Rapid Inactivation of Prostaglandin Endoperoxide Synthases by *N*-(Carboxyalkyl)maleimides†

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ABSTRACT: *N*-(Carboxyalkyl)maleimides were synthesized as potential inhibitors of prostaglandin endoperoxide synthase (PGHS). Inactivation of the cyclooxygenase and peroxidase activities of PGHS occurred in a biphasic manner with extremely rapid inactivation followed by slow, time-dependent inactivation. The carboxylic acid moiety was required for rapid inactivation. Optimal inhibition was observed with *N*-(carboxyheptyl)maleimide, which inhibited the cyclooxygenase activity of ovine PGHS-1 with an IC_{50} of 0.1 μM and the peroxidase activity with an IC_{50} of 3 μM. Inactivation of peroxidase activity was not prevented by pretreating the enzyme with the cyclooxygenase inhibitor indomethacin. *N*-(Carboxyheptyl)-succinimide inhibited neither enzyme activity, suggesting that covalent modification is critical for rapid as well as time-dependent inactivation. Shortening or increasing the alkyl chain by one methylene unit drastically reduced inhibitory potency. *N*-(Carboxyalkyl)maleimides also instantaneously inactivated the inducible form of PGHS (PGHS-2) from mouse and human sources but with higher IC_{50}'s (4.5 and 14 μM, respectively). *N*-(Carboxyheptyl)maleimide is the most potent covalent inactivator of PGHS yet described with an inhibitory potency 3–5 orders of magnitude greater than aspirin.

Prostaglandin endoperoxide synthase (PGHS)[1] catalyzes the first two steps of the arachidonic acid cascade. Its cyclooxygenase activity oxygenates arachidonic acid to PGG_2 and its peroxidase activity reduces PGG_2 to PGH_2 (Scheme 1) (Hamberg et al., 1974; Nugteren & Hazelhof, 1973). Two different PGHS proteins exist in human and animal tissue. Both PGHS's are heme proteins of approximately 70 kDa in size (Van Der Ouderaa et al., 1979; Sirois & Richards, 1992; Fletcher et al., 1992). The gene for PGHS-1 is expressed constitutively in cells whereas that for PGHS-2 is induced by growth factors, cytokines, etc. (Kujubu et al., 1991; Xie et al., 1991; O'Banion et al., 1991; Hla & Neilson, 1992). PGHS-1 appears to be responsible for synthesis of cytoprotective prostaglandins in the stomach, and PGHS-2 is a major contributor to prostaglandin synthesis in inflammatory cells (Masferrer et al., 1994).

Covalent modification of PGHS is responsible for enzyme inactivation by acetylsalicylic acid (Robinson & Vane, 1974; Rome et al., 1976), acylimidazoles (Wells & Marnett, 1992, 1993; Scherer et al., 1992), acyl-*N*-hydroxysuccinimides (Wells & Marnett, 1993), and *N*-alkylmaleimides (Kennedy et al., 1993). In all these cases, inactivation is time-dependent and occurs at ratios of inhibitor/enzyme of 100 or greater. In an attempt to target reactive functionalities to particular regions of the protein, we have tethered a series of substrate and inhibitor mimics to the maleimide group. We report herein that linkage of medium-chain fatty acids to the maleimide moiety produces a series of inactivators that exhibit nearly instantaneous as well as time-dependent inhibition. Furthermore, varying the fatty acid chain length reveals a remarkable sensitivity of PGHS to subtle alterations of inhibitor structure. The most potent inhibitor in this series inactivates cyclooxygenase at an inhibitor/enzyme ratio of 1/1 and is approximately 100 000 times more potent as an instantaneous inhibitor than aspirin.

