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MODIFICATION OF ANTI-INFLAMMATORY DRUG EFFECTIVENESS BY AMBIENT LIPID PEROXIDES

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Abstract—When the steady-state concentrations of peroxide in prostaglandin H synthase assay systems was lowered by added glutathione peroxidase, several agents (meclofenamic acid, mefenamic acid, acetamidophenol and phenylbutazone) became more potent inhibitors of the prostaglandin-forming cyclooxygenase reaction. Paradoxically, these agents stimulated oxygen incorporation in the absence of added peroxidase. On the other hand, dithiothreitol, ibuprofen, flurbiprofen and indomethacin all inhibited the reaction in a dose-dependent manner, and their inhibitory potencies were unaffected by the action of glutathione peroxidase. Aspirin, *dl*-gamma-tocopherol and salicylic acid were not inhibitory (without preincubation) under the assay conditions employed in this study. The findings demonstrate that the potencies of some anti-inflammatory agents may be diminished by high local peroxide concentrations.

Much attention has been focused on the inhibition of prostaglandin synthesis since Vane's report [1] that anti-inflammatory agents, such as aspirin, inhibit the prostaglandin-forming enzyme prostaglandin H (PGH) synthase (EC 1.14.99.1). A listing of the major nonsteroidal anti-inflammatory drugs (NSAIDs) includes compounds such as the salicylates, fenamates, arylacetic acids, and pyrazolones. These NSAIDs may act either as analgesics, antipyretics, anti-inflammatory or antiaggregatory agents; an individual drug may be more effective in treating one pathological state than another. Since all of the agents are inhibitors of cyclooxygenase activity, the reason for the observed selective effectiveness is not known.

The inflammatory condition involves a complex series of cellular responses. An integral part of the inflammatory response is the migration of neutrophilic polymorphonuclear leukocytes and monocytes to the site of inflammation [2]. Subsequently, the phagocytic cells can release arachidonic acid, prostaglandins and chemotactic agents and thus augment the response at the inflammatory site [3–5].

The formation of peroxides may be a significant chemical event associated with the appearance of phagocytic cells at inflammatory sites [6]. Lipid peroxides have been implicated in hyperalgesia associated with inflammation [7]. They are also required for the activation of the prostaglandin-forming cyclooxygenase [8, 9]. This requirement was first demonstrated when Smith and Lands [10] reported that the removal of peroxides by glutathione peroxidase (GSP) decreased prostaglandin formation *in vitro*. Further investigation demonstrated that the cyclooxygenase product, PGG_2, serves as an activator for the reaction and can act as the necessary activating hydroperoxide for assays utilizing the purified cyclooxygenase [11]. Hemler and Lands [12] later confirmed that cyclooxygenase activity had a continuous requirement for hydroperoxide because peroxide removal after initiation of the reaction could inhibit prostaglandin formation. They further demonstrated that phenol, which is capable of trapping free-radical intermediates, was an increasingly effective inhibitor in the presence of increased amounts of glutathione peroxidase.

Knowledge of this type of inhibition by phenol prompted us to investigate the possibility that some of the agents known to inhibit cyclooxygenase might be more effective inhibitors when the levels of the required peroxide activator(s) were decreased. The present report describes the manner in which the relative potency of certain cyclooxygenase inhibitors can be affected by ambient peroxide levels.

MATERIALS AND METHODS

Materials. Ibuprofen and flurbiprofen were gifts from the Upjohn Co. (Kalamazoo, MI), indomethacin from the Merck Co. (Rahway, NJ), *dl*-gamma-tocopherol from Hoffmann-LaRoche Inc. (Nutley, NJ), and meclofenamic and mefenamic acid from the Parke Davis Co. (Detroit, MI). Salicylic acid was purchased from the Malinckrodt Chemical Co. (St. Louis, MO), acetamidophenol from the Ruger Chemical Co. (Hillside, NJ), acetylsalicylic acid (aspirin) and ascorbic acid from the Merck Co., hemin chloride and butylated hydroxytoluene from CalBiochem (La Jolla, CA), ampholines for isoelectric focusing from Pharmacia Fine Chemicals (Piscataway, NJ), and ammonium sulfate from Schwarz-Mann (Orangeburg, NY). Phenylbutazone, diethylaminoethyl (DEAE) cellulose, sodium diethyl dithiocarbamate (DDC), arachidonic acid and Tween-40 were purchased from the Sigma Chemical Co. (St. Louis, MO), Fluorescamine from the Pierce Chemical Co. (Rockford, IL), and sheep vesicular glands from Roth Products. All other chemicals were

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reagent grade and obtained from common commercial sources.

