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INHIBITION OF MICROSOMAL CYTOCHROME *c* REDUCTASE ACTIVITY BY A SERIES OF α,β-UNSATURATED ALDEHYDES

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Abstract—α,β-Unsaturated aldehydes are reactive and cytotoxic compounds which occur in the environment and are also formed *in vivo*. Many of these aldehydes have been reported to inhibit hepatic cytochrome P-450. Our laboratory has shown that *trans,trans*-muconaldehyde (a possible metabolite of benzene) as well as acrolein and crotonaldehyde, when added to hepatic microsomes, decreased cytochrome P-450 (measured spectrophotometrically). Additional studies showed that several α,β-unsaturated aldehydes also inhibited hepatic microsomal NADPH–cytochrome *c* reductase. Acrolein, crotonaldehyde and *trans,trans*-muconaldehyde all decreased NADPH–cytochrome *c* reductase activity *in vitro*. Concentrations of 0.5, 1.0 and 1.5 mM acrolein decreased activity to 60, 26 and 11% of control respectively. Similar concentrations of *trans,trans*-muconaldehyde inhibited NADPH–cytochrome *c* reductase. Crotonaldehyde was a less effective inhibitor of this enzyme. Propionaldehyde, a saturated aldehyde, had no effect on NADPH–cytochrome *c* reductase activity. Time course experiments with acrolein over a period of 5–45 min suggest that the loss of NADPH–cytochrome *c* reductase activity is non-linear. The addition of reduced glutathione protected against the inhibition of reductase activity by acrolein. Formation of these aldehydes and their subsequent inhibition of these enzymes may have important consequences in xenobiotic metabolism.

Lipid peroxidation has been shown to be a causal factor in many types of cellular damage including carbon tetrachloride (CCl_4) induced liver injury [1]. One consequence of the peroxidation of unsaturated microsomal lipids is the inhibition of a number of enzymatic activities [2]. Lipid peroxidation initiated by either CCl_4 or NADPH·Fe^{2+} *in vitro* results in a decreased activity of the microsomal enzymes glucose-6-phosphatase (G-6-Pase) and aminopyrine demethylase, whereas NADPH–cytochrome *c* reductase activity remains unchanged or is enhanced slightly [3, 4]. The 4-hydroxyalk-2-enals, which are toxic end-products of lipid peroxidation *in vitro*, inhibit the same enzymes as are inhibited *in vitro* during both CCl_4 and NADPH·Fe^{2+} catalyzed lipid peroxidation [4]. The mechanism by which membrane bound enzymes are inhibited *in vitro* during lipid peroxidation is still not understood despite numerous investigations. Mechanisms proposed to explain this enzyme inhibition include (i) alterations in membrane structure [5], (ii) binding to active sites of enzymes by unsaturated aldehyde end-products of lipid peroxidation [6], and (iii) covalent binding of free radicals to critical membrane sites [7].

Acrolein, another α,β-unsaturated aldehyde, has been shown to inhibit hepatic microsomal cytochrome P-450 *in vitro* [8]. This compound is structurally similar to the alk-2-enals formed during lipid peroxidation [9]. Furthermore, similar concentrations of acrolein and 4-hydroxynonenal inhibit superoxide anion radical production in stimulated human neutrophils [10]. To further investigate the toxicity of these compounds, the present studies were undertaken to determine the effects of a series of α,β-unsaturated aldehydes on NADPH–cytochrome *c* reductase and cytochrome P-450, two major components of the hepatic mixed-function oxidase system. The effects of acrolein and crotonaldehyde as well as a reactive diene dialdehyde, *trans,trans*-muconaldehyde, on cytochrome P-450 and NADPH–cytochrome *c* reductase were studied.