MATERIALS AND METHODS

Arachidonic acid was purchased from Nu Chek Prep (Elysian, MN). Hematin, hydrogen peroxide, guaiacol, and indomethacin were purchased from Sigma Chemical Co. (St. Louis, MO). Ram seminal vesicles were purchased from Oxford Biomedical Research, Inc. (Oxford, MI). All other chemicals were reagent grade or better. All of the synthetic compounds were characterized by ^1H NMR, EI- or FAB-MS, and elemental analysis. Complete details of the procedures employed in the synthesis of the maleimide analogs will be reported elsewhere. PGHS-1 was purified from ram seminal vesicles as described earlier (Marnett et al., 1984). The specific activity of the protein was 20.9 μM O_2 min^{-1} mg^{-1}, and the percentage of holoenzyme was 13.5%. Apoenzyme was reconstituted by addition of hematin to the assay mixtures. Mouse and human PGHS-2 were generous gifts from J. Gierse, Monsanto (St. Louis, MO). The cyclooxygenase activity of the mouse PGHS-2 protein was 9.4 μmol of AA min^{-1} mg^{-1}, and that of the human PGHS-2 was ~18–20 μmol of AA min^{-1} mg^{-1}.

Cyclooxygenase Activity. Oxygen consumption was measured at 37 °C with a Gilson Model 5/6 oxygraph (Gilson Medical Electronics, Inc., Middleton, WI) equipped with a Clark electrode and a thermostated cuvette. Enzyme aliquots (20 μg) were added to 100 mM Tris-HCl at pH 8 containing 500 μM phenol and 1 μM hematin in a final volume of 1.3 mL. Oxygen uptake was initiated by the addition of 100 μM sodium arachidonate, and the initial reaction velocity was determined from the linear portion of the oxygen uptake curve.

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[1] Abbreviations: PGHS or PGH synthase, prostaglandin endoperoxide synthase (EC 1.14.99.1); AA or arachidonic acid, 5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid; NSAID, nonsteroidal antiinflammatory drug; FAB-MS, fast atom bombardment mass spectrometry; EI-MS, electron impact mass spectrometry; DMSO, dimethyl sulfoxide; AH_2, generic abbreviation for a peroxidase reducing substrate.

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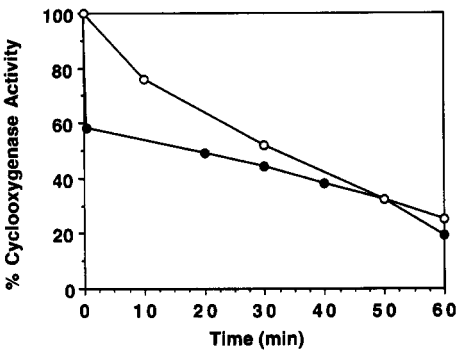


FIGURE 1: Inactivation of the cyclooxygenase activity of PGHS-1 by N-substituted maleimides. ApoPGHS (5 μM) in 100 mM sodium phosphate buffer, pH 7.2, containing 0.1% Tween 20 was treated with 1 equiv of hematin from a 500 μM stock solution in DMSO, and this mixture was allowed to stand at 25 °C for 5 min. The inhibitor in DMSO was added to this mixture, and 0.16 μM enzyme aliquots were assayed for remaining cyclooxygenase activity as described under Materials and Methods. Symbols: closed circles, *N*-(carboxydecyl)-maleimide; open circles, *N*-decylmaleimide.

Peroxidase Activity. Assays were performed at 25 °C on a Shimadzu UV 160U by measuring the initial rates of oxidation of guaiacol at 436 nm. Enzyme aliquots (20 μg) were added to 100 mM Tris-HCl (pH 8) containing 1 μM heme and 500 μM guaiacol in 1-mL disposable cuvettes. Reaction was initiated by the addition of 400 μM hydrogen peroxide.

RESULTS

Inactivation of Cyclooxygenase Activity. Incubation of hematin-reconstituted PGHS-1, purified from sheep seminal vesicles, with a series of *N*-(carboxyalkyl)maleimides led to a time-dependent loss of cyclooxygenase activity. The kinetics of inactivation observed with *N*-(carboxydecyl)maleimide was typical of those of other maleimides (Figure 1). Rapid inactivation occurred that was evident at the earliest time points practically measurable following simultaneous addition of inhibitor and arachidonic acid to the enzyme (~5 s). This was followed by a slower loss of the remaining activity. The percentage of activity that was lost in the initial phase was greater at higher concentrations of the *N*-(carboxyalkyl)-maleimide. The importance of the carboxylic acid for rapid inactivation was evident from comparison to the time course of inactivation by *N*-decylmaleimide, which exhibited only the slow inactivation typical of *N*-alkylmaleimides (Figure 1).