All drug solutions, with the exception of dithiothreitol (DTT), ascorbic acid and *dl*-gamma-tocopherol, were prepared in reagent grade acetone at concentrations that required the addition of no more than 60 μl to a 3.0 ml assay mixture to achieve the desired drug concentration. Ascorbic acid and DTT were dissolved in distilled water. Tocopherol was dissolved in toluene, and aliquots were evaporated to dryness in the reaction vessel with nitrogen prior to the addition of other assay components.

Preparation of arachidonic acid solutions. One hundred milligrams of arachidonate, dissolved in 3 ml of reagent grade methanol, was mixed with 0.2 g of sodium borohydride and allowed to stand for 1 hr at room temperature. Following this reductive treatment, 9 ml of distilled water was added to the mixture and acidified (pH 4.0) using 1.0 N HCl. The arachidonic acid was extracted twice with 4 ml portions of ethyl acetate, and the combined extracts were dried over 1 g of sodium sulfate for 30 min at 0°. The sodium sulfate was removed by filtration through a glass wool plug in a Pasteur pipette, and the ethyl acetate was evaporated under a stream of nitrogen. The remaining peroxide-free arachidonate was redissolved using 25 ml of toluene containing 0.1 mM butylated hydroxytoluene to provide a stock solution (approximately 15 mM).

Fresh aqueous dispersions of arachidonate were prepared by evaporating aliquots of the stock solution to dryness and resuspending in 0.1 M Tris–Cl (pH 8.5). These solutions were always used soon after preparation to avoid introducing excessive amounts of the hydroperoxide into the assay system.

Purification and treatment of cyclooxgenase. Homogeneous cyclooxgenase was purified using techniques that allow high yields as described previously [13]. Phenol, employed for enzyme stabilization during purification and storage [13], was removed by dialysis in 20 mM potassium phosphate (pH 7.0), 20% glycerol and 0.2% Tween-40. The phenol-free cyclooxygenase was stored at −70° and used within a day or two following dialysis. Protein concentrations were determined by either the method of Lowry *et al.* [14] or the Fluorescamine method of Bohlen *et al.* [15] using bovine serum albumin (fraction V, Sigma) as a standard.

Partial purification of glutathione peroxidase. Glutathione peroxidase was partially purified from rat liver by the method of Nakamura *et al.* [16] using the modifications and assay previously described [17]. For the assay of crude fractions prior to DEAE cellulose chromatography, the concentration of H_2O_2 was increased to 1 mM.

Assays of inhibitory potency. Oxygen consumption was monitored polarographically with a Yellow Springs 52 oxygen monitor [18] at 30° ± 0.1° kept constant with a Lauda K-2/R water bath (Brinkmann Instruments, Westbury, NY). Reaction vessels contained 40–60 μM arachidonate and 0.5 mM reduced glutathione in 3.0 ml of 0.1 M Tris–Cl buffer (pH 8.5). Other additions, with the exception of *dl*-gamma-tocopherol, were injected directly into the chamber, and the total volume of all the injections did not exceed 100 μl/assay. An electronic differentiator [19] was used to continuously monitor and record the rate of oxygen consumption (dO_2/dt) giving data similar to those illustrated in Fig. 1. Oxygenation reactions were routinely initiated by the addition of enzyme, and optimal velocities (maximum dO_2/dt) were obtained from the differentiator recording.

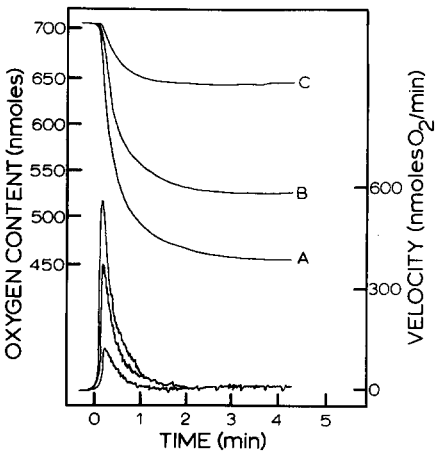

Fig. 1. Assay of cyclooxygenase activity in the presence of inhibitors. The oxygen content in the reaction mixtures is recorded in the upper half of the figure, and the continuous monitoring of the corresponding reaction velocity (dO_2/dt) is represented in the lower half. These reaction mixtures contained 50 μM arachidonate, 0.5 mM reduced glutathione and 2.0 mM phenylbutazone. The letters beside the oxygen content curves indicate the glutathione peroxidase units present in the assays (performed as described in Materials and Methods): (A) 0 units/ml, (B) 2000 units/ml, and (C) 13,000 units/ml.

