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Prostaglandin H Synthase: Perturbation of the Tyrosyl Radical as a Probe of Anticyclooxygenase Agents

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SUMMARY

EPR spectroscopy was used to study the effects of various nonsteroidal anti-inflammatory agents on the peroxidase-related tyrosyl radical present in prostaglandin H synthase (prostaglandin endoperoxide synthase; EC 1.14.99.1). Two types of perturbation of the tyrosyl radical by these anticyclooxygenase agents were observed. In the first case, aspirin, indomethacin, ibuprofen, (*S*)-flurbiprofen, and (*S*)-naproxen converted the doublet tyrosyl EPR signal seen on reaction of the uninhibited enzyme with ethyl hydroperoxide to a singlet bearing additional partially resolved hyperfine splittings. These compounds also decreased the maximum amount of radical generated, but they did not change the kinetics of formation and decay of the tyrosyl radical. In the second case, acetaminophen and three fenamate analogs (meclofenamate, flufenamate, and mefenamate) did not perturb the EPR line shape observed after reaction with hydroperoxide but did cause a more rapid decay of the tyrosine radical species. It would appear that, despite considerable variation in structure, the nonsteroidal anti-inflammatory agents may inhibit the cyclooxygenase activity of the synthase by two basic mechanisms.

The cyclooxygenase activity in PGH synthase catalyzes the first committed step in the biosynthesis of prostaglandins, thromboxanes, and prostacyclin (1). The pure synthase also has a heme-dependent peroxidase activity that is believed to initiate the cyclooxygenase reaction via the formation of a tyrosyl free radical (2, 3). A large number of inhibitors of the cyclooxygenase activity, termed nonsteroidal anti-inflammatory drugs because of their pharmacological effects, have been identified. These agents can be classified according to their effects on the cyclooxygenase reaction kinetics, as substrate analogs that interfere with the binding of arachidonate in either a reversible (e.g., ibuprofen) or an irreversible manner (e.g., aspirin and indomethacin) or radical-quenching agents (e.g., acetaminophen) that interfere with the initiation of the cyclooxygenase reaction (4). In studies using EPR spectroscopy, indomethacin has been found to cause structural changes in the tyrosyl radical similar to those seen after treatment of the synthase with tetranitromethane, an agent that selectively nitrates tyrosyl residues and that also inhibits the cyclooxygenase activity (3). This suggested that the mechanism of inhibition by indomethacin involved its action on the radical, and this has led us to examine the effects of a number of other anticyclooxygenase agents on the tyrosyl radical, using EPR spectroscopy. The results of this study indicate that, based on their actions on the tyrosyl radical, these agents can be grouped into two classes. The first, which includes indomethacin and aspirin, perturbed the structure of the tyrosyl radical, whereas the second, including several substrate analogs and acetaminophen, did not perturb the structure of the radical but, rather, led to a more rapid decrease in its concentration.

Experimental Procedures

Aspirin, heme, indomethacin, and acetaminophen were from Sigma Chemical Co., (St. Louis, MO). Sodium meclofenamate and ibuprofen were purchased from Biomol Research Laboratories, (Plymouth Meeting, PA). Flufenamic acid was from Aldrich Chemical Co., (Milwaukee, WI). The resolved enantiomers of flurbiprofen were generous gifts from the Upjohn Co., (Kalamazoo, MI). Mefenamic acid was a gift from the Parke-Davis Co. Naproxen and its inactive enantiomer were gifts from Syntex Inc. Arachidonic acid was from NuChek Preps Inc., (Elysian, MN). EtOOH was purchased as a 5% aqueous solution from Polysciences Inc., (Warrington, PA).

