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DNA strand cleavage as a sensitive assay for the production of hydroxyl radicals by microsomes: role of cytochrome P4502E1 in the increased activity after ethanol treatment

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There is increasing interest in the role of reactive oxygen radicals in the hepatotoxicity associated with ethanol consumption. Reactive oxygen intermediates interact with DNA and can cause single-strand breaks of supercoiled DNA. Experiments were carried out to evaluate the utility of this system as a sensitive assay for the detection of potent oxidants generated by rat liver microsomes isolated from pair-fed control rats and rats treated chronically with ethanol. DNA strand cleavage was assayed by monitoring the migration of the supercoiled and open circular forms in agarose. Microsomes catalysed DNA strand breakage with either NADPH or NADH as cofactors; iron was required to catalyse the reaction and various ferric complexes were effective in promoting the reaction. DNA strand cleavage was prevented by catalase, superoxide dismutase, GSH and hydroxyl-radical-scavenging agents, suggesting that a hydroxyl-radical-like species was the oxidant responsible for the breakage. This assay system proved to be much more sensitive in detecting hydroxyl radicals than are other methods, such as e.s.r. spectroscopy or oxidation of chemical scavenging agents with respect to the amount of microsomal protein and the nature and concentration of the iron catalyst required. Microsomes from ethanol-treated rats were more reactive than control microsomes in catalysing the DNA strand cleavage with either NADPH or NADH; increased catalytic activity was observed with various ferric complexes and was sensitive to the above antioxidants. Compared with pre-immune IgG, anti-(cytochrome P4502E1) IgG had no effect on DNA strand cleavage by the control microsomes, but completely prevented the NADPH- and the NADH-dependent increased activity found with microsomes from the ethanol-treated rats. Inhibitors of cytochrome P4502E1, such as diethyl dithiocarbamate and tryptamine, also lowered the extent of increase of DNA strand cleavage produced by microsomes from the ethanol-treated rats. These results indicate that DNA strand cleavage is a very sensitive assay for detecting the production of hydroxyl radicals by microsomes and to demonstrate increased activity by microsomes after chronic ethanol treatment. This increased activity with NADPH and NADH is due, at least in part, to induction of cytochrome P4502E1.

INTRODUCTION

The interaction of reactive oxygen intermediates with DNA has been studied in a variety of systems using photochemical or radiolysis methods to produce oxygen radicals. DNA can be degraded by reaction systems containing iron in the presence of either H_2O_2, GSH, ascorbate, anthracycline and other anti-tumour drugs, or xanthine plus xanthine oxidase (Bode, 1967; Braun and Fridovich, 1981; Muindi et al., 1985; Myers et al., 1987; Schneider et al., 1988, 1989; Sinha et al., 1988; Aruoma et al., 1989). In the presence of ferric-EDTA, NADPH and quinones, NADPH:cytochrome *P*-450 reductase was shown to catalyse single strand breakage of the plasmid pBR322 (Rumyantseva et al., 1989). The DNA scission is believed to be due to production of hydroxyl radicals (•OH) in these reaction systems. Supercoiled DNA is more compact than DNA and can be converted by single strand breaks into the relaxed open circular state or by double strand breaks to the linear form (Berlin and Haseltine, 1988; Schneider et al., 1989). These forms can be separated from each other and detected by monitoring their migration in agarose under the influence of an electric field (Schneider et al., 1988, 1989).

Microsomes isolated from the livers of rats treated chronically with ethanol have been shown to generate $O_2^{-\bullet}$ and H_2O_2 at elevated rates compared to pair-fed controls (Lieber and DeCarli, 1970; Thurman, 1973; Boveris et al., 1983; Ekstrom and Ingelman-Sundberg, 1989; Rashba-Step et al., 1993). In the presence of iron, microsomes from the ethanol-treated rats were more reactive in generating •OH and catalysing lipid peroxidation (Klein et al., 1983; Dicker and Cederbaum, 1987; Ekstrom and Ingelman-Sundberg, 1989; Krikun and Cederbaum, 1986, Puntarulo and Cederbaum, 1988a; Ekstrom et al., 1986). The goal of the current study was to evaluate the utility of DNA strand breakage as a sensitive assay system to detect the production of reactive oxygen species by microsomes and the effect of chronic ethanol treatment on oxygen radical production. Previous studies employing oxidation of •OH chemical scavengers or e.s.r. spectroscopy for the above purposes required the use of relatively large amounts of microsomal protein, e.g. 0.1–1 mg/assay (Klein et al., 1983; Dicker and Cederbaum, 1987; Rashba-Step et al., 1993; Ekstrom et al., 1986). The development of a more sensitive assay could be beneficial for studies in which a limited amount of biological material is available, e.g. surgical biopsy material or cell lines. Non-haem iron is required for the initiation of lipid peroxidation and for the generation of •OH-like species by Haber–Weiss or Fenton types of reactions (Aust

Abbreviations used: $O_2^{-\bullet}$, superoxide anion radical; •OH, hydroxyl radical; DDC, diethyl dithiocarbamate; SOD, superoxide dismutase; DTPA, diethylenetriamine penta-acetic acid; DMSO, dimethyl sulphoxide; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; 4-POBN, α-(4-pyridyl-1-oxide)-*N*-t-butyl nitrone; P4502E1, cytochrome P4502E1.