RESULTS

Table 1 lists twelve cyclooxygenase inhibitors that were tested to ascertain if their inhibitory potencies were increased when peroxide concentrations were lowered *in vitro*. Added glutathione peroxidase (GSP) was used to maintain lower peroxide concentrations in the assay. Phenol was excluded from the inhibitor assays to avoid its synergistic inhibition of the cyclooxygenase with GSP [12]. In the absence of either phenol or inhibitors, the amount of added glutathione peroxidase activity did not appreciably inhibit the oxygen incorporation by 120 nM cyclooxygenase.

The first four compounds listed in Table 1 gave greater inhibition of the cyclooxygenase when the peroxide level was lowered in the assay mixture. The rank order of inhibitory potency for those agents in the presence of 13,000 units GSP/ml was: mefenamic acid > meclofenamic acid > acetamidophenol > phenylbutazone. Lowering the GSP concentration to 2000 units/ml permitted higher steady-state peroxide levels in the assay and caused the agents to be less effective inhibitors. In the absence of GSP, these four agents were not inhibitory and actually increased the rate of oxygen incorporation above the rate observed when no inhibitor was present (252 nmoles O_2/min).

The remaining compounds shown in Table 1 did

Table 1. Influence of added glutathione peroxidase on the inhibitory effectiveness of anti-cyclooxygenase agents*

	0 units GSP	2000 units GSP/ml	13,000 units GSP/ml
Agent tested	V_{opt} (% inhib.)	V_{opt} (% inhib.)	V_{opt} (% inhib.)
Mefenamic acid			
10 μM	369 (0)		
15 μM	264 (0)		
50 μM	324 (0)	258 (20)	153 (44)
200 μM	303 (0)	216 (29)	0 (100)
Meclofenamic acid			
5 μM	291 (0)		
25 μM	237 (6)	342 (0)	
50 μM	381 (0)		
200 μM	417 (0)	139 (55)	150 (64)
1 mM	117 (64)	42 (83)	0 (100)
Acetamidophenol			
500 μM	609 (0)	591 (3)	450 (26)
2 mM	822 (0)	417 (49)	0 (100)
Phenylbutazone			
500 μM	582 (0)	456 (22)	312 (46)
2 mM	561 (0)	395 (33)	123 (78)
4 mM	189 (25)	87 (65)	0 (100)
Dithiothreitol			
10 μM	171 (32)	183 (27)	186 (27)
15 μM	159 (37)		
25 μM	135 (46)	153 (−36)	156 (36)
50 μM	108 (57)		
Ibuprofen			
10 μM	216 (14)	231 (8)	237 (6)
50 μM	245 (3)	220 (13)	215 (15)
Flurbiprofen			
10 μM	225 (11)	228 (9)	246 (2)
15 μM	195 (23)		
20 μM	159 (37)	180 (29)	174 (31)
Indomethacin			
10 μM	249 (1)	237 (6)	249 (1)
20 μM	225 (11)	210 (17)	225 (11)
30 μM	210 (17)		
Aspirin			
500 μM	249 (1)		
1 mM	249 (1)		
5 mM	255 (0)	267 (0)	258 (0)
10 mM	237 (6)	228 (9)	243 (4)
Salicylic acid			
750 μM	291 (0)		
1 mM	312 (0)		
5 mM	300 (0)	330 (0)	318 (0)
10 mM	246 (0)	267 (0)	267 (0)
Gamma-tocopherol			
500 μM	249 (0)		
1 mM	264 (0)		
5 mM	288 (0)	330 (0)	321 (0)
10 mM	252 (0)	291 (0)	321 (0)
Ascorbic acid			
5 mM	399 (0)	384 (4)	324 (19)
10 mM	414 (0)	417 (0)	318 (24)

* Assays contained 0.5 mM reduced glutathione, 120 nM cyclooxygenase, 50 μM arachidonate, and the indicated concentrations of GSP and inhibitor in 3.0 ml of 0.1 M Tris–Cl (pH 8.5). The optimal velocity in the absence of both GSP and inhibitors was 252 nmoles O_2/min, and some agents stimulated the activity above this control value. For experiments with GSP, the percent of inhibition is calculated from the rate observed in the absence of GSP (first column of results).

not increase in potency when GSP was included in the assay. Of these agents, dithiothreitol, ibuprofen, flurbiprofen and indomethacin inhibited the cyclooxygenase in a dose-dependent manner, and their rank order of potencies, based on their abilities to inhibit the cyclooxygenase reaction in the absence of GSP, was: dithiothreitol > ibuprofen > flurbiprofen > indomethacin. Aspirin, salicylic acid and gamma-tocopherol were relatively ineffective inhibitors under these assay conditions, and concentrations up to 10 mM were not appreciably inhibitory. It is not surprising that aspirin was ineffective because

this type of assay did not afford adequate incubation time for time-dependent inhibitors [20] to elicit their full effects.