N-(Carboxyheptyl)maleimide was the most potent inactivator of PGHS-1; its IC_{50} was 0.1 μM in an assay in which the protein concentration was 0.25 μM (Figure 2). All of the cyclooxygenase activity was lost instantaneously even at inhibitor concentrations below those of the enzyme. Thus, inactivation appeared to result from a stoichiometric reaction between *N*-(carboxyheptyl)maleimide and PGHS-1. Rapid inactivation of cyclooxygenase activity was also effected by other *N*-(carboxyalkyl)maleimides but at much higher concentrations (Table 1). The saturated analog of *N*-(carboxyheptyl)maleimide, *N*-(carboxyheptyl)succinimide, failed to inactivate the enzyme in either a rapid or a time-dependent fashion at inhibitor/enzyme ratios of 250/1. This suggests that covalent modification is required for both modes of inactivation.

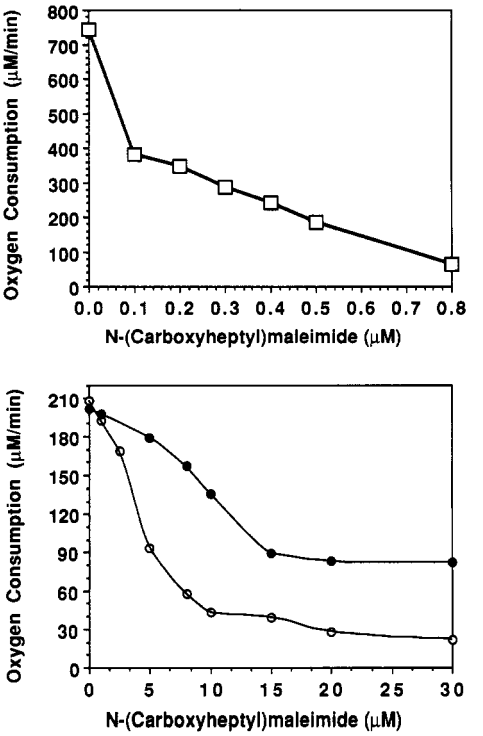
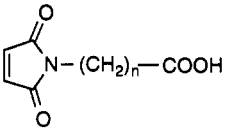


FIGURE 2: Inactivation of the cyclooxygenase activity of PGHS-1 and PGHS-2 by *N*-(carboxyheptyl)maleimide. ApoPGHS-1 (0.25 μM) and mouse or human apoPGHS-2 (0.21 μM) in 100 mM Tris-HCl, pH 8, were treated with 1 μM hematin and allowed to reconstitute for 1 min followed by simultaneous addition of the inhibitor and 100 μM sodium arachidonate. Key: upper panel, PGHS-1; lower panel, open circles, mouse PGHS-2; closed circles, human PGHS-2.

Table 1: IC_{50} Values for Instantaneous Cyclooxygenase Inactivation by *N*-(Carboxyalkyl)maleimides[a]

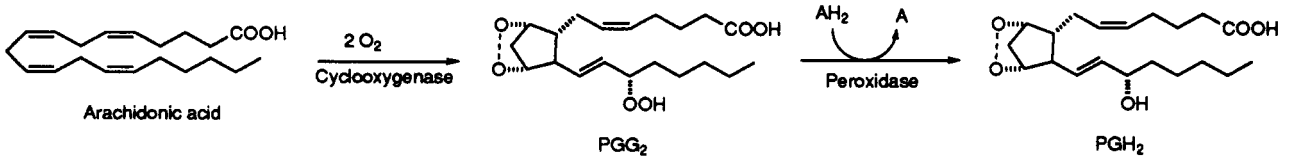


n	IC_{50} (μM)	*n*	IC_{50} (μM)
5	>800	9	37
6	570	10	40
7	0.1	11	140
8	26		

[a] Purified PGHS (250 nM) reconstituted with 1 μM hematin was treated with the maleimide, and the cyclooxygenase reaction was initiated by addition of 100 μM sodium arachidonate. No preincubation was allowed between enzyme and inhibitor. Each experiment was performed in duplicate, and all compounds were tested on a single day. The IC_{50} values were obtained from plots of oxygen consumption vs inhibitor concentration.