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et al., 1985; Halliwell and Gutteridge, 1985, 1986). It was considered that DNA strand cleavage could prove to be a sensitive assay system for detecting for the ability of different ferric complexes to catalyse production of 'OH by microsomes from ethanol-treated and control rats.

Increased production of reactive oxygen intermediates by microsomes after ethanol treatment may be due, at least in part, to the increased content of cytochrome P4502E1 (P4502E1), an isoform of cytochrome *P*-450 that displays elevated NADPH oxidase activity and which is poorly coupled to NADPH: cytochrome *P*-450 reductase (Gorsky et al., 1984; Ekstrom and Ingelman-Sundberg, 1989; Albano et al., 1991). A role for P4502E1 in the NADPH-dependent increased production of 'OH by microsomes of ethanol-treated rats has not been demonstrated. Microsomes from ethanol-treated rats are more reactive in generating 'OH, not only with NADPH as the microsomal reductant, but also with NADH (Dicker and Cederbaum, 1992). It is not known whether P4502E1 plays any role in the NADH-dependent increase in 'OH production by microsomes after ethanol treatment. Studies were carried out to evaluate the usefulness of DNA strand cleavage as a sensitive assay to investigate the role of P4502E1 in the increased production of 'OH by microsomes from ethanol-treated rats with NADPH and NADH as the microsomal reductants.

MATERIALS AND METHODS

Male Sprague–Dawley rats of starting weight 110–120 g were fed for 6–8 weeks with the Lieber–DeCarli liquid diet in which ethanol provided 36% of total energy; protein contributed 18%, fat 35% and carbohydrate 11% (Lieber and DeCarli, 1982). Pair-fed littermates consumed the same diet, except that carbohydrates isoenergetically replaced ethanol. Liver microsomes were prepared by minor modifications of the low-speed Ca^{2+} aggregation procedure described by Montgomery et al. (1974). Livers were perfused with 0.9% NaCl to remove blood, and homogenates were prepared in a buffer containing 0.25 M sucrose, 0.05 M Tris/HCl buffer, pH 7.4, 0.005 M $MgCl_2$, 0.05 mM desferrioxamine, 0.025 M KCl, and 0.008 M $CaCl_2$. The Ca^{2+}-aggregation method was used to remove ferritin from the microsomes (Montgomery et al., 1974), and desferrioxamine was added to chelate non-haem iron in the buffers, water or microsomes. The microsomes were washed twice with 0.15 M KCl at 100000 ***g*** for 60 min, resuspended in 0.15 M KCl, and stored at −70 °C. The buffers and water used to prepare all solutions were passed through columns containing Chelex-100 resin to remove metal contamination. Protein was determined by the method of Lowry et al. (1951). Induction of P4502E1 was validated by immunoblot analysis and by increased catalytic activity with substrates such as ethanol and *p*-nitrophenol. The source of the supercoiled DNA was plasmid pBluescriptIIKS(−). *Escherichia coli*, strain XLI Blue, was transformed with plasmid pBluescriptIIKS(−). using caesium chloride (Maniatis et al., 1982). Cells were spread on ampicillin (50 μg/ml) agar plates and incubated in a metabolic shaker overnight at 37 °C. The transformed *E. coli* cells were grown in Terrific Broth medium to a cell density of about 10^9 cells/ml, and were centrifuged in a Sorvall centrifuge at 4000 rev./min ($r_{av.}$ 10.8 cm) at 4 °C for 10 min. The supernatant was discarded and the pellet was suspended in 10 ml of a solution containing 50 mM glucose, 25 mM Tris, pH 8, and 10 mM EDTA, pH 8. Lysozyme (5 mg/ml) was added and the pellet was resuspended by pipetting up and down. The bacteria were lysed by addition of 80 ml of a solution of 0.2 M NaOH plus 1% SDS, followed by gentle mixing, and the mixture was left on ice for 10 min. Propan-2-ol (0.6 vol.) was added to each tube and, after standing at room temperature for 15 min, the suspension was centrifuged at 5000 rev./min for 5 min. The nucleic acid pellets were dried in a SpeedVac desiccator and dissolved in a total volume of 8 ml of 10 mM Tris/1 M EDTA, pH 8; 1 g of caesium chloride was added, followed by centrifugation at 3000 rev./min for 10 min. The supernatant was placed in a light-protected 13 ml Quick-Seal tube, 20 μl of ethidium bromide (10 mg/ml) was added, the tubes were sealed by heating and then centrifuged at 60000 rev./min ($r_{av.}$ 6.3 cm) at 20 °C for 48 h. The bottom band was removed with a syringe and a large-bore needle, dissolved in water and extracted five times with an equal volume of water-saturated butanol. Ethanol (100%) was added, and after the mixture had been left overnight at −20 °C, the DNA was pelleted by centrifugation at 5000 rev./min for 15 min. The pellet was dried in a SpeedVac desiccator and dissolved in 0.5 ml of sterile water. The concentration of DNA was determined from the absorbance at 260 nm.

