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Inhibition of microsomal-membrane bound and purified epoxide hydrolase by C_2–C_8 1,2-alkene oxides

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Alkene and arene oxides have been implicated as the ultimate toxic metabolites of many unsaturated hydrocarbons [1], and alkene oxides have been postulated to be obligatory intermediates in the metabolism of alkenes to glycols [2, 3]. Epoxide hydrolase (EC 4.2.1.63), a microsomal enzyme, catalyzes the conversion of epoxides to glycols and hydrodiols, which are generally less toxic than the parent compounds. Epoxide hydrolase may, therefore, be an important modulator of the toxicity of alkenes. Previous studies of the substrate [4–8] and inhibitor [9, 10] specificities of epoxide hydrolase have dealt primarily with arene oxides or substituted alkene oxides. As enzyme substrates are frequently competitive inhibitors of the activity of the enzyme, the potencies of 1,2-alkene oxides as inhibitors of epoxide hydrolase may indicate their relative susceptibilities to metabolism by this enzyme. The objective of the studies reported here was to determine the potencies of low molecular weight 1,2-alkene oxides as inhibitors of hepatic epoxide hydrolase.

Male F-344 rats (Charles River Breeding Laboratories, Wilmington, MA) were housed in hanging stainless steel cages and were allowed free access to water and food (Wayne Lab Blox) until 12 hr before being killed. Rats were pretreated by i.p. injection with 80 mg/kg sodium phenobarbital once a day for 3 days, the last dose being administered 24 hr prior to sacrifice. Rats were killed by cervical dislocation, and hepatic microsomes were prepared by the method of Dent *et al.* [11]. Microsomal epoxide hydrolase was prepared according to the method of Lu *et al.* [12]. The purified enzyme that gave one major band on sodium dodecylsulfate–polyacrylamide gel electrophoresis had a specific activity of 525 ± 15 (mean ± S.E.M., N = 10) nmoles benzo[a]pyrene 4,5-dihydrodiol, and 1715 ± 44 nmoles styrene glycol, produced per mg protein per min. Epoxide hydrolase activity was determined using [G-^{3}H]benzo[a]pyrene 4,5-oxide (Midwest Research Insitute) or [7-^{14}C]styrene 1,2-oxide (gift from Professor F. Oesch) as substrate. The assays were performed essentially as described by Oesch *et al.* [4] and Schmassman *et al.* [13]. The substrate concentrations employed were, respectively 200 μM and 2 mM for benzo[a]pyrene and styrene oxide. All assays were performed under conditions that provided linear product formation with time and protein concentration.

1,2-Alkene oxides were added to the incubation mixtures immediately prior to the addition of the substrates, except in some studies with ethylene oxide where a preincubation period of 30 min was employed. All water insoluble oxides were added as solutions in dimethylsulfoxide (DMSO); the volume of the solvent never exceeded 10 μl/ml incubation mixture. Ethylene oxide and propene oxide were added as solutions in 0.1 M Tris–HCl, pH 9.0. All solutions of the alkene oxides were prepared immediately before each experiment; any excess solution was disposed of by mixing with 5 N H_2SO_4. Solutions of ethylene oxide were prepared by bubbling the gas through 0.1 M Tris–HCl, pH 9.0. The concentration of ethylene oxide in solution was determined gravimetrically and was verified by gas chromatography at 110° on a 6-foot glass column packed with Tenax GC 60/80 mesh using a flame ionization detector with a carrier gas flow rate of 20 ml/min of N_2.

All of the alkene oxides inhibited both the microsomal and purified epoxide hydrolase (Table 1). As the chain lengths of the compounds increased their potencies as inhibitors of hydrolase activity increased, as reflected by the decreasing values of IC_{50}. The IC_{50} values obtained with octene oxide and hexene oxide were similar for both the purified and microsomally bound enzyme. With the lower molecular weight compounds (butene oxide, propene oxide and ethylene oxide), the purified enzyme was more sensitive than the microsomal enzyme to inhibition. The lower sensitivity of the microsomal enzyme probably resulted from the low molecular weight alkene oxides having reacted with microsomal proteins, effectively reducing the concentration of inhibitor that was available to the enzyme. As the chain length of the alkene oxide increased, the ratio of the IC_{50} with styrene oxide as substrate to the IC_{50} with benzo[a]pyrene 4,5-oxide as substrate (IC_{50} ratio) also increased. The IC_{50} ratio was approximately 19 for octene oxide with both the purified and the microsomal enzyme. The IC_{50} ratio decreased, as the chain length of the epoxide decreased, to a minimum of approximately 2 with ethylene oxide. The ratio of the concentration of styrene oxide to benzo[*a*]pyrene oxide in the assays was 10; the IC_{50} ratio of 19 for octene oxide indicates that this epoxide was a more effective inhibitor of the hydration of benzo[a]pyrene oxide than of styrene oxide. The decreasing IC_{50} ratio with decreasing chain length indicates that the shorter chain epoxides were relatively more efficient in inhibiting the hydration of styrene oxide than of benzo[a]pyrene oxide.

