FLAVOENZYMES INHIBITED BY INDOMETHACIN

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ABSTRACT

The effect of indomethacin on the activity of five different flavoenzymes, three dehydrogenases and six hydrosases, was determined. Indomethacin at concentration 1.0 mM inhibited the activity, in decreasing order of sensitivity, of the following flavoenzymes: D-amino acid oxidase (pig kidney), flavin-containing monooxygenases (pig liver microsomal), cyclohexanone monooxygenase (*Acinetobacter*), NADPH-quinone reductase (pig liver), and glutathione reductase (yeast), but it had no effect on the activity of glucose oxidase (*Aspergillus*) or liver microsomal NADPH-cytochrome P-450 reductase. Indomethacin was competitive with D-alanine for the D-amino acid oxidase (K_i=30 μM) and with NADPH for all other flavoenzymes sensitive to this compound (K_is 170-500 μM). While indomethacin also inhibited two of the three $NAD(P)^+$-dependent dehydrogenases tested, the K_is were relatively high (<1, 500 μM), and of the six different hydrolases tested only one, liver microsomal esterase, was inhibited by indomethacin (K_i=600 μM). Indomethacin also inhibited aminopyrine demethylation catalyzed by the liver microsomal P-450 monooxygenase (K_i=1,000 μM). Although the exact mechanism for the inhibition of functionally different flavoenzymes sensitive to indomethacin is not known, the inhibition is probably not due to the detergent properties of this drug.

KEY WORDS

indomethacin, flavoenzymes, D-amino acid oxidase, FMO, cyclohexanone monooxygenase

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INTRODUCTION

Indomethacin is an effective non-steroidal anti-inflammatory agent used clinically for the treatment of rheumatoid arthritis and osteoarthritis, but the high incidence of adverse side effects limits its chronic use. While inhibition of prostaglandin synthesis /1/ and perhaps of phospholipase /2/ by indomethacin is related to its anti-inflammatory activity the biochemical events responsible for many of the adverse reactions of this drug are largely unknown. In addition to the effects of indomethacin on the enzymes associated with the synthesis of prostaglandins, indomethacin has been reported to inhibit histidine decarboxylase /3/, phosphodiesterase /4/, tyrosine aminotransferase /5/, and serotonin N-acetyl transferase /6/.

In addition, Falzon *et al.* /7/ have shown that inhibition of microsomal NADH-cytochrome b_5 reductase, cytochrome P-450 monooxygenases and epoxide hydrolase by high concentrations of indomethacin *in vitro* may be a function of the detergent properties of the drug. Although detergent effects may account for some of the loss of cytochrome P-450 at pharmacological doses of indomethacin in rats /8/, this property is probably not responsible for adverse effects at therapeutic doses.

In the course of testing the effects of various xenobiotics on activities of microsomal monooxygenases, we observed that indomethacin inhibited reactions catalyzed by the microsomal flavin-containing monooxygenases. However, the inhibition was not selective and the data presented in this report indicate that indomethacin inhibits the activity of many, but not all, enzymes bearing FAD at K_is significantly lower than the concentrations required to elicit detergent effects.

MATERIALS AND METHODS

The nucleotides, indomethacin, and other biochemicals were purchased from Sigma Chemical Co., St. Louis, MO or from Aldrich Chem. Co., Milwaukee, WI. All other reagents were of the highest purity available from commercial sources. Alcohol dehydrogenase (yeast), acetylcholine esterase (bovine), catalase (bovine), carbonic anhydrase (bovine), β-glucuronidase (*Helix pomatia* type H-5), glutathione reductase (yeast), glucose oxidase (*Aspergillus*, type VII-

S), glucose-6-phosphate dehydrogenase (*L. mesenterodies* type XXIV), pepsin (porcine) and subtilisin (*B. licheniformis*) were obtained from Sigma Chem. Co. D-Amino acid oxidase /9/ and flavin-containing monooxygenase /10/ were isolated from pig kidney cytosol and pig liver microsomes, respectively, by the methods in the references cited. Cyclohexanone monooxygenase was isolated from *Acinetobacter* NCIMB 9871 as described by Donoghue *et al.* /11/. Pig liver cytosol and liver microsomes were used as sources of NAD(P)H-quinone reductase and NADPH-cytochrome P-450 reductase, respectively. Guinea-pig liver microsomes were used to determine the effects of indomethacin on microsomal esterases and NADPH-and O_2-dependent aminopyrine demethylase.