Liver microsomes from pair-fed controls or from ethanol-treated rats (typically used at an amount of 0.008 mg protein) were incubated at 37 °C in a shaking water bath with 0.5 μg of pBluescriptIIKS(−) in a reaction system containing 100 mM potassium phosphate buffer, pH 7.4, 50 μM ferric chelate, 1.5 mM NADH or 1.5 mM NADPH in a total volume of 50 μl. Samples were incubated in 1.5 ml Eppendorf plastic tubes covered by aluminium foil. Reactions were initiated with the cofactor and terminated by the addition of 3 μl of loading buffer (0.25% Bromophenol Blue/0.25% Xylene Cyanol/30% glycerol). The samples were treated with RNAase (1 μl) and proteinase K (1.5 μl) for 20 min at 37 °C to remove RNA and protein prior to electrophoresis. The samples were centrifuged at 15000 rev./min for 30 s and were then loaded on to a 1% agarose gel containing ethidium bromide and subjected to electrophoresis (58 V) for about 2 h to separate closed circular, superhelical (form I) DNA from DNA subjected to strand cleavage by oxidative damage. The DNA bands were revealed by fluorescence in a u.v. (Fotodyne 400) DNA transilluminator system and photographed. The negative of a Polaroid (type 55P/N) photograph of the gel was scanned with an ultrascan XL enhanced laser densitometer.

All experiments were carried out under aluminium foil to prevent photochemical reduction of the iron chelate. The numbers of experiments are indicated in the Table and Figure legends. Where indicated, results refer to means ± S.D., and statistical analysis was carried out by the paired *t* test. Results are reported as the ratio of form II (DNA modified by single strand breaks) to form I (supercoiled DNA). Ferric chelates were utilized at a ferric/chelate ratio of 1:2, except for ferric–ATP, which was utilized at a 1:20 ferric/chelate ratio. The ferric complexes were prepared by dissolving ferric ammonium sulphate in 0.1 M HCl and then diluting with the respective chelator to the appropriate stock concentration. The anti-P4502E1 IgG was an antibody raised in rabbits against P4502E1 purified from pyrazole-treated rats (Palakodety et al., 1988).

RESULTS

DNA strand cleavage by microsomes

Figure 1, lane 1, shows that the DNA in plasmid pBluescriptIIKS(−) migrates primarily as a single lower-M_r band (form I, the supercoiled form) with only a faint upper band (form II, the relaxed form). Incubation of the plasmid with a combination of microsomes plus NADPH plus iron (ferric–histidine) results in a loss of the supercoiled form coupled to an increase in the open circular form (Figure 1, lanes 2 and 3). Little or no conversion occurs if either microsomes or NADPH are omitted;

some conversion still occurs if the iron catalyst is omitted, most likely reflecting the presence of small amounts of iron in the microsomes themselves (Minotti, 1989). NADH can replace NADPH in catalysing DNA strand cleavage, although longer incubation periods are required (Figure 1, lanes 5 and 6). Microsomes isolated from rats treated with ethanol were more reactive than pair-fed control microsomes in catalysing this reaction with either cofactor (NADPH, lane 3 compared with lane 2, Figure 1; NADH, lane 6 compared with lane 5, Figure 1). In fact with NADPH, microsomes from the ethanol-treated rats catalysed DNA strand cleavage to the linear form III. With freshly prepared plasmid, about 90% of the total DNA exists as form I, while 10% is in the open form II; the ratio between the two forms (form II/form I) is about 0.10. Incubating the plasmid with microsomes plus iron plus either NADPH or NADH results in a decrease in arbitrary units of form I, coupled to a corresponding increase in form II. For the control microsomes, incubation with NADPH or NADH increased the percentage of form II to values of 62 and 39% respectively; the percentage of form II was elevated to values of 86 (NADPH) and 73% (NADH) when microsomes from ethanol-fed rats were used. The ratio between the forms for the experiment shown in Figure 1 was: plasmid, 0.10; NADPH, control microsomes, 2.01; NADPH, ethanol microsomes, 5.03; NADH, control microsomes 0.63; NADH, ethanol microsomes, 2.67. Whether expressed as loss of form I or gain of form II in arbitrary units, or percentage conversion of the two forms or the ratio between the two forms, the microsomes from the ethanol-treated rat were more reactive in catalysing this DNA strand cleavage than were pair-fed control microsomes. For ease of presentation, results will be reported as the ratio of form II/form I, the higher the ratio being indicative of increased generation of reactive oxygen species.