MATERIALS AND METHODS

Chemicals. Acrolein (99% pure, 1% H_2O), crotonaldehyde (99% pure, 1% H_2O), propionaldehyde 97% pure, 3% H_2O) and β-naphthoflavone (BNF) were purchased from the Aldrich Chemical Co. (Milwaukee, WI). Ferricytochrome *c*, NADPH and reduced glutathione (GSH) were purchased from the Sigma Chemical Co. (St. Louis, MO). Sodium dithionite was obtained from Fisher Scientific (Springfield, NJ) and 7-ethoxyresorufin was purchased from Pierce (Rockford, IL). Pure *trans,trans*-muconaldehyde was synthesized in our laboratory by Louise Latriano.

Animals and animal treatment. Male Sprague–Dawley rats weighing 150–200 g (Taconic Farms, Taconic, NY) were housed in groups of six and acclimated for 5 days in our animal facilities prior to any treatment. To induce hepatic microsomal enzymes, animals were injected intraperitoneally

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with either sodium phenobarbital (PB) at a dose of 60 mg/kg (0.1 ml/100 g body weight) for 3 days or with BNF in corn oil at a dose of 80 mg/kg (0.1 ml/100 g body weight) for 3 days. Control rats were injected with equal volumes of corn oil.

Microsome preparation. Hepatic microsomes were isolated using the method of Thomas *et al.* [11]. Briefly, the microsomes were prepared in 0.05 M Tris buffer, pH 7.4, containing 0.15 M KCl. These microsomes were washed in 0.15 M KCl with 10 mM EDTA and stored at −80° in 0.25 M sucrose. Microsomal samples were not reused after thawing. The microsomal protein concentration was determined by the Biuret method [12] using bovine serum albumin as the reference standard.

Preincubation studies. In all inhibition studies, microsomes were preincubated with the aldehydes in 0.1 M phosphate buffer, pH 7.4, containing 0.15 M KCl, at 37° for 15 min. For the cytochrome P-450 spectral studies and the NADPH–cytochrome *c* reductase studies, microsomes (5.6 mg protein/ml) were incubated with the aldehydes at various concentrations (as listed in the tables) in tightly capped test tubes which were partially submerged in a shaking water bath. Since NADPH has been shown to have no effect on the acrolein-mediated decrease in cytochrome P-450 [8], NADPH was not added to the preincubation mixture. For the GSH protection studies, microsomes were preincubated with both acrolein (1.0 mM) and GSH (0.5 to 8.0 mM). For 7-ethoxyresorufin *O*-deethylase (EROD) assays, microsomes (10 mg protein/ml) were incubated with acrolein at various concentrations (as shown in table 4). The aldehydes were dissolved in anhydrous ethanol with the exception of propionaldehyde which was diluted in 0.1 M phosphate buffer, pH 7.4.

Enzyme assays. Cytochrome P-450 was determined with difference spectroscopy by the method of Omura and Sato [13] using an extinction coefficient of 91 mM^{-1} cm^{-1}. Each cuvette contained 2.8 mg of aldehyde-pretreated protein in a volume of 2.5 ml. A Pye Unicam SP-1800 recording spectrophotometer was used for all spectrophotometric measurements.

NADPH–cytochrome *c* reductase activity was measured using a modification of the method of Phillips and Langdon [14] using an extinction coefficient of 21.1 mM^{-1} cm^{-1} for reduced cytochrome *c*. All assays were carried out at 21°. The reaction mixture contained 2.7×10^{-4} M ferricytochrome *c*, 0.56 mg aldehyde-pretreated microsomal protein (100 μl of the aldehyde-pretreated sample) and 2 mM KCN in a final volume of 3.2 ml of 0.1 M phosphate buffer, pH 7.4, containing 0.15 M KCl. The reaction was started with the addition of 3×10^{-5} M NADPH (final concentration), and the initial rate of formation of reduced cytochrome *c* was monitored at 550 nm.