PGH synthase was purified to electrophoretic homogeneity from sheep seminal vesicles, as described earlier (5). The protein concentration was determined by a modification of the Lowry method (14) or by the absorbance at 279 nm ($A^{1\%}$ = 16.5). The procedures for assay of cyclooxygenase and peroxidase activities have also been described (3). The cyclooxygenase specific activity of the synthase preparations used in this study was 100–125 μmol of O_2/min/mg of protein. The holoenzyme was reconstituted by addition of heme and treatment with DEAE-

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ABBREVIATIONS: PGH, prostaglandin H; EtOOH, ethyl hydrogen peroxide.

cellulose (6). In the holoenzyme used for EPR, the heme concentration was 15–27 μM (extinction coefficient at 411 nm of 165 mM^{-1} cm^{-1}).

The aspirin-acetylated synthase was prepared by incubation of the synthase (88% in holoenzyme form) with 0.3 mM aspirin at room temperature for 65 min (<3% of the initial cyclooxygenase activity remained), followed by removal of excess reagent by chromatography on a desalting column (Bio-Rad 10DG) equilibrated and eluted with 50 mM potassium phosphate, pH 7.4, 30% glycerol, 0.05% octyl glucoside. About 90% of the initial peroxidase activity remained. For examination of the effects of meclofenamate, flufenamate, mefenamate, (*S*)-flurbiprofen, (*R*)-flurbiprofen, ibuprofen, indomethacin, acetaminophen, (*S*)-naproxen, and (*R*)-naproxen, the agent [at 0.6 mol/mol of synthase subunit, except for ibuprofen and acetaminophen, which were 100 μM, and (*R*)-flurbiprofen, which was tested at 0.3, 0.6, and 1.2 mol/mol of synthase subunit] was incubated with PGH synthase holoenzyme at room temperature for at least 15 min.

Each sample was placed in a quartz EPR tube and equilibrated in a water/ethylene glycol bath at −10° before injection of EtOOH (in a 3–5-μl aliquot) to give a final peroxide concentration 10 times that of synthase heme. After immediate manual mixing for about 5 sec with a cooled stirrer made of nichrome wire, the incubation was continued at −10° for a total of 15 sec (shorter times where indicated) before the tube was plunged into a dry ice/acetone bath to freeze the sample in 1–2 sec; the frozen samples were transferred to a liquid nitrogen bath for storage until their EPR spectra could be recorded. When longer reaction times were desired, a tube initially reacted at −10° for 15 sec was removed from liquid nitrogen and placed back in the −10° water bath; the additional reaction time was calculated from the point at which the sample was about half thawed (typically 35 sec). Kinetic data obtained in this manner did not appear to be different from our previous data that used separate samples for each time point (3). The present procedure minimized the need to calibrate the sample tubes and considerably conserved the supply of enzyme.

EPR spectra were recorded with a Varian E-6 spectrometer equipped with an Air Products flexible transfer line for cooling with liquid helium. The $g = 2$ region of the spectrum was recorded using a microwave power of 10 μW and a modulation amplitude of 2 G; the frequency was 9.29 GHz and the temperature was 12°K. Quantitation of the intensity of the hydroperoxide-induced radicals was done by double integration, with reference to a cupric sulfate standard; the values were corrected for variations in the diameter of the EPR sample tubes.

Results

Addition of EtOOH to the resting synthase resulted in the rapid generation of an EPR doublet in the $g = 2$ region (Fig. 1, *left, spectrum a*), followed by the slower conversion of the doublet to a singlet signal with similar overall peak to trough width (Fig. 1, *right, spectrum a*); very similar signals have been reported before and ascribed to a tyrosyl free radical (2, 3). The intensity of the radical signal, obtained by double integration, peaked after about 150 sec, with about 0.6 spin/heme (Fig. 2A).

Acetylation of the synthase by aspirin before reaction with EtOOH resulted in dramatic changes in the EPR spectrum; only a narrower (peak to trough width of 26 G) singlet with clear hyperfine features was seen throughout the reaction (Fig. 1, *left* and *right, spectrum i*). The spectrum of the radical obtained with the acetylated synthase is reminiscent of that observed with the indomethacin-synthase complex (3) (Fig. 1, *left* and *right, spectrum g*). The maximal intensity of the radical signal was lower in the acetylated synthase, peaking at 0.3 spin/heme; the rate of decline of the radical intensity was not greatly different from that of the control (Fig. 2).