A time-course experiment for conversion of form I into form II by microsomes is shown in Table 1. Two different iron catalysts were utilized: ferric–EDTA, which is very reactive in catalysing microsomal generation of ·OH-like species (Winston et al., 1984; Puntarulo and Cederbaum, 1988b) and ferric–ATP, which is much less reactive than ferric–EDTA, but could be representative of the small pools of non-haem iron present in cells. The ability of NADH to catalyse this reaction was also evaluated and compared with the effectiveness of NADPH. There

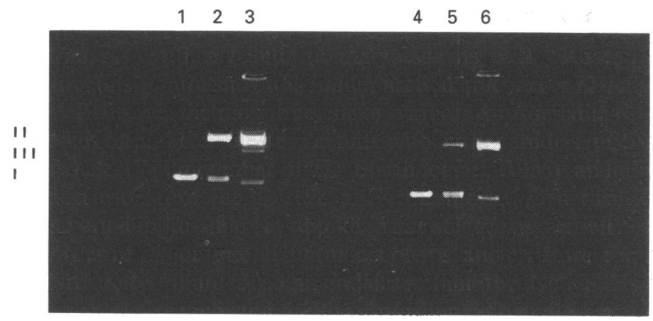

Figure 1 Gel showing DNA cleavage by rat liver microsomes

Experiments were carried out as described in the Materials and methods section. Lanes 1 and 4 contain the plasmid alone, lanes 2 and 5 show results with microsomes from control rats with either NADPH or NADH as reductant, and lanes 3 and 6 show results with microsomes from ethanol-treated rats with either NADPH or NADH as reductant. Experiments with NADPH (lanes 2 and 3) were carried out for 5 min, while experiments with NADH (lanes 5 and 6) were carried out for 45 min. Ferric–histidine was utilized as the iron catalyst. Roman numerals refer to: I; form I DNA, supercoiled form; II, form II DNA, open circular relaxed DNA; III, form III DNA, linear DNA.

was an increase in the form II/form I ratio as a function of time with microsomes from control and ethanol-treated rats, with both iron complexes and with both reductants. Ferric–EDTA was more reactive than ferric–ATP in catalysing this conversion with either NADPH or NADH. NADPH was more effective than NADH in catalysing the conversion with either ferric–EDTA or ferric–ATP. With either reductant or ferric catalyst, microsomes from ethanol-fed rats were significantly more reactive than pair-fed control microsomes in catalysing this conversion (Table 1).

Effect of iron on DNA strand cleavage by microsomes

When microsomes from control or ethanol-treated rats were incubated for 2.5 min with NADPH in the absence of added iron, but in the presence of desferrioxamine to chelate any adventitious iron present, the form II/form I ratio was similar (0.18, 0.24) to that of the plasmid itself (0.20) (Table 2), i.e., there

Table 1 Time course characterizing DNA strand cleavage by rat liver microsomes

Experiments were carried out as described in the Materials and methods section using either NADPH or NADH as reductant, and either 50 μM ferric–EDTA or ferric–ATP as iron catalyst. Results are means ± S.D. for four or five pairs of rats. Statistical significance: $^{*}P < 0.05$; $^{**}P < 0.02$.

		Ratio form II/form I			
		NADPH		NADH	
Ferric–chelate	Time (min)	Control	Ethanol	Control	Ethanol
Ferri–EDTA	2.5	1.02 ± 0.16	1.66 ± 0.27**		
	5	2.25 ± 0.95	4.68 ± 0.81**	0.57 ± 0.06	0.59 ± 0.25
	15	4.04 ± 1.90	11.14 ± 3.05**	0.91 ± 0.33	1.55 ± 0.36*
	30			1.76 ± 0.60	3.93 ± 0.82*
Ferric–ATP	2.5	0.61 ± 0.06	1.05 ± 0.53		
	5	1.03 ± 0.10	2.28 ± 0.57*	0.32 ± 0.09	0.33 ± 0.13
	15	1.81 ± 0.43	3.20 ± 0.67*	0.36 ± 0.08	0.42 ± 0.05
	30			0.46 ± 0.04	0.86 ± 0.25*
	45			1.42 ± 0.60	2.78 ± 0.27*
	60			3.29 ± 0.88	5.64 ± 0.53*

Table 2 Effect of different ferric complexes on DNA strand cleavage by rat liver microsomes

Experiments were carried out for 2.5 min with NADPH as the reductant or 30 min with NADH as reductant. The concentrations of ferric–EDTA and ferric–DTPA were 5 μM ferric (1:2 chelate), while the iron concentration of the other four complexes was 50 μM ferric ion. Desferrioxamine was present at a final concentration of 0.02 mM. Results are from three pairs of rats. Statistical significance: $^{*}P < 0.05$; $^{**}P < 0.02$.

