Stimulation of NADH-dependent microsomal DNA strand cleavage by rifamycin SV

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Rifamycin SV is an antibiotic anti-bacterial agent used in the treatment of tuberculosis. This drug can autoxidize, especially in the presence of metals, and generate reactive oxygen species. A previous study indicated that rifamycin SV can increase NADH-dependent microsomal production of reactive oxygen species. The current study evaluated the ability of rifamycin SV to interact with iron and increase microsomal production of hydroxyl radical, as detected by conversion of supercoiled plasmid DNA into the relaxed open circular state. The plasmid used was pBluescript II KS(−), and the forms of DNA were separated by agarose-gel electrophoresis. Incubation of rat liver microsomes with plasmid plus NADH plus ferric–ATP caused DNA strand cleavage. The addition of rifamycin SV produced a time- and concentration-dependent increase in DNA-strand cleavage. No stimulation by rifamycin SV occurred in the absence of microsomes, NADH or ferric–ATP. Stimulation occurred with other ferric complexes besides ferric–ATP, e.g. ferric–histidine, ferric–citrate, ferric–EDTA, and ferric–$(NH_4)_2SO_4$. Rifamycin SV did not significantly increase the high rates of DNA strand cleavage found with NADPH as the microsomal reductant. The stimulation of NADH-dependent microsomal DNA strand cleavage was completely blocked by catalase, superoxide dismutase, GSH and a variety of hydroxyl-radical-scavenging agents, but not by anti-oxidants that prevent microsomal lipid peroxidation. Redox cycling agents, such as menadione and paraquat, in contrast with rifamycin SV, stimulated the NADPH-dependent reaction; menadione and rifamycin SV were superior to paraquat in stimulating the NADH-dependent reaction. These results indicate that rifamycin SV can, in the presence of an iron catalyst, increase microsomal production of reactive oxygen species which can cause DNA-strand cleavage. In contrast with other redox cycling agents, the stimulation by rifamycin SV is more pronounced with NADH than with NADPH as the microsomal reductant. Interactions between rifamycin SV, iron and NADH generating hydroxyl-radical-like species may play a role in some of the hepatotoxic effects associated with the use of this anti-bacterial antibiotic.

INTRODUCTION

Rifamycin SV is a naphthohydroquinone with antibiotic activity isolated from the micro-organism *Nocardia mediterranel* which has been especially useful for the treatment of tuberculosis (Sensi et al., 1960; Timbol, 1960; Lester, 1972). This drug has been reported to have anti-viral, anti-inflammatory and immunosuppressive properties (Subak-Sharpe et al., 1969; Kasik and Monick, 1981; Arora and Main, 1984; Caruso et al., 1993). Both the hydroquinone moiety and the long aliphatic bridge of rifamycin SV are responsible for its pharmacochemical behaviour. Rifamycin SV can undergo autoxidation when exposed to air for a long period of time (Scrutton, 1977; Kono, 1982). This aerobic oxidation is accelerated by certain metals (Kono, 1982). Reactive oxygen species (ROS) including $O_2^{-\cdot}$, H_2O_2 and hydroxyl radical ($^{\cdot}OH$) are produced in the presence of rifamycin SV, oxygen and metals (Kono, 1982; Kono and Sugiura, 1982; Quinlan and Gutteridge, 1987, 1991; Saez et al., 1991). Oxidative destruction of DNA by rifamycin SV and copper has been related to the metal-dependent production of ROS (Quinlan and Gutteridge, 1987, 1991). The bactericidal activity of this antibiotic has been suggested to be due to its ability to generate ROS (Kono, 1982; Kono and Sugiura, 1982; Quinlan and Gutteridge, 1987, 1991; Saez et al., 1991).