Activities of all enzymes, except pepsin, were measured at pH 7.4, 37°C in 0.1 M phosphate buffer. Pepsin activity was measured at pH 1.5 by following the release of acid soluble peptides absorbing at 280 nm from denatured hemoglobin. Activities of all other commercial hydrolases were determined by minor modifications (all measured only at pH 7.4) of the procedures described in the references cited: acetylcholine esterase /12/, carbonic anhydrase /13/, β-glucuronidase /14/, and subtilisin /15/. Activities of $NAD(P)^+$-dependent dehydrogenases were measured by following enzyme and substrate-dependent changes in absorbance at 340 nm with a Hewlett Packard 8452A Diode Array Spectrophotometer fitted with a stirred cell maintained at 37°C. Changes in absorbance from 300-400 nm were recorded every 0.5 sec and initial rates calculated from changes at 340 nm relative to an isosbestic point. Activities of glutathione reductase, NADPH-cytochrome P-450 reductase, and NAD(P)H-quinone reductase were measured spectrophotometrically by following, respectively, GSSG-dependent NADPH oxidation, reduction of cytochrome *c*, and dicumarol-sensitive reduction of 2,6-dichloroindophenol. Activities of flavin-containing and P-450-dependent monooxygenases were determined by methods described in references /16/ and /17/. Activities of glucose oxidase, D-amino acid oxidase and catalase were measured polarigraphically by following substrate-dependent O_2 reduction with the oxidases and H_2O_2-dependent O_2 release by catalase.

The effects of 1.0 mM indomethacin on activities of the enzymes listed above were initially tested at concentrations of substrates 10 to 20% below saturation. All enzymes detectably affected by this

concentration of indomethacin were then tested plus and minus 1.0 mM indomethacin at five to six concentrations of substrate selected such that half the values were below K_m. After calculating K_i the assays were repeated with concentrations of indomethacin near the initially calculated K_i. With bi- and ter-molecular reactions, the concentrations of the second and third substrates were fixed at or above ten times their respective K_ms.

Inhibition constants were calculated from double reciprocal plots of substrate vs initial velocities without and with the two different concentrations of indomethacin. Both competitive (K_i comp) and uncompetitive (K_i uncomp) constants were calculated for reactions in which indomethacin affected both the slope and the intercepts of the double reciprocal plots.

RESULTS AND DISCUSSION

Five of the seven flavoenzymes tested were inhibited by indomethacin. In this group the D-amino acid oxidase was the most sensitive, whereas activities of glucose oxidase and NADPH-cytochrome P-450 reductase were not affected at all by indomethacin (Table 1). While two of the $NAD(P)^+$-dependent dehydrogenases were also inhibited by indomethacin, the concentrations required for 50% inhibition were quite high and perhaps of little consequence at normal therapeutic doses. In agreement with the report of Falzon *et al.* /7/, the demethylation of aminopyrine catalyzed by a P450-dependent monooxygenase was also inhibited by indomethacin although again only at relatively high concentrations. On the other hand the hydrolysis of p-nitrophenolacetate catalyzed by microsomal esterases was competitively inhibited by indomethacin at a K_i (600 μM). This was the only activity not catalyzed by a flavoprotein that was sensitive at concentrations apparently unrelated to the detergent properties of indomethacin. Other enzymes not involved in the synthesis or release of prostaglandins that are also inhibited by indomethacin at relatively low concentrations include tyrosine amino transferase /5/, cyclic AMP phosphodiesterase /4/, and acyl-CoA: lysolecithin: acyltransferase /18/. These reports, along the data summarized in Table 1, suggest that indomethacin at concentrations in the mid to low micromolar range inhibits a rather diverse group of enzymes that appear to have little in common. Other than its apparent predilection for flavoenzymes (Table

TABLE 1

Indomethacin inhibition constants for various enzymes

Enzyme activities measured by the procedures described under Methods were initially screened with and without 1.0 mM indomethacin. All enzymes detectably affected at 1.0 mM were then examined with two different concentrations of indomethacin against variable concentrations of substrate.