All inhibitors, whose potencies were enhanced by GSP, stimulated oxygen consumption when GSP was not present in the assay. The observed stimulation of cyclooxygenase activity was biphasic, with stimulated activity at lower concentrations and inhibition at higher levels of added agent. Mefenamic acid was the most effective GSP-assisted inhibitor, yet it caused the least amount of observable stimulation. Conversely, acetamidophenol was the most stimulatory agent in the absence of GSP and one of the least potent GSP-enhanced inhibitors. Ascorbic acid, gamma-tocopherol, and salicylic acid were slightly stimulatory in the absence of GSP but not to the extent observed with the GSP-enhanced inhibitors.

Figure 2 demonstrates, for the case of phenylbutazone, the dose–response relationship that is characteristic of the type of compound that exhibited GSP-enhanced inhibition. Stimulation was observed when the steady-state levels of peroxide were elevated or only slightly lowered. Figure 2 also illustrates how GSP-enhanced agents were sensitive to the ratio of GSP to cyclooxygenase activity. As increase in this ratio increased the *in vitro* effectiveness of phenylbutazone as an inhibitor. Figure 2A shows the effects of increasing phenylbutazone concentration with 85 nM cyclooxygenase (enzyme subunit concentrations are based on a molecular weight of 70,000). Maximal stimulation of the optimal rate in the absence of GSP was observed with 300 μM phenylbutazone and represented approximately a 50% increase over the control value. Only a slight stimulation by the drug was seen when the steady-state peroxide level was lowered by 1700 units/ml GSP, and the rate decreased in a dose-dependent manner with concentrations exceeding 100 μM phenylbutazone. The apparent IC_{50} value (taken as the concentration of inhibitor required to decrease the rate to one-half that observed under the same conditions without any inhibitor) for phenylbutazone was 375 μM at this concentration of GSP. A 3-fold increase in GSP concentration abolished any stimulation and lowered the IC_{50} to 100 μM. No IC_{50} for phenylbutazone could be obtained in this assay system in the absence of added peroxidase.

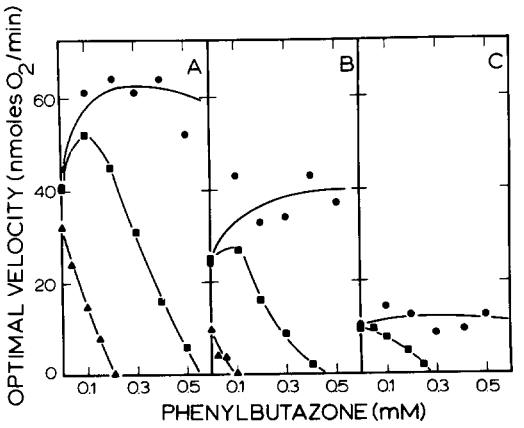

Fig. 2. Increased phenylbutazone inhibition with increased ratios of glutathione peroxidase to cyclooxygenase activity. All assays contained 3.0 ml of 100 mM Tris–Cl buffer (pH 8.5), 20 μM arachidonate, 0.5 mM reduced glutathione, and the designated amounts of GSP and phenylbutazone. Reactions were initiated by the addition of cyclooxygenase; the enzyme concentrations in the assay were 85 nM for A, 55 nM for B, and 35 nM for C. Glutathione peroxidase concentrations were 0 units/ml (●–●–), 1700 units/ml ((–■–■–), and 5000 units/ml (–▲–▲–).

Table 2. Effect of increased ratios of glutathione peroxidase to cyclooxygenase activities on the apparent IC_{50} of phenylbutazone*

GSP/Cyclooxygenase (units·ml^{-1}·nM^{-1})	Apparent IC_{50} (μM)
20	375
31	250
50	170
60	100
90	40

* Values are calculated from results displayed in Fig. 2.