In a previous study designed to characterize the interaction of rifamycin SV with rat liver microsomes (Kukiełka and Cederbaum, 1992), the antibiotic was shown to increase microsomal production of $O_2^{-\cdot}$ 3-fold with NADPH as cofactor, and more than 5-fold with NADH; rates of H_2O_2 production were elevated 2-fold with NADPH and 8-fold with NADH. Oxidation of 2-oxo-4-thiomethylbutyrate, taken as a reflection of the production of $^{\cdot}OH$-like species, was also elevated by rifamycin SV, especially with NADH as the microsomal reductant (Kukiełka and Cederbaum, 1992). These results indicate that rifamycin SV stimulates microsomal production of ROS, and that, in contrast with results with most redox cycling agents, e.g. paraquat or menadione, is quite effective with NADH as the cofactor.

The current study was carried out to evaluate the stimulation of microsomal $^{\cdot}OH$ production by rifamycin SV in a more sensitive system than previously employed. Iron is required to catalyse microsomal $^{\cdot}OH$ production, and, in contrast with NADPH, NADH is much less reactive in catalysing $^{\cdot}OH$ production with most iron complexes, except for ferric–EDTA (Kukiełka and Cederbaum, 1989; Kukiełka et al., 1989; Kukiełka and Cederbaum, 1992). Indeed, in a previous study (Kukiełka and Cederbaum, 1992), rates of microsomal production of $^{\cdot}OH$ assessed by oxidation of 2-oxo-4-thiomethylbutyric acid were low and difficult to quantify with most ferric complexes and most studies were carried out with ferric–EDTA. A recent study (Kukiełka and Cederbaum, 1994) indicated that DNA strand scission catalysed by rat liver microsomes was a very sensitive assay for the detection of $^{\cdot}OH$ production as catalysed by a variety of ferric complexes.

The interaction of ROS with DNA has been studied in a variety of systems (Braun and Fridovich, 1981; Muindi et al.,

Abbreviations used: ROS, reactive oxygen species; DTPA, diethylenetriaminepenta-acetic acid; SOD, superoxide dismutase.

1985; Myers et al., 1987; Berlin and Heseltine, 1988; Schneider et al., 1988; Sinha et al., 1988; Aruoma et al., 1989; Rumyantseva et al., 1989). DNA scission is believed to be due to production of ·OH in these reaction systems. Supercoiled DNA is more compact than circular DNA and can be converted by single-strand breaks into the relaxed open circular state or by double-strand breaks into the linear form (Berlin & Haseltine, 1988; Rumyantseva et al., 1989; Schneider et al., 1988, 1989). These forms can be separated from each other and detected by following their migration in agarose under the influence of an electric field. Using this sensitive assay system, the ability of rifamycin SV to interact with ferric complexes to catalyse microsomal production of ·OH was evaluated.

MATERIALS AND METHODS

The source of supercoiled DNA was plasmid pBluescript II KS(−). *Escherichia coli*, strain XLI Blue, was transformed with plasmid pBluescript II KS(−) using caesium chloride (Maniatis et al., 1982) as previously described (Kukielka and Cederbaum, 1994). DNA concentration was determined from the absorbance at 260 nm.

Liver microsomes were prepared from male Sprague–Dawley rats by minor modifications of the low speed Ca^{2+}-aggregation procedure of Montgomery et al. (1974). Livers were perfused with 0.9% NaCl to remove blood, and homogenates were prepared in buffer containing 0.25 M sucrose, 0.05 M Tris/HCl, 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 micromes. The microsomes were washed twice with 0.15 M KCl, centrifuged 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).

Rat liver microsomes (typically used at a protein concentration of 8 μg) were incubated at 37 °C with 0.5 μg of pBluescript II KS(−) in a reaction mixture consisting of 100 mM potassium phosphate buffer, pH 7.4, 50 μM ferric chelate, 1.5 mM NADH, and in the presence or absence of 0.4 mM rifamycin SV in a total volume of 50 μl. Reactions were initiated by addition of the NADH and terminated by the addition of 3 μl of loading buffer (0.25% Bromophenol Blue, 0.25% xylene cyanol, and 30% glycerol). The samples were treated with RNAase and proteinase for 20 min to remove RNA and protein before electrophoresis. They were then centrifuged and 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 that had undergone strand cleavage caused by oxidative damage. The DNA bands were visualized by fluorescence in a u.v. (Fotodyne 400) DNA transilluminator system and photographed. The negative photograph of the gel was scanned with an ultrascan XL enhanced laser densitometer. All experiments were carried out under the cover of aluminium foil to prevent photochemical reduction of the iron chelate. Results are reported as the ratio of form II (DNA modified by single-strand breaks) to form I (supercoiled DNA).

