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INDOMETHACIN INHIBITION OF GLUTATHIONE S-TRANSFERASES

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Indomethacin inhibited rat liver glutathione S-transferases (EC 2.5.1.18). Its inhibition was non-competitive with respect to 3,4-dichloronitrobenzene with an apparent K_i of 5.3×10^{-5} M and uncompetitive with respect to glutathione with an apparent K_i of 4.0×10^{-5} M. 4-Chlorobenzoic acid and 5-methoxy-2-methylindole-3-acetic acid, two metabolites of indomethacin, were weak inhibitors of the enzymes. On the other hand, meclofenamic acid was a competitive inhibitor of the enzymes with an apparent K_i of 3.0×10^{-4} M. Possible significance of these findings in arachidonic acid metabolism is discussed.

Glutathione S-transferases (EC 2.5.1.18) catalyze transfer of the glutathionyl group to an electrophilic acceptor. When the acceptor contains a reactive double bond or epoxy group, an addition reaction takes place. The reaction is considered an initial step in converting the electrophile to a mercapturic acid for excretion [1,2]. Recently, Hammarstrom et al. [3] and Parker et al. [4] suggested that glutathione S-transferases catalyze the conversion of leukotriene A_4 to leukotriene C_4, a constituent of SRS. If confirmed, these enzymes will assume an important role in the lipoxygenase pathway of arachidonic acid metabolism.

Indomethacin, 1-(p-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid, was identified first by Vane [5] to be an inhibitor of prostaglandin biosynthesis. A comparative study of the inhibitory effect of non-steroidal anti-inflammatory drugs including indomethacin on prostaglandin synthetase from different sources followed shortly [6]. More recently, Siegel et al. [7] showed that indomethacin among others inhibited 12-HPETE peroxidase activity in human platelets.

Abbreviations: CBA, 4-chlorobenzoic acid; DCNB, 3,4-dichloronitrobenzene; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); GSH, reduced glutathione; 12-HPETE, 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid; MMIA, 5-methoxy-2-methylindole-3-acetic acid; SRS, slow reacting substance (of anaphylaxis).

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Hence, indomethacin inhibits enzymes in both pathways of arachidonic acid metabolism.

This report describes our observations on the inhibition by indomethacin of rat liver glutathione S-transferases and its possible implications on SRS formation.

Materials and Methods

Chemicals. CBA, MMIA, and DCNB were purchased from Aldrich Chemical Co., GSH and DTNB from Sigma Chemical Co. Indomethacin and meclofenamic acid (sodium salt), respectively, were gifts of Merck Sharp & Dohme and Warner Lambert - Parke Davis. All other chemicals were of reagent grade.

Enzyme preparation. Livers from two white male rats were removed after decapitation under light anesthesia to drain off as much blood as possible. The livers were immediately minced and homogenized in ice-cold distilled water to give approximately 250 mg/ ml. All subsequent steps were carried out at 4°. The homogenate was centrifuged at 10,000 x g for 1 hr. The supernatant fluid was introduced into a 2.5 x 7.0 cm DEAE-cellulose column previously equilibrated with 10 mM Tris buffer, pH 8.0. The enzymes were eluted with the same buffer and fractions with the enzyme activity were pooled. This chromatographic step followed the procedure of Habig et al. [8]. $(NH_4)_2SO_4$ was added to 50% saturation and the precipitate was discarded. $(NH_4)_2SO_4$ was again added to the supernatant liquor to 65% saturation. The precipitate was dissolved in 5 ml of 10 mM potassium phosphate, pH 6.7, and dialyzed against two changes of the same buffer with a total volume of 1 liter. The dialyzed preparation had a specific activity of 0.12 μmoles of DCNB conjugated/ min/ mg protein and was used in the following experiments without further treatment.