The possible interaction of acrolein with cytochrome *c* was examined. A sample of acrolein (1.5 mM) was preincubated in the absence of microsomes as described above. An aliquot (100 μl) of this sample was added to a solution of ferricytochrome *c* (2.7×10^{-4} M), and the spectrum of the solution was recorded over a range of 350–700 nm. The ferricytochrome *c* was then reduced with sodium dithionite, and a similar spectrum was recorded. Control spectra were recorded in the absence of acrolein.

The continuous spectrophotometric assay of Klotz *et al.* [15] was used to determine EROD activity at 26°–28° using an extinction coefficient of 73.1 mM^{-1} cm^{-1} for resorufin. The reaction mixture contained 1 μM 7-ethoxyresorufin and 1 mg of microsomal protein (100 μl of the aldehyde-pretreated sample) in a final volume of 2.5 ml of 0.1 M Tris–Cl, pH 8.0, containing 0.1 M NaCl. The reaction was initiated by the addition of 0.5 mM NADPH (final concentration), and the formation of resorufin was monitored at 572 nm.

RESULTS

Table 1 summarizes the effects of the α,β-unsatu-

Table 1. Effects of α,β-unsaturated aldehydes on rat liver Cytochrome P-450

Compound	Pretreatment concentration (mM)	(nmoles P-450*/mg microsomal protein)	% of Control
Acrolein	0.0	1.15 ± 0.06	100
	1.5	0.86 ± 0.03	75
	6.0	0.74 ± 0.03	64
	12.0	0.72 ± 0.03	62
Crotonaldehyde	0.0	1.20 ± 0.11	100
	15.0	0.78 ± 0.07	65
	30.0	0.77 ± 0.11	64
	60.0	0.70 ± 0.06	59
Trans,trans-muconaldehyde	0.0	1.13 ± 0.11	100
	0.5	0.96 ± 0.12	85
	1.0	0.80 ± 0.06	71
	1.5	0.85 ± 0.08	75

Microsomal protein (5.6 mg/ml) from PB-induced rats was preincubated with reactive aldehydes as described in Materials and Methods. Microsomes pretreated with propionaldehyde (40–120 mM) did not result in a loss of cytochrome P-450 (96% of control activity).

* Values are $\bar{X} \pm SD$.

Table 2. Effects of α,β-unsaturated aldehydes on NADPH–cytochrome *c* reductase activity in rat liver microsomes

Compound	Pretreatment concentration (mM)	NADPH–cytochrome *c* reductase activity* (nmoles product formed/ min/mg microsomal protein)
Acrolein	0.0	122.2 ± 4.5 (100)
	0.5	73.3 ± 11.2 (60)
	1.0	31.7 ± 5.8 (26)
	1.5	13.8 ± 4.2 (11)
Trans,trans-muconaldehyde	0.0	103.4 ± 10.6 (100)
	0.25	73.6 ± 22.4 (73)
	0.5	51.8 ± 9.3 (50)
	1.0	34.2 ± 6.1 (33)
	1.5	12.8 ± 6.4 (12)
Crotonaldehyde	0.0	128.0 ± 1.6 (100)
	5.0	98.6 ± 1.6 (76)
	10.0	83.5 ± 4.2 (65)
	15.0	60.5 ± 7.4 (47)

Microsomal protein (5.6 mg/ml) from PB-induced rats was preincubated with reactive aldehydes as described in Materials and Methods. Microsomes pretreated with propionaldehyde (5–30 mM) did not result in a loss of cytochrome *c* reductase activity (98% of control).

* Values are $\bar{X} \pm SD$. The results were obtained from at least two separate experiments performed in duplicate. Values in parentheses are percent of control activity.

rated aldehydes on rat hepatic cytochrome P-450. Treatment with acrolein, crotonaldehyde and *trans,trans*-muconaldehyde decreased cytochrome P-450 as determined spectrophotometrically. The concentration of *trans,trans*-muconaldehyde (1.5 mM, preincubation concentration) which decreased the cytochrome P-450 values by 25% was similar to that for acrolein, whereas a 10-fold greater concentration of crotonaldehyde (15 mM, preincubation concentration) was needed to produce a similar decrease. Treatment of microsomes with the saturated aldehyde, propionaldehyde (40–120 mM), had no effect on cytochrome P-450 concentrations. A concomitant increase in the amount of cytochrome P-420 was noted when the cytochrome P-450 concentration decreased.