Ferric chelates were utilized as a 1:2 complex except for ferric–ATP which was utilized as a 1:20 complex. The ferric complexes were prepared by dissolving ferric–$(NH_4)_2SO_4$ in 0.1 M HCl and then diluting with the respective chelator to the appropriate stock concentration. Results are means ± S.E.M.; the number of experiments is indicated in the Table legends. Statistical analysis was carried out by Student's *t* test for unpaired data.

RESULTS

DNA strand cleavage by microsomes

Figure 1 (lane 1) shows that the DNA in plasmid pBluescript II KS(−) migrates primarily as a single lower-molecular-mass band (form I, supercoiled form) with only a faint upper band (form II, relaxed form). Incubation of the plasmid with microsomes, NADH or low concentrations of iron has little or no effect on the configuration of DNA. However, incubation of the plasmid with a combination of microsomes plus iron (5 μM FeATP) plus NADH results in a decrease in the supercoiled form, coupled to an increase in the open circular form (lane 2 of Figure 1). No such conversion occurred with microsomes plus ferric–ATP in the absence of NADH, or NADH plus ferric–ATP in the absence of microsomes; some conversion occurred with microsomes plus NADH in the absence of ferric–ATP, probably reflecting the presence of small amounts of iron in the microsomes. The ratio between the two forms was 0.16 for lane 1 and 0.34 for lane 2. The addition of 0.05–0.4 mM rifamycin SV produced an increase in DNA strand cleavage, with form II/form I ratios of 0.58, 0.93 and 0.95 obtained from lanes 3–5 respectively. There was no effect of rifamycin SV in the absence of microsomes, or of NADH or the ferric–ATP catalyst. The ability of rifamycin SV to stimulate DNA strand cleavage can be observed 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. Subsequent results will be reported as the ratio of form II/form I, higher ratios being indicative of increased generation of ROS.

A time-course experiment for conversion of form-I DNA into form II by microsomes in the presence of either NADH or NADPH as the reductant is shown in Figure 2. Ferric–ATP was present as the iron catalyst. In the absence of ferric–ATP, DNA strand cleavage was low, and this low activity was stimulated by 0.4 mM rifamycin SV by only 30%. In the presence of ferric–ATP, there was an increase in the form II/form I ratio as a

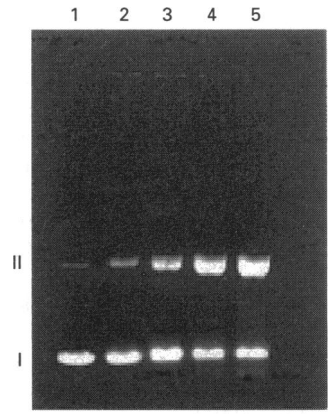

Figure 1 Gel showing the effect of ferric–ATP in the absence and presence of rifamycin on DNA strand cleavage

Experiments were carried out for 30 min using 5 μM ferric–ATP as the iron catalyst and NADH as the reductant. Lane 1, plasmid; lane 2, microsomes plus ferric–ATP plus NADH plus plasmid; lane 3, same as lane 2 plus 0.05 mM rifamycin SV; lane 4, same as lane 2 plus 0.10 mM rifamycin SV; lane 5, same as lane 2 plus 0.40 mM rifamycin SV. Gels were scanned with a densitometer and the intensity of the bands expressed as arbitrary units.