	Substrate	Kinetic constants (mM)*		
		K_m	K_i(comp)	K_i(uncomp)
FLAVOENZYMES				
D-Amino acid oxidase	D-alanine	1.7	0.030±0.010	—
FMO**	Methimazole	0.0050	—	1.4±0.3
	NADPH	0.0090	0.17±0.06	—
CMO**	Methimazole	0.065	—	0.28+0.05
	NADPH	0.030	0.34±0.07	—
Gluthathione reductase	GSSG*	0.15	1.9±0.3	1.7±0.4
	NADPH	0.015	0.52±0.10	—
NAD(P)H-Quinone reductase	DCIP*	0.030	1.7±0.3	—
	NADPH	0.028	0.52±0.10	1.5±0.5
NADPH-DEPENDENT DEHYDROGENASES				
Alcohol dehydrogenase	NADH	0.15	1.3±0.4	—
	Acetaldehyde	1.4	2.8+1.1	—
Glucose-6-phosphate dehydrogenase	$NADP^+$	0.022	—	8.1+1.2
	glucose-6-phos.	1.9	8.6+2.0	—
P450-DEPENDENT MONOOXYGENASE				
Aminopyrine demethylase	aminopyrine	2.7	0.94±0.10	4.0±0.8
HYDROLASES				
Microsomal esterase	4-nitrophenyl acetate	0.35	0.60+0.10	—

Enzyme activities not affected by indomethacin were:
FLAVOENZYMES - NADPH-cytochrome P-450 reductase and glucose oxidase
NADPH-DEPENDENT DEHYDROGENASES -Isocitrate dehydrogenase
HYDROLASES - Acetylcholine esterase, carbonic anhydrase, β-glucuronidase, pepsin and subtilisin.

*Inhibition constants are average ± SEM of no less than 6 determinations at each concentration of indomethacin.

**Abbreviations are: FMO-pig liver flavin-containing monooxygenase, CMO-*Acinetobacter* cyclohexanone monooxygenase, GSSG-glutathionine disulfide, DCIP-2,6-dichloroindophenol.

1) the inhibition appears independent of the type of reaction catalyzed. For instance, both D-amino acid oxidase and glucose oxidase are FAD-dependent enzymes that catalyze reduction of molecular oxygen by the organic substrate, but the former was the most sensitive of all the enzymes tested whereas activity of glucose oxidase was not affected at all. Other differences in the nature of the inhibition are also observed in that indomethacin is not only competitive with NADPH for the flavoenzyme monooxygenases and reductases (Table 1), but it also competes with substrates for enzymes that do not require NADPH (e.g. D-amino acid oxidase, microsomal esterases, phosphodiesterase /4/, and tyrosine amino transferase /5/). It is difficult to postulate an active site that can accommodate such structurally diverse compounds and it would appear that the molecular basis for inhibition is more complex than simple competition of indomethacin for the same site on an enzyme. While indomethacin and the competitive substrate must interact with the same form of the enzyme, they may interact at different sites. How indomethacin interferes with substrate binding cannot be determined from the data available.

Serum concentrations of indomethacin probably do not exceed 3-6 μM in patients treated with therapeutic doses of the drug /19/ and the observed *in vitro* inhibition of most of the enzymes listed in Table 1 is probably of little consequence *in vivo*. On the other hand, the rather low K_i of indomethacin for the D-amino acid oxidase from pig kidney suggests that significant inhibition of this flavoenzyme *in vivo* by therapeutic doses of indomethacin is possible. Although the exact function of this enzyme has not been fully defined, it has the properties of an enzyme of detoxification /20/ and accepts a few xenobiotic sulfur compounds as well as D-amino acids. Because indomethacin competes with the substrate, the oxidation of xenobiotic substrates at concentrations one-tenth or less than their K_m values could be significantly inhibited by 3 μM indomethacin. While the effect of indomethacin on the activity of this flavoprotein in an intact animal has not been determined, indomethacin may be a potential tool for examining in greater detail the role of the D-amino acid oxidase in the metabolism of xenobiotics.

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