Enzyme assay. The spectrophotometric assay of Habig et al. [8] was used. The reaction mixture contained 4.5 mM GSH, 0.5 mM DCNB and a suitable amount of the enzyme preparation in 0.1 M potassium phosphate buffer, pH 7.4, so that the increase in absorbance at 345 nm and 25° was less than 0.05 A/ min. The rate of the reaction was monitored in a Beckman DU-5 spectrophotometer. Under the conditions used, non-enzymic conjugation was negligible. When a compound was sparingly soluble in water, it was first dissolved in ethanol. The final concentration of ethanol in the assay mixture, however, never exceeded 3.3%. Occasionally, the rate of reaction was estimated by determining decrease in GSH concentration at 25° with DTNB [9] in a Zeiss PMQII spectrophotometer. Protein was determined by the procedure of Lowry et al. [10] with bovine serum albumin as standard.

Results and Discussion

Indomethacin inhibited glutathione S-transferases from rat liver. Its inhibition was non-competitive with respect to DCNB with an apparent K_i of 5.3×10^{-5} M and the apparent K_m was 4.0×10^{-4} M. Its inhibition was uncompetitive with respect to GSH with an apparent K_i of 4.0×10^{-5} M and the apparent K_m was 3.0×10^{-4} M. These results are shown graphically in Figs. 1 and 2 by double reciprocal plots. The enzyme preparation used in the present

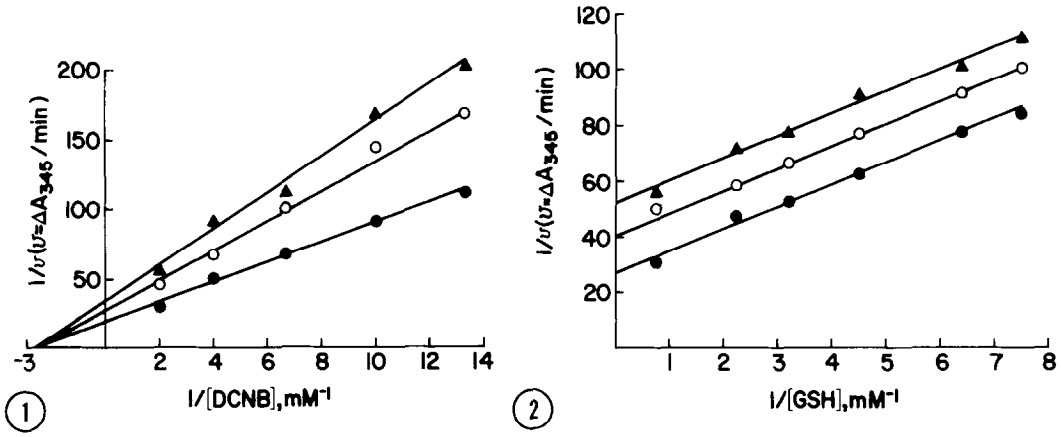

Fig. 1. Non-competitive inhibition by indomethacin. The concentration of DCNB varied, while that of GSH was held constant at 4.5 mM. Indomethacin was added at 20 (O) and 40 (▲) μM.

Fig. 2. Uncompetitive inhibition by indomethacin. The concentration of GSH varied, while that of DCNB was held constant at 0.5 mM. Indomethacin was tested at 20 (O) and 40 (▲) μM.

study was partially purified only and it contained most of the enzymic forms found in liver cells [8]. Kinetic parameters determined with such a preparation could represent the "weighted" averages of all contributing enzymic forms and thus might reflect more closely the situation in vivo than with a preparation of a single enzymic form. However, rigorous kinetic treatments have been compromised.