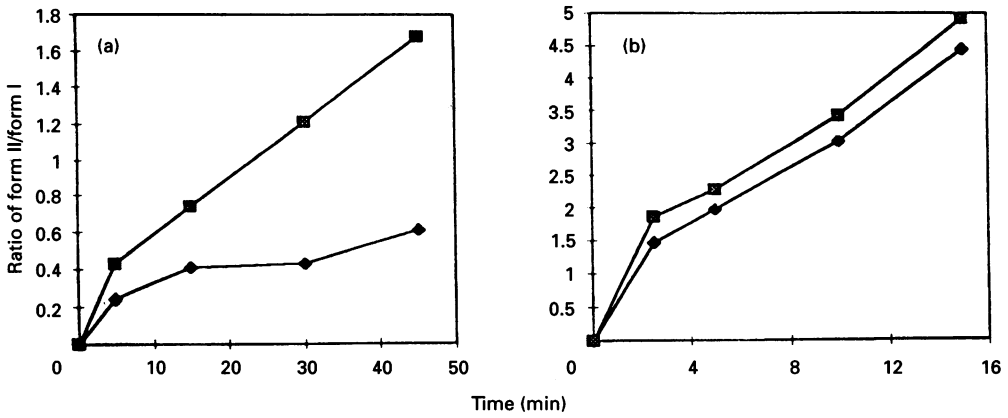

Figure 2 Time course characterizing the effect of rifamycin SV on NADH- and NADPH-dependent DNA strand cleavage

The plasmid was incubated with microsomes plus 20 μM ferric–ATP in the absence (◆) or presence (▩) of 0.4 mM rifamycin SV. Experiments were carried out with either NADH (**a**) or NADPH (**b**) as the microsomal reductant. Results are expressed as the ratio of form II/form I.

Table 1 Concentration-dependent stimulation of DNA strand cleavage by rifamycin SV

DNA strand cleavage by rat liver microsomes was assayed as described in the Materials and methods section in the presence of NADH and the indicated concentrations of rifamycin SV. Experiments were carried out for 15 min in the absence of added iron, in the presence of 5 μM ferric–ATP or 5 μM ferric–EDTA. Results are expressed as the ratio of form II/form I and are means ± S.E.M. from four experiments, except for the 0.4 mM rifamycin SV concentration, for which results are from six experiments. Values in parentheses are percentage increases caused by rifamycin SV. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with the value obtained with no added iron.

Rifamycin SV concentration (mM)	DNA strand cleavage		
	No added iron	Ferric–ATP	Ferric–EDTA
0	0.42 ± 0.03	0.41 ± 0.03	1.07 ± 0.33
0.01	0.44 ± 0.03 (+5)	0.57 ± 0.05 (+39)	2.69 ± 0.40 (+151)*
0.05	0.48 ± 0.07 (+14)	0.72 ± 0.04 (+76)**	3.25 ± 0.23 (+204)**
0.10	0.52 ± 0.04 (+23)	1.00 ± 0.07 (+144)***	6.17 ± 0.41 (+477)***
0.40	0.48 ± 0.06 (+14)	1.03 ± 0.04 (+151)***	7.19 ± 1.11 (+572)**
1.00	0.45 ± 0.08 (+7)	1.04 ± 0.08 (+154)***	4.91 ± 0.52 (+359)***

function of time (Figure 2). NADPH was more effective than NADH in promoting microsomal DNA strand cleavage (Figure 2a compared with Figure 2b). The addition of the rifamycin SV resulted in a large increase in NADH-dependent DNA strand cleavage, whereas no effect of rifamycin SV was found with the NADPH-dependent system (Figure 2).

The concentration-dependence of rifamycin stimulation of NADH-dependent DNA strand cleavage in the absence of added iron or with either ferric–EDTA or ferric–ATP as the iron catalyst is shown in Table 1. In the absence of added iron, rifamycin, even at concentrations as high as 1 mM, did not significantly increase NADH-dependent DNA strand cleavage. However, in the presence of either ferric–ATP or ferric–EDTA, rifamycin produced a concentration-dependent stimulation of DNA strand cleavage, with significant increases being found at 0.01 to 0.05 mM rifamycin, and maximal increases occurring at 0.4 mM rifamycin. Ferric–EDTA, as expected, was a more powerful catalyst of microsomal DNA strand cleavage then was ferric–ATP, in both the absence and presence of rifamycin.