Several other anticyclooxygenase agents were also examined for their effects on the EPR spectrum of the EtOOH reaction product. Ibuprofen and the more inhibitory isomers of flurbiprofen and naproxen each resulted in the singlet EPR signal seen with both the acetylated enzyme and the indomethacin-synthase complex; the shape of these spectra did not change throughout the reaction (Fig. 1, *left* and *right, spectra e, f,* and *h*). Addition of the less inhibitory *R*-isomer of flurbiprofen (at 0.3 or 0.6 mol/subunit) before the hydroperoxide resulted in spectra that appeared to be combinations of the control doublet and the singlet seen with the active isomer. At 1.2 mol/subunit (*R*)-flurbiprofen, the resulting spectrum was indistinguishable from that with the active *S*-isomer at 0.6 mol/subunit (data not shown). The inactive noninhibitory isomer of naproxen had no effect on the EPR spectrum of the hydroperoxide reaction product or the intensity of the radical signal (data not shown).

Surprisingly, three potent anticyclooxygenase agents, meclofenamate, flufenamate, and mefenamate, had no effect on the shape of the spectrum of the hydroperoxide reaction product, with the doublet appearing at short reaction times and the singlet at longer reaction times, just as in the control (Fig. 1, *left* and *right, spectra b–d*). However, these agents each caused a much more rapid decay in the intensity of the radical species than seen in the control (Fig. 2). Acetaminophen also had no effect on the shape of the spectrum of the hydroperoxide reaction product (data not shown) but led to a much smaller accumulation of the radical, <0.2 spin/heme, and to a very rapid decline in its intensity, with almost no signal remaining after 90 sec (Fig. 2A).

Discussion

Several lines of evidence implicate a hydroperoxide-induced tyrosyl radical species in cyclooxygenase catalysis by PGH synthase. (a) Reaction of the synthase with hydroperoxide generates a free radical species whose EPR spectrum is similar to that observed for the tyrosyl free radical in ribonucleotide reductase (2). (b) Tetranitromethane treatment leads to nitration of about two tyrosyl residues in the synthase, loss of cyclooxygenase activity, and alteration of the EPR spectrum of the hydroperoxide-induced radical (3). (c) Replacement of Tyr-385 by phenylalanine by site-directed mutagenesis results in the selective loss of cyclooxygenase activity (7). (d) Treatment of the synthase with aspirin, indomethacin, flurbiprofen, ibuprofen, or naproxen results in inhibition of the cyclooxygenase activity and the alterations in the EPR spectrum of the hydroperoxide-induced radical (see Results and Ref. 3). In contrast, peroxidase activity in the synthase is not significantly affected by the anticyclooxygenase agents that clearly perturb the hydroperoxide-induced radical species (8). Thus, either the radical species has no role in the peroxidase catalytic cycle itself or peroxidase catalysis tolerates changes in the radical structure that cyclooxygenase catalysis does not. The differential effects on the cyclooxygenase and peroxidase activities are quite consistent with the proposed function of the tyrosyl radical as a mechanistic link between the two activities (2, 3).