Rapid inactivation of cyclooxygenase activity by *N*-(carboxyheptyl)maleimide was also demonstrated with

Scheme 1



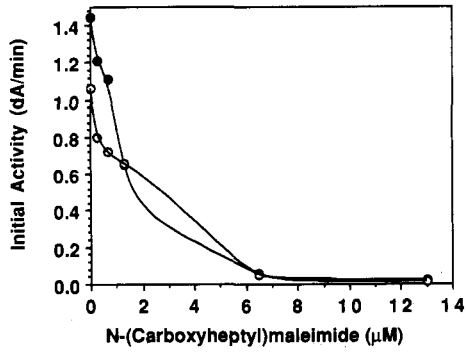


FIGURE 3: *N*-(Carboxyheptyl)maleimide-dependent inactivation of the peroxidase activity of PGHS-1 in the presence of indomethacin. An incubation mixture containing PGHS-1 (5 μM) reconstituted with 1 μM hematin was treated with indomethacin (15 μM) and allowed to incubate at 25 °C for 15 min. Under these conditions, the cyclooxygenase activity was inhibited by greater than 90% whereas normal peroxidase levels were maintained. Instantaneous inactivation of the peroxidase activity utilizing the guaiacol peroxidase assay was analyzed by addition of various concentrations (0.2–13 μM) of the maleimide to aliquots of the incubation mixture in the UV cuvette followed immediately by addition of hydrogen peroxide. Symbols: closed circles, without indomethacin; open circles, with indomethacin.

PGHS-2 derived from mouse and human sources. Incubation of heme-reconstituted enzymes (0.18 μM) with *N*-(carboxyheptyl)maleimide led to inactivation at IC_{50} values of 4.5 μM (mouse) and 14 μM (human) (Figure 2).

Inactivation of the Peroxidase Activity of PGHS. The peroxidase activity of PGHS-1 was also subject to kinetically biphasic inactivation by *N*-(carboxyalkyl)maleimides. The IC_{50}'s and time courses for the slower, time-dependent inactivation were identical to those for inactivation of cyclooxygenase activity. However, rapid inactivation of peroxidase activity required higher concentrations of *N*-(carboxyalkyl)maleimides than required for rapid inactivation of cyclooxygenase activity. For example, the IC_{50} for the peroxidase inactivation of PGHS-1 by *N*-(carboxyheptyl)-maleimide was 3 μM, which is 30-fold higher than the IC_{50} for loss of cyclooxygenase activity. Thus, at intermediate concentrations (~1 μM), cyclooxygenase activity was completely inactivated but peroxidase activity was not.

Rapid inactivation of the peroxidase activity of PGHS-1 by *N*-(carboxyheptyl)maleimide occurred in the presence of indomethacin, which selectively inhibits the cyclooxygenase activity. PGHS-1 preincubated with 3 equiv of indomethacin was treated with *N*-(carboxyheptyl)maleimide. Peroxidase inactivation of the indomethacin-treated enzyme by *N*-(carboxyheptyl)maleimide displayed a similar dose–response to inactivation of peroxidase activity in the absence of indomethacin (Figure 3).

DISCUSSION

Reaction of PGHS with N-substituted maleimides results in a complex series of events that lead to loss of cyclooxygenase and peroxidase activities. A reaction leading to rapid, nearly instantaneous, inactivation occurs concomitant with a reaction(s) leading to slower time-dependent inactivation. Time-dependent inactivation has been observed previously with *N*-ethylmaleimide and was observed in the present study with longer chain *N*-alkylmaleimides (Kennedy et al., 1993). Saturation of the maleimide double bond produces a molecule with no inhibitory potency, implying that rapid as well as slow inactivation results from covalent modification of the protein. Previous studies from our laboratory have shown that all three free cysteines (Cys^{313}, Cys^{512}, and Cys^{540}) in ovine PGHS-1 are modified to varying extents following treatment with *N*-ethylmaleimide (Kennedy et al., 1993). Whether cysteine modification by these reagents is responsible for enzyme inactivation is uncertain.