Lowering the prostaglandin synthase concentration lowered both the degree of stimulation and the IC_{50} values (Fig. 2B). The increased ratio of GSP activity to cyclooxygenase activity lowered the IC_{50} for phenylbutazone to 250 and 40 μM when 1700 and 5000 units GSP/ml, respectively, were present in the assay. Phenylbutazone was even more effective as an inhibitor when the enzyme concentration was lowered further to 35 nM (Fig. 2C). The drug had an observed IC_{50} of 170 μM at 1700 units GSP/ml at this enzyme concentration, and the degree of stimulation was the lowest observed for the three enzyme concentrations used. Also, at this prostaglandin synthase concentration, 5000 units GSP/ml was sufficient to completely inhibit the reaction even in the absence of phenylbutazone. Table 2 summarizes the way in which a 4.5-fold increase in the ratio of glutathione peroxidase activity to cyclooxygenase activity caused nearly a 10-fold decrease in the apparent IC_{50} of phenylbutazone.

DISCUSSION

The action of hydroperoxides, based on their ability to stimulate cyclooxygenase activity *in vitro* [9], has been suggested as being important in the physiologic regulation of prostaglandin biosynthesis [21]. Cellular repression of peroxides appears vital to the integrity of the living cell, and the very low levels of lipid peroxides that occur *in vivo* may be inadequate to permit maximal rates of prostaglandin formation. Indeed, bovine vesicular glands contain a cytosolic factor, presumed to be glutathione peroxidase, that represses prostaglandin synthesis *in vitro* [21].

Our results now make it apparent that decreasing the peroxide concentration can alter the effectiveness of certain anti-cyclooxygenase agents *in vitro*. The manner by which the peroxidase-sensitive agents acquire this inhibitory behavior may be explained by a hypothetical mechanism proposed by Hemler and Lands [12] for the PGH synthase complex. This hypothesis proposes a requirement for a low con-

centration of hydroperoxide (10^{-8}–10^{-7} M [22]) to initiate and maintain the free-radical cyclooxygenase reaction. However, the peroxidase activity of the synthase may also form metastable intermediates that are inactive with respect to cyclooxygenase activity. In this condition, added peroxidase cosubstrates may stimulate further cyclooxygenase activity by converting the enzymic-peroxidase intermediate complexes back to a form of enzyme capable of catalyzing the cyclooxygenase reaction. Agents that remove radical intermediates essential to the cyclooxygenase reaction are more effective inhibitors of cyclooxygenase activity when the concentration of activating hydroperoxide is decreased to very low values. As levels of hydroperoxide activator increase, the cyclooxygenase reaction accelerates and overcomes the ability of these agents to remove radical intermediates as rapidly as they form. Thus, as Table 2 indicates, the ratio of peroxide-removing activity to peroxide-forming activity in the assay becomes important in determining the effectiveness of this class of cyclooxygenase inhibitors.

The paradoxical ability of the peroxide-sensitive agents to both stimulate and inhibit the oxygenation reaction depends upon the ambient peroxide concentration and parallels the reported behavior of phenol as both a stimulator and synergistic inhibitor of the cyclooxygenase [23]. The capacity for synergistic inhibition described for prostaglandin peroxidase cosubstrates correlated with their ability to stimulate the cyclooxygenase reaction. This result led Egan *et al.* [23] to suggest that "the effect which contributes to cyclooxygenase stimulation may also result in increased sensitivity to these agents" and that this behavior "could result from stimulator-induced changes in the binding of inhibitors. . . possibly resulting from a conformational change in the enzyme." Application of our finding that added glutathione peroxidase enhanced the inhibitory effectiveness of the fenamates tested and several other agents leads to a unifying hypothesis for all the observations. The stimulators of cyclooxygenase act as cosubstrates of the peroxidase associated with PGH synthase. The resulting increase in peroxidase activity can be expected to reduce the steady-state level of activating lipid peroxide, in a manner similar to the addition of glutathione peroxidase. Only when the hydroperoxide level (and initiation of the cyclooxygenase reaction) is below a critical value does the radical-trapping ability of agents such as phenylbutazone, acetamidophenol or the fenamates become apparent and cause significant inhibition. It is important to note that inhibition by indomethacin, flurbiprofen or ibuprofen was not augmented by phenol [23] or by added glutathione peroxidase. These drugs act by competitive reversible and irreversible mechanisms [24], and their binding seems uninfluenced by ambient peroxidase. Thus, accounting for the synergistic inhibition reported earlier [23] does not require invoking conformational changes in the enzyme, but merely recognition of the impact of lowered availability of hydroperoxide activator upon the inhibitory effectiveness of radical trapping or antioxidant-like agents.

The observation that the *in vitro* potency of certain anti-inflammatory drugs was increased when the concentrations of hydroperoxides were lowered provides two benefits: (1) a clearer interpretation of the mode of inhibition by these agents, and (2) a rationale for the variation in different theraputic effectiveness of a given drug when treating different pathophysiological states.

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