The observation that the changes in the cyclooxygenase activity and in the radical spectrum seen with the anticyclooxygenase agents like aspirin and indomethacin are similar to those that accompany covalent modification with tetranitromethane (see Results and Ref. 3) suggests that these compounds inhibit the cyclooxygenase activity by perturbing the structure of the tyrosyl radical. The marked similarity of the

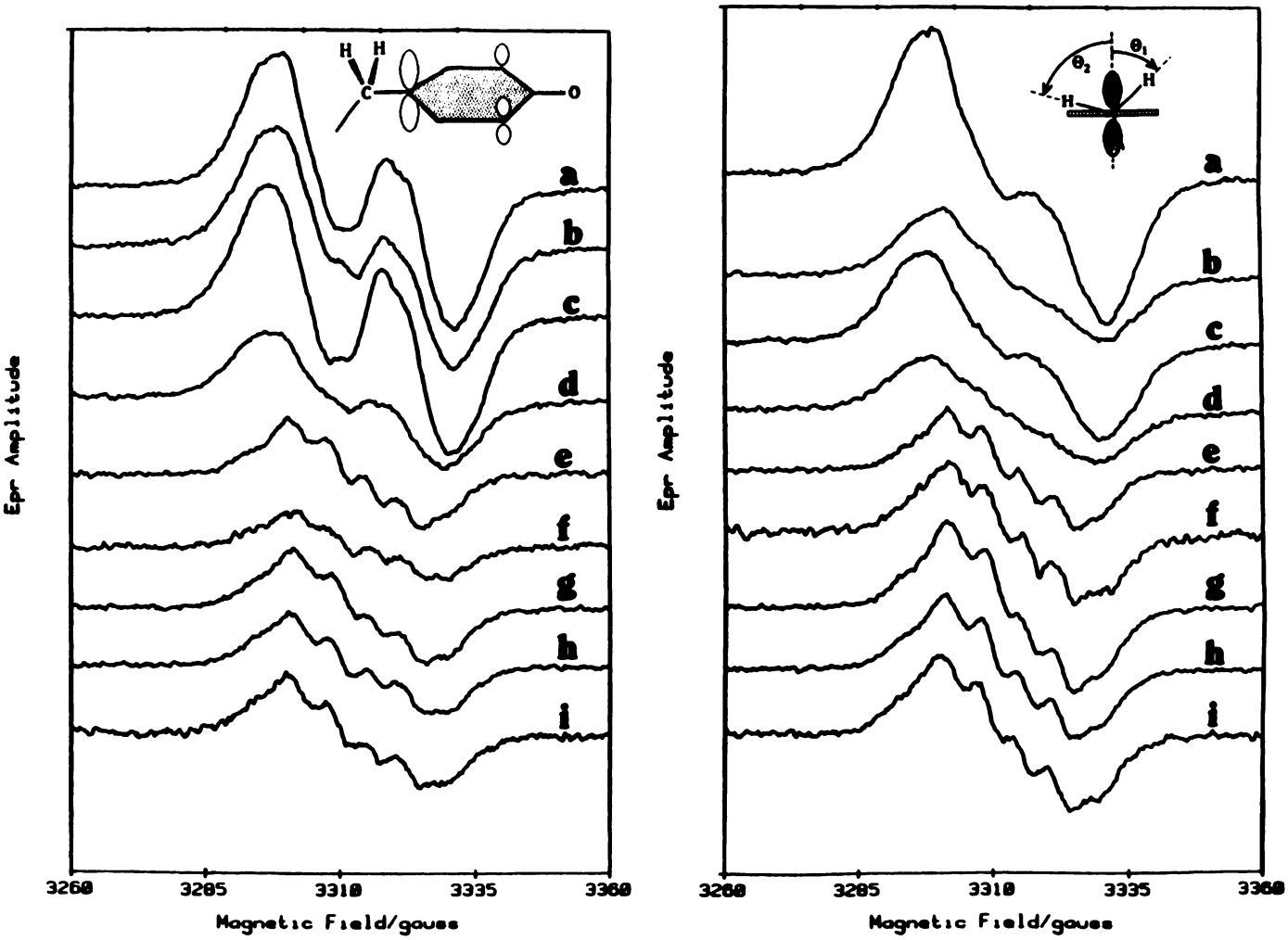

Fig. 1. Effects of anticyclooxygenase agents on the EPR spectrum of the synthase tyrosyl radical. The synthase holoenzyme was pretreated with the agents before reaction at −10° with a 10-fold molar excess of EtOOH, and the spectra were recorded as described in Experimental Procedures. *Left*, spectra in the $g = 2$ region (normalized to represent contributions from the same synthase heme concentration) obtained after short (5–10-sec) reaction periods; *right*, those obtained after longer reactions (75–120 sec). The individual spectra are from the control (*a*) and from synthase pretreated with meclofenamate (*b*), flufenamate (*c*), mefenamate (*d*), ibuprofen (*e*), (*S*)-naproxen (*f*), indomethacin (*g*), (*S*)-flurbiprofen (*h*), and aspirin (*i*). *Insets*, schematic of phenyl ring conformation in the tyrosyl radical. *Left*, ovals above and below the plane of the phenyl ring at C1, C3, and C5 represent p_z orbitals. *Right*, a view along the carbon-carbon bond between the methylene group and the phenyl ring (shown edge on); the dihedral angles to the two methylene protons from the p_z orbital axis are indicated.

EPR spectra of the tyrosyl radicals in synthase pretreated with aspirin, indomethacin, (*S*)-flurbiprofen, ibuprofen, and naproxen suggests that the same structural alteration is induced by all of these reagents. This conclusion is somewhat surprising, given the widely different structures of these agents (Fig. 3) and the differences in their inhibitory effects; ibuprofen and naproxen are reversible agents, flurbiprofen and indomethacin have time-dependent irreversible (but probably noncovalent) effects, and aspirin acetylates a serine residue on the synthase (see Table 1).