	Ratio form II/form I			
	NADPH		NADH	
Ferric–chelate	Control	Ethanol	Control	Ethanol
Desferrioxamine	0.18 ± 0.10	0.24 ± 0.08	0.30 ± 0.16	0.39 ± 0.14
Ferric–histidine	1.31 ± 0.35	2.51 ± 0.42*	0.56 ± 0.25	1.87 ± 0.45*
Ferric–citrate	1.60 ± 0.25	2.95 ± 0.50*	1.19 ± 0.21	2.28 ± 0.35*
Ferric–ATP	1.11 ± 0.30	2.00 ± 0.15*	0.97 ± 0.08	1.77 ± 0.27**
Ferric ammonium sulphate	1.07 ± 0.35	3.66 ± 0.25**	1.32 ± 0.44	3.69 ± 0.52*
Ferric–EDTA	1.37 ± 0.15	2.05 ± 0.20*	2.88 ± 0.85	11.44 ± 3.15*
Ferric–DTPA	0.73 ± 0.22	1.91 ± 0.45*	1.18 ± 0.25	1.62 ± 0.37

Table 3 Ferric–histidine titration curve for catalysis of DNA strand cleavage

DNA strand cleavage by microsomes from ethanol-treated rats and pair-fed controls was assayed in the presence of the indicated concentrations of ferric–histidine (1:2 ferric/histidine ratio). Reactions were carried out for 5 min with NADPH as reductant, and for 45 min with NADH as reductant. Results are averages for two pairs of rats.

	Ratio form II/form I			
	NADPH		NADH	
[Ferric–histidine] (μM)	Control	Ethanol	Control	Ethanol
0	0.84	1.09	0.34	0.48
0.5	1.08	2.60		
1	1.33	2.92		
2.5	1.73	3.99	0.39	0.61
3.3	2.24	4.78	0.49	0.84
5.0			0.57	0.96
10.0	2.76	4.38	0.71	1.35

was no conversion in the 'absence' of iron by either microsomal preparation. The form II/form I ratio was elevated in the presence of several ferric complexes; ferric–histidine, –citrate, –ATP, –EDTA and –DTPA, as well as with ferric ammonium sulphate. With all ferric complexes, microsomes from ethanol-fed rats were 2–3-fold more reactive in catalysing the conversion compared with control microsomes (Table 2).

Essentially similar results were observed for the NADH-dependent system. The various ferric complexes were all reactive in catalysing the form I-to-form II conversion, and microsomes from the ethanol-treated rats were more reactive than control microsomes with all ferric complexes evaluated (Table 2).

An iron concentration curve, using ferric–histidine as the iron catalyst, is shown in Table 3. With NADPH as the reductant, an increase in the form II/form I ratio was observed even at the lowest concentration of ferric–histidine (0.5 μM), with further increases as the concentration of ferric–histidine was elevated. Higher concentrations of ferric–histidine were required to catalyse significant increases in the form II/form I ratio with NADH as compared with NADPH with both microsomal preparations, although microsomes from the ethanol-treated rats were again more reactive than control microsomes (Table 3).

Table 4 Effect of antioxidants on DNA strand cleavage

DNA strand cleavage by microsomes from ethanol-treated rats and pair-fed controls was assayed in the presence of the indicated additions. Ferric–histidine was the iron catalyst and reactions were carried out for 5 min with NADPH as reductant or 45 min with NADH as reductant. Results are from two pairs of rats.

		Ratio form II/form I			
Addition		NADPH		NADH	
Amount or concn.	Compound	Control	Ethanol	Control	Ethanol
–	None	2.54	4.24	3.07	4.90
50 units	SOD	1.55	1.67	0.60	0.51
1300 units	Catalase	0.39	0.28	0.43	0.27
0.1 mM	Trolox	1.83	5.36	3.92	4.40
5 mM	GSH	0.51	0.51	0.46	0.27
50 mM	DMSO	0.21	0.36	0.21	0.19
50 mM	Mannitol	0.61	1.51	0.78	0.64
50 mM	Ethanol	0.78	1.59	1.12	0.92
100 mM	Glycerol	0.72	0.58	0.62	0.27
30 mM	DMPO	0.42	0.64	0.47	0.25
30 mM	POBN	0.57	0.61	0.76	0.49

Effect of antioxidative agents on DNA strand cleavage by microsomes

Catalase was an effective inhibitor of the increased form II/form I ratio produced by both microsomal preparations with NADPH or NADH (Table 4). SOD also proved to be inhibitory. Trolox, which is a powerful inhibitor of microsomal lipid peroxidation, had no effect on the DNA strand cleavage produced by either microsomal preparation. GSH was a very effective inhibitor of the DNA strand cleavage, as were a variety of agents (DMSO, mannitol, ethanol, glycerol, DMPO, and 4-POBN) which react with $^{\cdot}OH$ (Table 4).