Cyclohexene oxide (CCHO) and 1,1,2-trichloropropene 2,3-oxide (TCPO) inhibited both the purified and the

Table 1. IC_{50} Values for inhibition of epoxide hydrolase by 1,2-alkene oxides

Inhibitor	IC_{50} (μM)		IC_{50} (SO)
	Benzo[*a*]pyrene 4,5-oxide (BPO)	Syrene oxide (SO)	IC_{50} (BPO)
Microsomal epoxide hydrolase*			
Ethylene oxide	930,000	2,050,000	2.2
1-Propene oxide	235,300	921,600	3.9
1-Butene oxide	8800	37,000	4.2
1-Hexane oxide	1000	5100	4.3
1-Octene oxide	140	2750	19.6
Cyclohexane oxide	350	1500	4.3
1,1,2-Trichloropropene 2,3-oxide	7.5	70	9.3
Purified epoxide hydrolase			
Ethylene oxide	575,000	1,600,000	2.8
1-Propene oxide	62,500	450,000	7.2
1-Butene oxide	3500	27,000	7.7
1-Hexane oxide	900	4600	5.1
1-Octene oxide	145	2825	19.5
Cyclohexane oxide	325	1750	5.4
1,1,2-Trichloropropene 2,3-oxide	16.6	245	14.7

* Uninhibited activities of the microsomal hydrolase were 16.6 ± 0.7 (mean ± S.E.M.) nmoles benzo[*a*]pyrene 4,5-dihydrodiol, and 22.0 ± 0.8 nmoles styrene glycol, produced per mg protein per min.

microsomal enzyme. In constrast to the alkene oxides, the concentration of TCPO required to produce 50 per cent inhibition of the pure enzyme was slightly higher than the IC_{50} with the microsomal enzyme.

It has been suggested that CCHO acts as a non-competitive inhibitor of styrene oxide hydration [8]. More recently, however, Levin *et al.* [14] demonstrated that the type of inhibition produced by CCHO is dependent on the substrate used, and these data along with the data on the effects of other modifiers of epoxide hydrolase activity suggest that more than one form of epoxide hydrolase exists. Recently, Guengerich *et al.* [15, 16] isolated several forms of hydrolase from both rat and human liver microsomes. In contrast to CCHO, TCPO has been shown to be a potent uncompetitive inhibitor of epoxide hydrolase when styrene oxide is the substrate [8]. Thus, the increase in IC_{50} ratio with increasing carbon chain length of the alkene oxides could be a reflection of either a change in the type of inhibition or differing sensitivities of the forms of hydrolase present; the data presently available do not allow us to distinguish between these possibilities.

The concentration of ethylene oxide required to produce 50 per cent inhibition of the microsomal hydrolase was respectively, 1000 and 5000 times the substrate concentration for styrene oxide and benzo[a]pyrene oxide. Although more potent than ethylene oxide, propene oxide and butene oxide were also very poor inhibitors of the hydrolase (Table 1). It has been demonstrated previously that octene oxide is a good substrate for epoxide hydrolase [6]. In addition, a correspondence between ability to inhibit epoxide hydrolase and metabolism by the enzyme has been demonstrated for the 1,2-alkene oxides between C_7 and C_{16} [10]. It may be concluded that the degree of inhibition of epoxide hydrolase by a 1,2-alkene oxide with a carbon chain of less than 7 will be a good reflection of the potential as a substrate for this enzyme. Thus, the high concentrations of the ethylene oxide, propene oxide, and butene oxide, required to inhibit the enzyme, indicate that these compounds would be poor substrates for the hydrolase.