One possibility for a common structural action by these different agents is suggested by the recent results of Barry *et al.* (9). The EPR spectra of model tyrosyl radicals free in alkaline solution are very similar in shape to those shown in Fig. 1 for the radical in synthase treated with indomethacin, aspirin, flurbiprofen, naproxen, and ibuprofen. Barry *et al.* (9) calculated that the most stable conformation of the model tyrosyl side chain has the methylene protons oriented at angles of 45° and −75° to the normal to the plane of the aromatic ring. By assuming no change in electron distribution (9), the wider hyperfine splitting found in the tyrosyl radical signal in the untreated synthase implied a less stable conformation, with the methylene protons oriented at angles of 35° and −85°. Agents like indomethacin and aspirin would appear to release the tyrosyl radical from this strained conformation, thus leading to a more relaxed conformation incompatible with catalytic activity. This attractive interpretation requires that the perturbation of the EPR signal by the anticyclooxygenase agents and tetranitromethane does not reflect a redistribution of electron density in the radical; there are as yet no data that would invalidate this basic assumption (9).

A model in which the tyrosyl radical can adopt either an active or an inactive conformation might explain why synthase acetylated by aspirin has no detectable cyclooxygenase activity, whereas the tight but noncovalent complex of the synthase with indomethacin is still capable of significant cyclooxygenase catalysis (4% of the initial velocity, 100% of the reaction extent before self-inactivation). Acetylation of Ser-506 by aspirin