Role of P4502E1 in the increased DNA strand cleavage after ethanol treatment

To evaluate a role for P4502E1 in the DNA strand cleavage catalysed by microsomes, and particularly, in the increased catalytic activity by microsomes from ethanol-fed rats, the effect of anti-P4502E1 IgG as well as chemical inhibitors such as DDC and tryptamine were studied. A gel obtained after electrophoresis showing the effect of anti-P4502E1 IgG on NADPH-dependent and NADH-dependent DNA strand cleavage by microsomes from an ethanol-treated rat is shown in Figure 2. For this experiment, prolonged incubation times were carried out to promote extensive conversion, thereby allowing clear evaluation of the effect of the antibody. Pre-immune IgG did not significantly prevent the NADPH-catalysed conversion of form I into form II; however, anti-P4502E1 IgG was inhibitory against this conversion. At a low concentration of protein (2.33 mg of IgG/mg of microsomal protein), pre-immune IgG had no effect on the NADH-catalysed conversion (Figure 2, lane 9 compared with lane 8). However, the same protein concentration of anti-P4502E1 IgG protected against conversion (Figure 2, lane 10, compared with lanes 8 and 9).

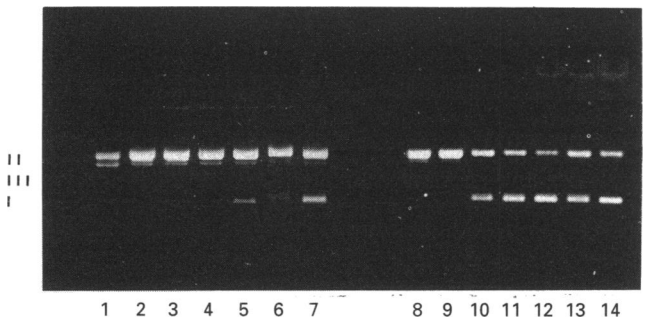

Figure 2 Gel showing the effect of pre-immune or anti-P4502E1 IgG on DNA strand cleavage catalysed by microsomes from ethanol-treated rats

Lanes 1–7 refer to experiments carried out with NADPH (20 min incubation), while NADH (75 min incubation) was the reductant for the experiments shown in lanes 8–14. Ferric–histidine was the iron catalyst. Lanes 1 and 8, no IgG added; these show the extensive conversion of form I to form II by the microsomes. Lanes 2 and 9, 2.3 mg pre-immune IgG/mg of microsome protein; lanes 3 and 10, 2.3 mg of anti-P4502E1 IgG/mg of protein; lanes 4 and 11, 4.6 mg of pre-immune IgG; lanes 5 and 12, 4.6 mg of anti-P4502E1 IgG; lanes 6 and 13, 9.2 mg of pre-immune IgG; lanes 7 and 14, 9.2 mg of anti-P4502E1 IgG.

With control microsomes, the NADPH-dependent DNA strand cleavage was inhibited by anti-P4502E1 IgG; however, at these protein concentrations, pre-immune IgG proved to be equally inhibitory (Figure 3a). Inhibition by the pre-immune IgG could reflect non-specific interaction of protein with the oxidants (·OH) responsible for the DNA strand cleavage. With microsomes from the ethanol-treated rats, pre-immune IgG was also inhibitory; however, anti-P4502E1 IgG was much more effective (Figure 3a). Most of the increase in DNA strand cleavage found after ethanol treatment was prevented by anti-P4502E1.

With control microsomes, pre-immune IgG and anti-P4502E1 IgG were equally effective in preventing NADH-dependent DNA strand cleavage (Figure 3b). Results with microsomes from the ethanol-treated rats were more complicated, as pre-immune IgG was much more inhibitory in this system (also shown in Figure 2) than the NADPH-dependent system, perhaps a reflection of the weaker activity of the NADH system. At an IgG concentration of 0.88 mg/mg of microsomal protein, anti-P4502E1 IgG produced a 63% reduction in the form II/form I ratio, whereas pre-immune IgG produced a 33% reduction (Figure 3b); in fact at this narrow window of IgG concentration, most or all of the increase in NADH-dependent DNA strand cleavage found after ethanol treatment was prevented by anti-P4502E1 IgG.

DDC has been shown to be an effective inhibitor of P4502E1-catalysed oxidation of substrates such as nitrosamines (Guengerich et al., 1991; Brady et al., 1991). Over the concentration range of 0.1–1.0 mM, DDC had only a small effect (10–30% inhibition) on the NADPH-dependent DNA strand cleavage catalysed by control microsomes, but inhibited the reaction with microsomes from ethanol-treated rats (Table 5).