The data in Table 2 show that NADPH-cytochrome *c* reductase activity was also decreased by reactive aldehydes. Acrolein (0.5 mM, preincubation concentration) and *trans,trans*-muconaldehyde (0.5 mM, preincubation concentration) decreased the reductase activity by 40 and 50%, respectively, whereas a 10-fold greater concentration of crotonaldehyde decreased this activity by only 25%. The saturated aldehyde, propionaldehyde, had no effect on reductase activity at concentrations up to 30 mM (preincubation concentration). Acrolein had no effect on either reduced or oxidized cytochrome *c* content as detected by the spectral studies in the absence of microsomes. There was also no interference by acrolein with the reduction of ferricytochrome *c* by sodium dithionite.

The effect of acrolein on NADPH–cytochrome *c* reductase activity in hepatic microsomes from rats treated with various inducing agents is summarized in Table 3. Acrolein at a concentration of 1.5 mM (preincubation concentration) decreased NADPH–cytochrome *c* reductase activity to 11, 9 and 14%

Table 3. Effect of acrolein on NADPH–cytochrome *c* reductase activity in hepatic microsomes from rats treated with phenobarbital, β-naphthoflavone and corn oil

Acrolein* (mM)	NADPH-cytochrome *c* reductase activity† (nmoles product formed/min/mg microsomal protein)		
	Phenobarbital	β-Naphthoflavone	Control‡
0.0	122.2 ± 4.5 (100)	65.6 ± 1.6 (100)	73.6 ± 1.6 (100)
0.5	73.3 ± 11.2 (60)	38.7 ± 2.2 (58)	52.8 ± 1.6 (72)
1.0	31.7 ± 5.8 (26)	15.4 ± 3.8 (24)	28.8 ± 2.2 (39)
1.5	13.8 ± 4.2 (11)	5.8 ± 0.0 (9)	10.6 ± 1.3 (14)

Microsomal protein (5.6 mg/ml) from rats treated with the various inducing agents was preincubated with reactive aldehydes as described in Materials and Methods.

* Acrolein concentrations are pretreatment concentrations.

† Values are $\bar{X} \pm SD$. The values are from at least two separate experiments performed in duplicate. The values in parentheses are percent of control activity.

‡ Controls were treated with corn oil.

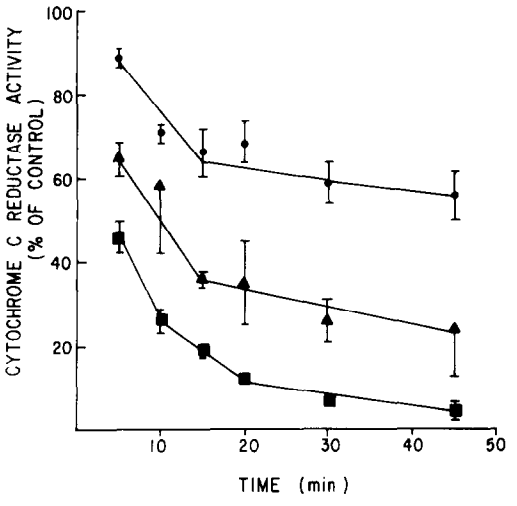

Fig. 1. Time course of the inhibition of NADPH–cytochrome *c* reductase by acrolein. Microsomal protein (5.6 mg/ml) from PB-induced rats was preincubated with 0.5 (●—●), 1.0 (▲—▲) and 1.5 (■—■) mM acrolein for the various time periods, and NADPH–cytochrome *c* reductase activity was determined as described in Materials and Methods. Control values for the three concentrations of acrolein were: 119, 112 and 118 nmoles of product formed/min/mg microsomal protein.

of control in PB, BNF and untreated microsomes respectively. Thus, the different types of microsomes appeared to be equally sensitive to these reactive aldehydes.