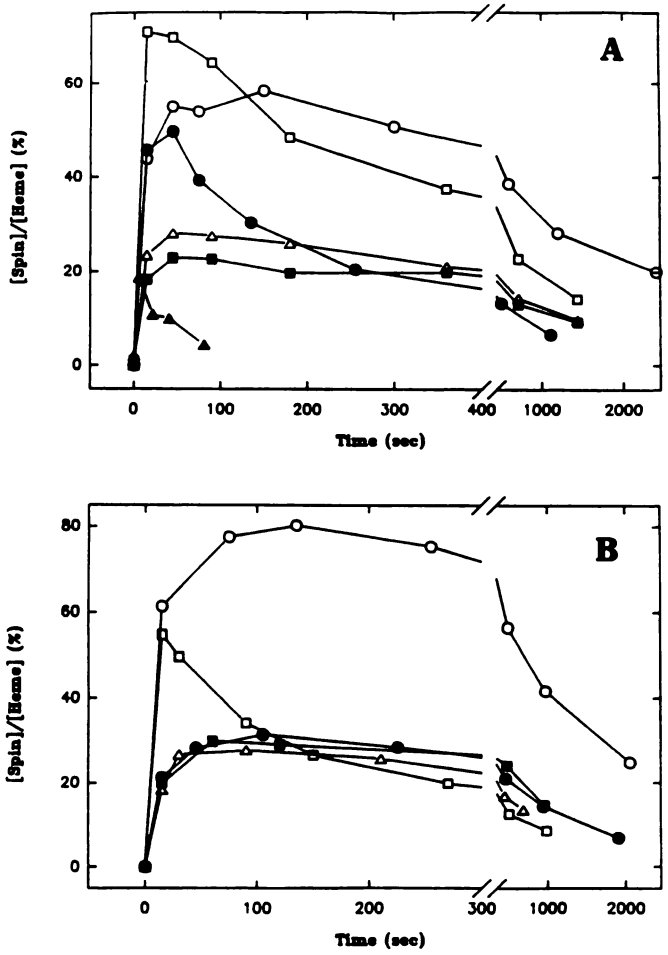

Fig. 2. Effects of anticyclooxygenase agents on the concentration of the EtOOH-induced tyrosyl radical. The synthase holoenzyme was preincubated with the agents before reaction with EtOOH, and the concentration of the tyrosyl radical after the indicated periods of reaction at −10° was determined by double integration of the radical EPR signal, as described in Experimental Procedures. Two different batches of the synthase were used; results with one batch are presented in A and those with the other batch in B. The individual samples in A are the control (○) and the synthase treated with flufenamate (□), ibuprofen (△), mefenamate (●), naproxen (■), and acetaminophen (▲). The samples in B are the control (○) and the synthase treated with meclofenamate (□), (*S*)-flurbiprofen (△), indomethacin (●), and aspirin (■).

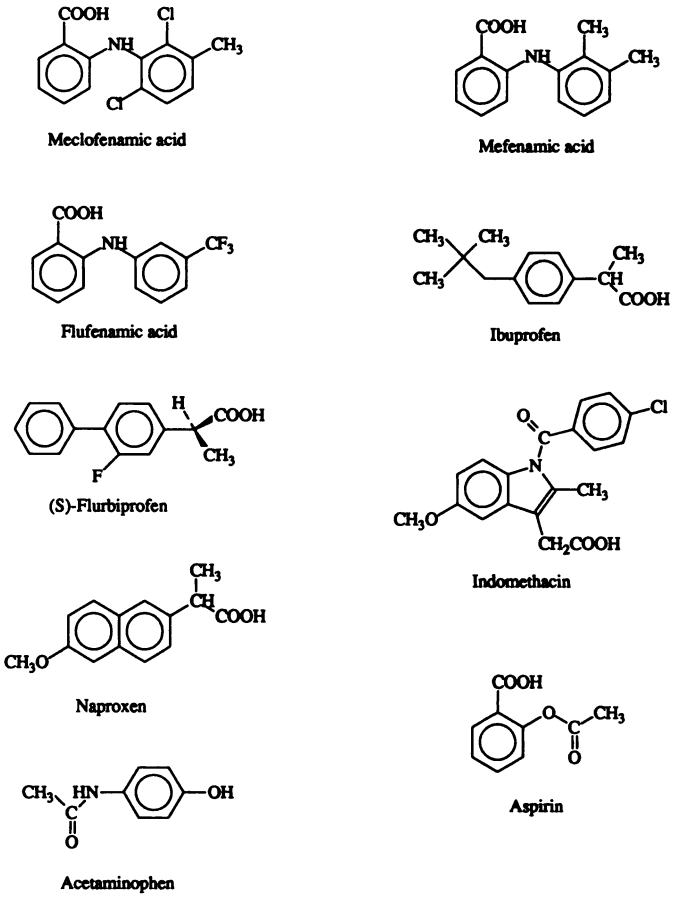

Fig. 3. Structures of the anticyclooxygenase agents used in this study.

