaglandin synthase.

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