We next determined possible inhibition by CBA and MMIA, because they are major metabolites of indomethacin [11]. Despite their structural dissimilarities, both CBA and MMIA inhibited the transferases non-competitively, but they were only about 1% as effective as indomethacin. Table 1 shows the results. Since these two compounds together make up the entire indomethacin molecule, we

Table 1. Inhibition of rat liver glutathione S-transferases

Compound	Highest concentration tested, mM	Type of inhibition	Apparent K_i, mM
CBA	0.89	Non-competitive	4.7
MMIA	0.88	Non-competitive	3.3
Aspirin	3.4	Not inhibitory	
Benzoic acid	4.3	Not inhibitory	
Meclofenamic acid	0.44	Competitive	0.30

determined the total inhibition by equimolar mixtures of these two compounds. We found that the inhibition was additive, but it was still far less than that by indomethacin alone (data not shown). These results would seem to suggest that CBA and MMIA occupy adjacent sites on the enzyme, but their topographic arrangement may be different from that in the indomethacin molecule. Consequently, when indomethacin takes up these two adjacent sites on the enzyme, it causes a greater conformational change of the latter than can be accounted for by either CBA or MMIA or both.

As can be seen in the same table, neither aspirin nor benzoic acid inhibited glutathione S-transferases from rat liver in the range of concentrations used. Comparison of these two compounds with CBA suggests that the chlorine atom of the latter plays a role in binding to the enzyme. Perhaps the binding involves a displacement of the chlorine atom, but the resulting complex with the enzyme does not dissociate. Further, the lack of inhibition of the transferases by aspirin is probably not surprising, since earlier inhibition studies with prostaglandin synthetase [6] have shown that aspirin was less effective than indomethacin by a factor of roughly two orders of magnitude depending on the source of the enzyme.

Meclofenamic acid, which is a slightly more potent prostaglandin synthetase inhibitor than indomethacin [6], inhibited rat liver glutathione S-transferases. The inhibition was competitive with respect to DCNB with an apparent K_i of 3.0×10^{-4} M. Hence, meclofenamic acid was about one-tenth as effective as indomethacin as inhibitor of the transferases.

It would be of interest to know whether tissues and cells that are potential sites of SRS formation contain glutathione S-transferase activity and how it is inhibited by indomethacin. In preliminary experiments, we found that the enzyme activity in guinea pig lung and human leukocytes was lower than that in rat liver, but the activity was inhibited by indomethacin also. However, since the several enzymic forms of the transferases have differing catalytic activities [8] and since the distribution of the enzymic forms varies from one tissue to another [12], enzyme activity of a tissue assayed with DCNB as substrate (based on a displacement reaction) may not necessarily reflect its SRS synthesizing capacity (requiring an addition reaction). These considerations deserve further studies.

Granting that glutathione S-transferases catalyze SRS formation, we should like to comment on the possible significance of our findings in arachidonic

acid metabolism. Since indomethacin inhibits not only prostaglandin synthetase [6], but also 12-HPETE peroxidase [7] and glutathione S-transferases, we can expect the drug to have a direct effect on both cyclooxygenase and lipoxygenase pathways. To what extent each pathway in a given type of cells will be affected by a given concentration of indomethacin depends on the relative concentrations of the synthetase, the peroxidase, and the transferases and the K_i values for the three enzyme systems in these cells. Moreover, unlike the synthetase and the peroxidase, glutathione S-transferases have activity toward a wide spectrum of substrates, and leukotriene A_4 is but one of them. These potential substrates must compete with one another for the several enzymic forms depending on their concentrations and K_m values. Additionally, there probably is variable access of indomethacin to these enzymes located in different anatomical sites in vivo. Hence there is a host of factors that can modulate the inhibitory potency of indomethacin on the two pathways in vivo.

The multiple loci of indomethacin action in arachidonic acid metabolism would preclude a simple explanation for its effect on a cellular level. Hence, studies such as those of Falkenhein et al. [13], which reported that indomethacin at a concentration of 10 μg/ ml (2.8×10^{-5} M) did not result in a change in either 5-hydroxy-6,8,11,14-eicosatetraenoic acid or SRS formation in rat basophilic leukemia cells, can be better understood and interpreted when fuller information about the enzyme systems and their inhibition by indomethacin becomes known.

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