could be considered to lock the tyrosyl radical in the inactive conformation, whereas indomethacin, because it is not covalently bound, might be able to adopt a range of binding orientations on the synthase surface, some of which permit the protein to maintain the tyrosyl radical in the active conformation and continue cyclooxygenase catalysis; if 4% of the tyrosyl radical was in the active conformation, it would not be easy to detect in the EPR spectrum.

Three of the anticyclooxygenase agents examined (meclofenamate, flufenamate, and mefenamate) did not alter the structure of the hydroperoxide-induced radical species, as judged by the EPR spectra (Fig. 1). These agents did significantly decrease the lifetime of the radical species (Fig. 2), presumably by acting as reductants. Flufenamate and meclofenamate have been shown to act as reducing cosubstrates for the peroxidase activity of the synthase (10); meclofenamate and mefenamate were found to impair initiation of the cyclooxygenase (11). All three agents belong to the fenamate class of anticyclooxygenase agents and share the same basic chemical structure (Fig. 3). It would appear likely that inhibition of the cyclooxygenase by this class of agents is due to their ability to decrease the concentration of the tyrosyl radical rather than to perturb its structure.

Meclofenamate is of particular interest. It exhibited a rapid time-dependent inhibitory effect at stoichiometric levels, just as seen with indomethacin and flurbiprofen (12), and thus presumably forms a tight complex with the synthase. The time-dependent effect was lost when the carboxyl group of meclofenamate was methylated; the same was true for indomethacin and flurbiprofen (13). The initial binding of meclofenamate,

TABLE 1
Characteristics of the anticyclooxygenase agents used

Compound	Competitive binding[a]	Time-dependent inhibition	Impaired initiation[b]	Ref.
Aspirin	Yes	Yes	No	13
Indomethacin	Yes	Yes	No	12, 13
Flurbiprofen	Yes	Yes	No	12, 13
Ibuprofen	Yes	No	No	13
Naproxen	Yes	No	No	4
Meclofenamate	Yes	Yes	Yes	4, 11, 12, 13
Mefenamate	Yes	No	Yes	4, 11
Flufenamate	Yes	No	ND[c]	4
Acetaminophen	No	No	Yes	11

[a] Competition with arachidonic acid binding, indicated by decreased inhibitory potency with increases in arachidonic acid concentration.
[b] Indicated by increased inhibitory potency when hydroperoxide levels were suppressed by glutathione peroxidase.
[c] ND, not determined.

indomethacin, and flurbiprofen was competitively inhibited by docosahexaenoic acid (12), suggesting that the agents occupied part of the substrate binding site. In spite of all these similarities, the lack of an effect of meclofenamate on the shape of the tyrosyl radical EPR signal (Fig. 1) indicates that there are significant structural differences between the complexes of the synthase with the other time-dependent agents and that with meclofenamate.

There were no obvious characteristics in the EPR spectra shown in Fig. 1 that distinguished agents known to have time-dependent inhibitory effects on the cyclooxygenase (e.g., indomethacin and meclofenamate) from those whose effects are reversible (e.g., ibuprofen and mefenamate). The present results thus cannot define a molecular basis for the progressive and essentially irreversible effects of the former group of compounds. However, the apparent lack of distinguishing features in the EPR would be consistent with the involvement of areas of the protein that do not have immediate interactions with the residue carrying the unpaired electron.