Rapid inactivation of cyclooxygenase activity appears specific to *N*-(carboxyalkyl)maleimides as it was not observed with *N*-alkylmaleimides (e.g., *N*-ethyl- or *N*-octylmaleimide). The most potent inhibition was observed with *N*-(carboxyheptyl)maleimide, which inactivated the enzyme at stoichiometric concentrations. Inhibitory potency was exquisitely sensitive to the length of the carboxyalkyl chain. Shortening or lengthening the carbon chain by one methylene unit (~1.5 Å) decreased the inhibitory potency by approximately 4 and 2 orders of magnitude, respectively (Table 1).

Reaction of PGHS-1 with stoichiometric amounts of *N*-(carboxyheptyl)maleimide abolished cyclooxygenase activity but not peroxidase activity. Similar behavior is exhibited by aspirin, which covalently modifies PGHS, and by several other NSAID's, which do not covalently modify the protein (e.g., indomethacin, flurbiprofen) (DeWitt et al., 1990; Meade et al., 1993; Egan et al., 1980). All of these inhibitors contain a carboxylic acid and bind to the protein in the arachidonic acid access channel, which is well removed spatially from the peroxidase active site (Picot et al., 1994). Solution of the structure of a PGHS–flurbiprofen cocrystal revealed ion pairing between the carboxylate of flurbiprofen and Arg^{120}, which is the only positively charged residue in the fatty acid binding channel (Picot et al., 1994). The importance of Arg^{120} for inactivation by *N*-(carboxyheptyl)maleimide is uncertain, but it is important to note that the distance between the maleimide double bond and the carboxyl oxygen in the fully extended form of *N*-(carboxyheptyl)maleimide is 11.5 Å. Thus, a nucleophile 13–15 Å from Arg^{120} could provide a site of covalent attachment, and ion pairing with Arg^{120} could fix the covalently bound maleimide in the arachidonic acid binding channel. If rapid inactivation results from cysteine modification, the only likely candidates are Cys^{512} and Cys^{540}, which are contained on opposite ends of an α-helix that crosses the fatty acid binding channel (Picot et al., 1994). Cys^{512} is 16.5 Å from Arg^{120} in the crystal structure, and Cys^{540} is 17.3 Å away. However, it is also possible that inactivation results from modification of other residues besides cysteine. For example, Tyr^{385}, which is believed to play a role in arachidonic acid oxidation, is 12.5 Å from Arg^{120} (Picot et al., 1994; Shimokawa et al., 1990).

Raising the concentration of *N*-(carboxyheptyl)maleimide leads to inactivation of peroxidase activity (IC_{50} ~ 3 μM). Since *N*-(carboxyheptyl)maleimide is an extremely rapid inactivator of PGHS, inactivation of the peroxidase moiety may reflect the same covalent modification that leads to time-dependent inactivation of peroxidase activity by the other maleimides. If so, this modification does not appear to be in the fatty acid binding channel because it is not prevented by pretreatment of the protein with indomethacin.

N-(Carboxyheptyl)maleimide is the most potent covalent inactivator of PGHS described to date. Aspirin, the only clinically used NSAID that covalently inactivates PGHS exhibits an IC_{50} for instantaneous inactivation of cyclooxygenase activity in excess of 10 mM and an IC_{50} for time-dependent inactivation of ~100 μM (DeWitt et al., 1990). Thus, *N*-(carboxyheptyl)maleimide is between 5 and 3 orders of magnitude more potent than aspirin, depending on the time of preincubation. The exquisite sensitivity of cyclooxygenase inhibition to the structure of the substituted maleimides is consistent with our previous suggestion that the sensitivity of

Ser^{530} to acetylation by aspirin is dependent on complementary interactions between the salicylate moiety and PGHS (Wells & Marnett, 1992). Furthermore, it raises the possibility that other covalent inactivators could be designed to differentially inhibit the two isoforms of PGHS. *N*-(Carboxyheptyl)-maleimide appears to be more selective for PGHS-1 than PGHS-2 by a factor of 30–100. However, by alteration of the electrophilic moiety and the alkyl group tethered to it, it may be possible to reverse the selectivity.

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