Figure 1 shows the degree of NADPH–cytochrome *c* reductase inhibition resulting from preincubation of microsomes with acrolein for periods of 5–45 min. At all three acrolein concentrations (0.5, 1.0 and 1.5 mM) the loss of NADPH–cytochrome *c* reductase activity was not linear with time since a break in linearity occurred between 15 and 20 min. NADPH–cytochrome *c* reductase activity was stable to incubation at 37° for periods of 15–90 min when microsomes were incubated with buffer alone (data not shown).

Table 4. Effect of acrolein on 7-ethoxyresorufin *O*-deethylase activity in rat liver microsomes

Acrolein* (mM)	Ethoxyresorufin *O*-deethylase activity† (pmoles product formed/min/mg microsomal protein)	% of Control
0.0	318.5 ± 70.2	100
0.13	304.2 ± 117.0	96
0.25	275.1 ± 88.4	86
0.5	212.7 ± 72.8‡	67

Microsomal protein (10 mg/ml) from β-naphthoflavone-induced rats was preincubated with acrolein as described in Materials and Methods.

* Acrolein concentrations are pretreatment concentrations.

† Values are $\bar{X}$ ± SD. The results were obtained from at least two separate experiments performed in duplicate.

‡ Statistically different from control by a paired Student's *t*-test ($P < 0.01$).

Table 4 summarizes the effects of acrolein on EROD activity. The range of acrolein concentrations (0.13 to 0.5 mM, pretreatment concentrations) which decreased EROD activity (96–67% of control) was similar to that which decreased NADPH–cytochrome *c* reductase activity (Table 3). In a separate experiment, 1.0 mM acrolein decreased EROD activity to 43% of control (data not shown).

As shown in Table 5, addition of reduced glutathione (GSH) to the acrolein–microsome preincubation mixture prevented the loss of reductase activity. GSH (0.5 to 8.0 mM), when added to the microsomal preincubation mixture in the absence of acrolein, decreased NADPH–cytochrome *c* reductase activity by 10% (data not shown). Therefore, in samples treated with acrolein and GSH the 8.0 mM GSH control was used to calculate percent of control values. At a molar ratio of 1:2 (GSH:

Table 5. Protection by reduced glutathione of the acrolein-mediated inhibition of rat liver microsomal NADPH–cytochrome *c* reductase

Acrolein (1 mM)	Glutathione (mM)	NADPH-cytochrome *c* reductase activity* (nmoles product formed/min/mg microsomal protein)	% of Control†
−	0.0	128.0 ± 7.0	
−	8.0	117.8 ± 4.2	
+	0.0	44.8 ± 4.8	35‡
+	0.5	87.4 ± 2.2	74
+	1.0	114.9 ± 10.6	98
+	2.0	115.5 ± 13.4	98
+	4.0	123.2 ± 10.2	105
+	8.0	121.0 ± 8.3	103

Microsomal protein (5.6 mg/ml) from PB-treated rats was incubated with acrolein and reduced GSH as described in Materials and Methods.

* Values are $\bar{X}$ ± SD. The values were obtained from at least two separate experiments performed in duplicate.

† Calculated using the control activity in the presence of 8.0 mM GSH.

‡ Calculated using the control activity in the absence of 8.0 mM GSH.

acrolein) the reductase activity was inhibited by only 26% compared with a 65% inhibition in the absence of GSH. The inhibition was prevented completely when there was a 1:1 molar ratio of reactive aldehyde to GSH.