In one group of experiments, microsomes and purified enzyme were preincubated for 30 min with various concentrations of ethylene oxide prior to initiation of the reaction with substrates. This preincubation did not alter the IC_{50} values with either microsomes or purified enzyme for ethylene oxide. This observation is of particular interest in light of two other reports. DuBois *et al.* [17] provided evidence that histidine is involved in the active site of epoxide hydrolase. Ehrenberg *et al.* [18] have shown that, in animals exposed to ethylene oxide, *N*-hydroxy ethyl histidine residues result from alkylation of histidine in the hemoglobin. If ethylene oxide reacts readily with histidine residues, and if histidine is an essential component of the active site of epoxide hydrolase, then preincubation of microsomes or purified enzyme with ethylene oxide would be expected to cause a significant inhibition of the hydrolase. This is clearly not the case. The failure of preincubation with ethylene oxide to increase the extent of inhibition of the hydrolase suggests that the reaction of ethylene oxide with nitrogen in the hisitidine at the hydrolase active site occurs very slowly, if at all. These results clearly demonstrate the extremely low affinity of ethylene oxide for the hydrolase.

These studies demonstrate that low molecular weight (C_2–C_8) 1,2-alkene oxides are able to inhibit epoxide hydrolase. The shorter chain-length compounds in this series, however, inhibit the hydrolase only at very high concentrations. These data suggest that ethylene oxide, propene oxide and butene oxide are poor substrates for epoxide hydrolase. It is therefore unlikely that epoxide hydrolase plays a major role in the detoxification of these compounds. It has been demonstrated in this laboratory (unpublished data) that ethylene oxide rapidly depletes glutathione in isolated rat liver hepatocytes. Together with the data presented here, this suggests that glutathione conjugation of low molecular weight alkene oxides may be a more important route of metabolism for these compounds than hydrolysis catalyzed by the hydrolase.

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Effect of nonsteroidal anti-inflammatory drugs on sublethal retinoic acid toxicity in Swiss mice

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Administration of excessive amounts of retinol (vitamin A) and of certain derivatives and synthetic analogs (retinoids) produces a characteristic set of symptoms, including headache, vomiting, pruritus, skeletal pain, and bone resorption in experimental animals [1, 2] and man [3–6]. In animals, administration of retinyl esters [2] or retinoic acid (vitamin A acid) [7] at sufficiently high doses can result in lethality. The symptoms of retinoid toxicity are similar to reported physiologic effects of prostaglandins [8–11] so it seemed reasonable to us that retinoid toxicity may be mediated in some way by prostaglandins. If this were the case, co-administration of an inhibitor of prostaglandin synthesis might interfere with the toxicity resulting from retinoid administration. We have demonstrated that concurrent administration of aspirin, an inhibitor of prostaglandin synthesis [8, 12], protects mice against the toxicity resulting from administration of retinoic acid at the LD_{50} [13] or LD_{10} [14]. This study was designed to compare the abilities of nonsteroidal anti-inflammatory (NSAI) drugs of known potency as inhibitors of prostaglandin synthetase to protect mice against retinoic acid toxicity.

Retinoids have been recommended for use in man as cancer chemopreventive agents, that is, to arrest or reverse the development of cancer [15]. As such, retinoids would be used for long periods in individuals who are generally healthy but who have a greater than average risk of developing cancer [15,16]. Because clinical use of retinoids is anticipated, studies that examine interference with the effects of lethal doses of retinoid are less practical than those concerned with sublethal doses of these drugs. We have shown that retinoid-induced bone fractures in mice are dose-related and that they reliably indicate the comparative toxicity of retinoids at lethal and sublethal doses [7]. Consequently, we have chosen the occurrence of bone fractures as a useful end point for sublethal retinoid toxicity.

NSAI drugs that are more potent inhibitors than aspirin of prostaglandin synthesis *in vitro* [17] are currently available. In addition to these, ascorbic acid decreases the weight loss and lethality resulting from excess vitamin A [18] and exhibits weak anti-inflammatory properties [19]. Moreover, ascorbic acid inhibits prostaglandin synthestase *in vitro* [20, 21]. We report here the effect of concurrent administration of NSAI drugs or ascorbic acid on bone fractures occurring in mice treated with a sublethal dose of retinoic acid.

Methods

Materials. All-*trans*-retinoic acid (RA) was suspended in an aqueous solution of 8% Cremophor EL (Sigma Chemical Co., St. Louis, MO) and 10% propylene glycol; these