Extensive examinations of the inhibitory kinetics of the anticyclooxygenase agents (for review, see Ref. 4) have previously identified three categories of inhibitor: classic competitive inhibitors of substrate binding (like ibuprofen), competitive inhibitors that exhibited an additional time-dependent inhibition (like aspirin, indomethacin, and meclofenamate), and radical-quenching agents (like acetaminophen). The results of the present EPR study of these agents' effects on the synthase tyrosyl radical have brought the focus closer to possible molecular mechanisms of cyclooxygenase inhibition. Although a cause-effect relationship between perturbation of the tyrosyl radical and inhibition of the cyclooxygenase has yet to be established, this new perspective should prove useful in the elucidation of the catalytic mechanism of this complex enzyme and in an understanding of the pharmacological effects of the nonsteroidal anti-inflammatory agents at a molecular level.

References

1. Samuelsson, B., M. Goldyne, E. Granstrom, M. Hamberg, S. Hammarstrom, and C. Malmsten. Prostaglandins and thromboxanes. *Annu. Rev. Biochem.* **47**:997–1029 (1978).
2. Karthein, R., R. Dietz, W. Nastainczyk, and H. H. Ruf. Higher oxidation states of prostaglandin H synthase. *Eur. J. Biochem.* **171**:313–320 (1988).
3. Kulmacz, R. J., Y. Ren, A.-L. Tsai, and G. Palmer. Prostaglandin H synthase: spectroscopic studies of the interaction with hydroperoxides and with indomethacin. *Biochemistry* **29**:8760–8771 (1990).
4. Lands, W. E. M., and A. M. Hanel. Inhibitors and activators of prostaglandin biosynthesis, in *Prostaglandins and Related Substances* (C. Pace-Asciak and E. Granstrom, eds.). Elsevier Science Publishing, New York, 203–223 (1983).
5. Kulmacz, R. J., and W. E. M. Lands. Cyclo-oxygenase: measurement, purification and properties, in *Prostaglandins and Related Substances: A Practical Approach* (C. Benedetto, R. G. McDonald-Gibson, S. Nigam, and T. F. Slater, eds.). IRL Press, Washington DC, 209–227 (1987).
6. Kulmacz, R. J., A.-L. Tsai, and G. Palmer. Heme spin states and peroxide-induced radical species in prostaglandin H synthase. *J. Biol. Chem.* **262**:10524–10531 (1987).
7. Shimokawa, T., R. J. Kulmacz, D. L. DeWitt, and W. L. Smith. Tyrosine 385 of prostaglandin endoperoxide synthase is required for cyclooxygenase catalysis. *J. Biol. Chem.* **265**:20073–20076 (1990).
8. Mizuno, K., S. Yamamoto, and W. E. M. Lands. Effects of non-steroidal anti-inflammatory drugs on fatty acid cyclooxygenase and prostaglandin hydroperoxidase activities. *Prostaglandins* **23**:743–757 (1982).
9. Barry, B. A., M. K. El-Deeb, P. O. Sandusky, and G. T. Babcock. Tyrosine radicals in photosystem II and related model compounds. *J. Biol. Chem.* **265**:20139–20143 (1990).
10. Markey, C. M., A. Alward, P. E. Weller, and L. J. Marnett. Quantitative studies of hydroperoxide reduction by prostaglandin H synthase. *J. Biol. Chem.* **262**:6266–6279 (1987).
11. Hanel, A. M., and W. E. M. Lands. Modification of anti-inflammatory drug effectiveness by ambient lipid peroxides. *Biochem. Pharmacol.* **31**:3307–3311 (1982).
12. Kulmacz, R. J., and W. E. M. Lands. Stoichiometry and kinetics of the interaction of prostaglandin H synthase with anti-inflammatory agents. *J. Biol. Chem.* **260**:12572–12578 (1985).
13. Rome, L. H., and W. E. M. Lands. Structural requirements for time-dependent inhibition of prostaglandin biosynthesis by anti-inflammatory drugs. *Proc. Natl. Acad. Sci. USA* **72**:4863–4865 (1975).
14. Peterson, G. L. Determination of total protein. *Methods Enzymol.* **91**:95–119 (1983).

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