DISCUSSION

The present *in vitro* studies indicate that reactive aldehydes which have an α,β-unsaturated functional group decrease both microsomal cytochrome P-450 content and NADPH–cytochrome *c* reductase activity. The observed inhibition of cytochrome P-450 by acrolein when measured spectrophotometrically was similar to that reported by Gurtoo *et al.* [8] who studied the effects of acrolein, a metabolite of cyclophosphamide, on cytochrome P-450 *in vitro*. Other α,β-unsaturated aldehydes such as the 4-hydroxyalk-2-enals have also been reported to inhibit cytochrome P-450 *in vitro* [4]. In our studies, acrolein and *trans,trans*-muconaldehyde were equipotent in decreasing cytochrome P-450, whereas crotonaldehyde was one-tenth as effective. These same aldehydes inhibited NADPH–cytochrome *c* reductase in a pattern similar to their inhibition of cyrochrome P-450. This pattern of inhibition correlates well with the reactivity of these compounds towards nucleophiles, a reaction which proceeds more readily the greater the positive charge on the beta carbon.

In our studies, GSH when added to the microsomal preincubation mixture in the presence of acrolein (1:1 molar ratio) prevented the inhibition of NADPH–cytochrome *c* reductase. One explanation might be that the sulfhydryl group of a soluble low molecular weight thiol is more accessible to the aldehyde than that of the reductase. Since the hepatic cytoplasmic concentration of GSH is approximately 10 mM, there is a large pool of a potential detoxifying agent [16]. However, if the α,β-unsaturated aldehyde is generated within the lipid matrix of the endoplasmic reticulum, cytoplasmic GSH may not readily interact with the endogenously generated reactive aldehyde. Further studies are necessary to determine whether the inactivation of NADPH–cytochrome *c* reductase by acrolein is the result of an interaction of the α,β-unsaturated aldehyde with the enzyme sulfhydryl group. Gurtoo *et al.* [8] found that the addition of GSH (1.4 mM) to microsomes treated with acrolein (0.7 mM) also protects against the loss of cytochrome P-450 content *in vitro*. When microsomes are treated *in vitro* with CCl_4, cysteine (2.5 mM) does not protect against the loss of either cytochrome P-450 content or G-6-Pase activity [17]. Our data along with the findings of Waller and Recknagel [17] support the conclusion that the α-β-unsaturated aldehydes might not be the primary source of inhibition in the CCl_4-mediated decrease in both cytochrome P-450 content and G-6-Pase activity.

Preincubation with concentrations of acrolein (0.13 to 1.0 mM), which were lower than those concentrations which decreased P-450 in spectral experiments, inhibited 7-ethoxyresorufin O-deethylase (EROD). Acrolein (0.5 mM, preincubation concentration) decreased EROD activity to 67% of control. This decrease was similar to results of Ferrali *et al.* [4] who found that synthetic 4-hydroxynonenal or carbonyl compounds derived from lipid peroxidation (0.45 mM) decrease aminopyrine demethylase activity to 67% of control. Ferrali *et al.* [4] reported that these alk-2-enals do not inhibit microsomal NADPH–cytochrome *c* reductase significantly. In the present study, 0.5 mM acrolein decreased NADPH–cytochrome *c* reductase activity to 58% of control. This difference could be explained by dissimilar mechanisms of action for acrolein compared with the alk-2-enals and the lipid peroxidation products. Another explanation may be differences in experimental procedures between laboratories. Studies in our laboratory have shown that the alk-2-enals are unstable with time in aqueous solution and are best prepared in ethanol and used fresh (unpublished results).

The inhibition of EROD activity by acrolein may be a direct effect on cytochrome P-450. Another possibility, however, is that the inhibition of EROD activity involves the inhibition of the NADPH–cytochrome *c* reductase, an enzyme which catalyzes the rate-limiting step in the mixed-function oxidase system. Since acrolein decreased both EROD and NADPH–cytochrome *c* reductase activities, further studies are necessary to determine if this inhibition is mediated by a direct effect by the aldehyde on cytochrome P-450, or is mediated indirectly by affecting NADPH–cytochrome *c* reductase, or a combination of both. Studies using a reconstituted mixed-function oxidase system could address this question.

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