At low concentrations, tryptamine has been shown to be a good inhibitor of the NADPH oxidase activity and generation of reactive oxygen species associated with P4502E1 (Albano et al., 1991). At a concentration of 1 mM, tryptamine had no effect on the NADPH-dependent DNA strand breakage catalysed by control microsomes, but caused a 36% decrease in the reaction catalysed by microsomes from the ethanol-treated rats (Table 5). Higher concentrations of tryptamine produced correspondingly greater inhibition of the conversion catalysed by both microsomal

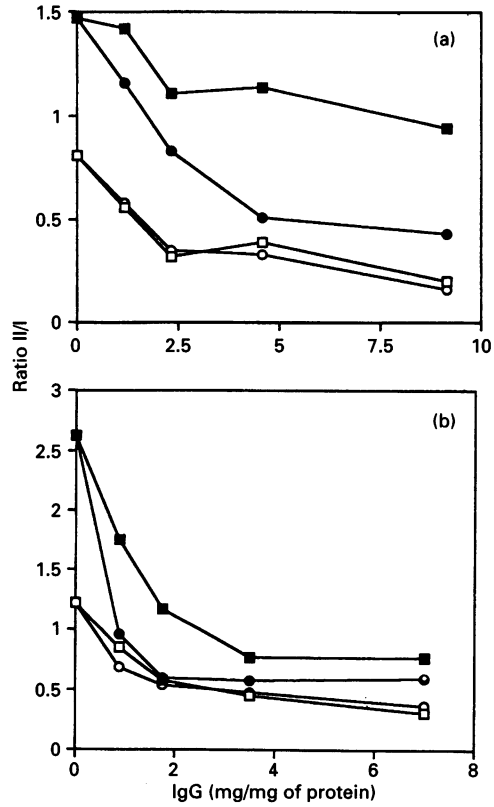

Figure 3 Effect of anti-P4502E1 IgG on DNA strand cleavage by rat liver microsomes from control and ethanol-treated rats

Ferric–histidine was the iron catalyst and either NADPH (**a**) or NADH (**b**) was the reductant. Experimental conditions were: ○, control microsomes, anti-P4502E1 at the indicated concentrations; □, control microsomes, pre-immune IgG at the indicated concentrations; ●, alcohol microsomes, anti-P4502E1, IgG; ■, alcohol microsomes, pre-immune IgG. Reactions were carried out for 5 min with NADPH or 45 min with NADH. Results are from two to four pairs of rats.

Table 5 Effect of tryptamine and DDC on DNA strand cleavage

Experiments were carried out in the presence of the indicated concentrations of tryptamine or DDC. Ferric–histidine was the iron catalyst and reaction times were 5 min with NADPH or 45 min with NADH. Results are from two pairs of rats.

		Ratio form II/form I			
Addition		NADPH		NADH	
Compound	Concn. (mM)	Control	Ethanol	Control	Ethanol
None	–	1.33	2.95	7.63	11.85
Tryptamine	1	1.27	1.88	9.42	10.00
	3	0.71	1.47	8.05	9.05
	5	0.62	1.08	9.95	6.16
DDC	0.1	1.14	2.98		
	0.5	1.22	1.88		
	1.0	0.91	1.26		

preparations. Tryptamine over a concentration range of 1–5 mM had no effect on the NADH-catalysed DNA strand cleavage by control microsomes, but produced progressive inhibition of the

NADH-dependent reaction catalysed by microsomes from the ethanol-treated rats (Table 5).

DISCUSSION

DNA strand cleavage proved to be a very sensitive assay system (a) to detect for the production of ·OH by isolated rat liver microsomes with either NADPH or NADH as cofactor; (b) to study the ability of different ferric complexes to catalyse production of ·OH by microsomes; (c) to evaluate the effect of chronic ethanol treatment on microsomal production of ·OH; and (d) to assess the role of P4502E1 in the elevated production of ·OH after ethanol treatment. Previous results using oxidation of chemical scavengers or e.s.r. spectroscopy to detect for the production of ·OH by microsomes required the use of relatively high amounts of microsomal protein (0.1–1 mg per assay) (Klein et al., 1983; Dicker and Cederbaum, 1987; Rashba-Step et al., 1993). Stimulation of ·OH production was readily observed with powerful ferric complexes such as ferric–EDTA, but was more difficult to study with ferric chelates such as ferric–ATP, citrate, or histidine, which are of more physiological or toxicological significance. This was especially notable with NADH as the microsomal reductant (Dicker and Cederbaum, 1992; Kukielka and Cederbaum, 1989). DNA strand cleavage was readily catalysed by as little as 0.008 mg of microsomal protein over a 2.5–10 min reaction with NADPH as cofactor or a 10–60 min time period with NADH as reductant. This increased sensitivity should prove useful for studies with limited amounts of biological material. The DNA-strand-cleavage assay also proved to be very sensitive in evaluating the catalytic effectiveness of various ferric complexes in promoting ·OH production by microsomes with either NADPH or NADH as reductants, and in demonstrating increased ·OH production by microsomes after ethanol treatment. Moreover, concentrations of ferric–histidine as 'low' as 0.5 μM were catalytically reactive in the NADPH-dependent reaction system, and increased activity by microsomes after ethanol treatment could be detected in the presence of such low concentrations of iron.

The oxidant responsible for the DNA strand cleavage appears to be ·OH. DNA strand cleavage was prevented by catalase and by SOD, indicating that H_2O_2 and $O_2^{-\cdot}$ played roles in the overall reaction pathway. DNA strand cleavage was also prevented by ·OH-scavenging agents such as GSH, DMSO, mannitol, ethanol and glycerol. Although GSH inhibits microsomal lipid peroxidation (Burk, 1983), the lack of effect of trolox suggests that GSH is probably working as a radical scavenger rather than as an anti-oxidant against lipid peroxidation. While trolox may under certain conditions, display a pro-oxidant effect (Aruoma et al. 1990), other anti-oxidants such as propyl gallate and butylated hydroxytolouene also failed to protect against DNA strand cleavage (results not shown). The effect of the various antioxidants suggests that the DNA strand cleavage is mediated by ·OH generated via an iron-catalysed Haber–Weiss or Fenton-type of reaction. Microsomes from controls and ethanol-treated rats display the same sensitivity to the various antioxidants, suggesting similar mechanism for ·OH production.

Several of the metabolic effects of ethanol on the liver have been ascribed to reduction of the NAD^+/NADH redox state during oxidation of ethanol by alcohol dehydrogenase (Krebs, 1968; Williamson et al., 1969). The DNA-strand-cleavage assay demonstrates that NADH can support microsomal production of reactive oxygen species, although not as effectively as NADPH at longer incubation times (Table 1) and higher concentrations of the iron catalyst are required with NADH (Table 3). Since NADPH is the preferred cofactor for donating electrons (at least the first electron) to cytochrome *P*-450, the increased effectiveness of NADPH compared with NADH is not surprising. Nevertheless, the ability of NADH to react with microsomes and various ferric chelates to catalyse the production of ·OH may be significant as a reflection of an acute effect produced as a consequence of ethanol oxidation.

The increase in production of oxygen radicals by microsomes after ethanol treatment is assumed to be due to induction of P4502E1; for example, the increase in lipid peroxidation can be blocked by anti-P4502E1 IgG (Ekstrom and Ingelman-Sundberg, 1989; Castillo et al., 1992). There is no direct evidence implicating P4502E1 in the increased production of ·OH by microsomes after ethanol treatment with NADPH as cofactor, nor is it known whether P4502E1 plays a role in increases found when NADH is the reductant. With NADPH as the reductant, anti-P4502E1 was not any more effective than pre-immune IgG in preventing DNA strand cleavage catalysed by pair-fed control microsomes, suggesting that P4502E1 does not play a major role in catalysing ·OH production by these microsomes. Presumably other cytochrome *P*-450 isoforms participate in the overall pathway and/or the reductase makes an important contribution under these conditions. Consistent with the lack of an effect by anti-P4502E1 is the inability of DDC or 1 mM tryptamine to prevent the DNA strand cleavage with pair-fed control microsomes. Anti-P4502E1 IgG did inhibit DNA strand cleavage in the microsomes from the ethanol-treated rats (over the inhibition produced by pre-immune IgG) and nearly completely prevented the increased activity found after ethanol treatment. DDC also inhibited DNA strand cleavage and lowered the extent of increase with the microsomes from the ethanol-treated rats. Relatively high concentrations of DDC were required to inhibit DNA strand cleavage (0.5 or 1 mM, whereas P4502E1-catalysed oxidation of substrates such as *p*-nitrophenol can be inhibited by 0.1 mM DDC); however, these high concentrations had little effect with microsomes from the pair-fed controls. It is possible that concentrations of inhibitors which block P4502E1-catalysed substrate oxidation differ from concentrations required to block P4502E1-catalysed production of reactive oxygen intermediates. At a concentration of 1 mM, tryptamine produced some inhibition of DNA strand cleavage with microsomes from ethanol-treated rats without any effect with microsomes from the pair-fed controls. Higher concentrations of tryptamine appeared to be not specific for only P4502E1, as inhibition of DNA strand cleavage was observed with both microsomal preparations. The results with anti-P4502E1 IgG, DDC and low concentrations of tryptamine support a role for P4502E1 in catalysing the elevated NADPH-dependent production of ·OH by microsomes after chronic ethanol treatment.

There also appears to be a role for P4502E1 in the increased DNA strand cleavage by microsomes from ethanol-treated rats with NADH as the cofactor. Although anti-P4502E1 IgG was not any more effective than pre-immune IgG in preventing DNA strand cleavage with control microsomes (similar to results with NADPH), lower concentrations of the antibody completely prevented the NADH-dependent increase in DNA strand cleavage by microsomes after ethanol treatment. Tryptamine also produced inhibition with microsomes from the ethanol-fed rats but not with control microsomes.

In summary, these results indicate that DNA strand cleavage is a very sensitive assay to detect for the production of ·OH by microsomes with either NADPH or NADH as reductants, and with various ferric complexes as iron catalysts. Microsomes from rats treated chronically with ethanol are more reactive than controls in catalysing DNA strand cleavage with either reductant and with various ferric complexes. This increased effectiveness is

due, at least in part, to induction of cytochrome P4502E1, and it appears that this cytochrome *P*-450 isoform plays an important role in the increased production of reactive oxygen intermediates by microsomes after ethanol treatment.

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