Effect of ferric complexes on DNA strand cleavage

The concentration-dependence of the ability of ferric–ATP to catalyse NADH-dependent DNA strand cleavage in the absence and presence of rifamycin is shown in Table 2. Rifamycin significantly stimulated conversion at ferric–ATP concentrations as low as 2–5 μM; stimulation became more pronounced as the

Table 2 Concentration-dependent ferric–ATP stimulation of DNA strand cleavage in the absence and presence of rifamycin SV

DNA strand cleavage by microsomes was assayed in the absence or presence of 0.4 mM rifamycin SV and the indicated concentrations of ferric–ATP. Reactions were carried out for 15 min using 6 μg of microsomal protein and NADH as reductant. Results are expressed as the ratio of form II/form I and are means ± S.E.M. from three experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with the value in the absence of rifamycin SV.

Concentration of Ferric–ATP (μM)	DNA strand cleavage		Effect of rifamycin SV (%)
	− Rifamycin SV	+ Rifamycin SV	
0	0.32 ± 0.05	0.34 ± 0.04	+6
2	0.40 ± 0.05	0.55 ± 0.05*	+38
5	0.40 ± 0.06	0.71 ± 0.02**	+78
10	0.42 ± 0.03	0.93 ± 0.04***	+121
20	0.42 ± 0.09	1.14 ± 0.08**	+171
50	0.41 ± 0.05	2.55 ± 0.33**	+522

Table 3 Effect of ferric complexes on DNA strand cleavage in the absence and presence of rifamycin SV

DNA strand cleavage was assayed in the absence or presence of 0.4 mM rifamycin SV and the indicated additions. Final concentrations of the ferric complexes were 50 μM ferric. Desferrioxamine was added to a final concentration of 20 μM. Reactions were carried out for 15 min. Results are expressed as the ratio of form II/form I and are from two to five experiments. * $P < 0.01$, compared with the value obtained in the absence of rifamycin SV.

Addition	DNA strand cleavage		Effect of rifamycin SV (%)
	−Rifamycin SV	+Rifamycin SV	
None	0.44 ± 0.04	0.58 ± 0.08	+32
Desferrioxamine	0.32 ± 0.02	0.35 ± 0.04	+9
Ferric–ATP	0.45 ± 0.09	1.13 ± 0.16*	+151
Ferric–citrate	0.63	2.33	+270
Ferric–histidine	0.61	3.88	+536
Ferric–$(NH_4)_2SO_4$	0.88	3.55	+303
Ferric–EDTA	1.99	16.07	+708
Ferric–DTPA	1.93	3.12	+62

concentration of ferric–ATP was elevated. A variety of other ferric complexes besides ferric–ATP were also studied (Table 3). DNA strand cleavage in the absence of added iron was partially inhibited by desferrioxamine suggesting a role for iron in the system or microsomes participating in the reaction. Ferric–citrate, ferric–histidine, ferric–ATP, and ferric–$(NH_4)_2SO_4$ were all effective at promoting NADH-dependent microsomal DNA strand cleavage. The most effective catalysts were ferric–EDTA and ferric–diethylenetriaminepenta-acetic acid (DTPA). Rifamycin slightly stimulated the form II/form I ratio in the absence of added iron; this stimulation was completely blocked in the presence of desferrioxamine, indicating that it was dependent on iron present in the reaction system. Rifamycin stimulated DNA strand cleavage with ferric–ATP (2.5-fold), ferric–citrate (3.7-fold), ferric–$(NH_4)_2SO_4$ (4-fold), ferric–histidine (6.4-fold) and ferric–EDTA (8-fold). However, stimulation by rifamycin with ferric–DTPA was poor (1.6-fold), although this ferric complex in the absence of rifamycin is as powerful a catalyst for DNA strand cleavage as is ferric–EDTA.

Table 4 Effect of SOD and catalase on DNA strand cleavage in the presence of rifamycin SV

DNA strand cleavage by microsomes was assayed in the presence of 0.4 mM rifamycin SV and NADH as reductant. Experiments were carried out for 30 min in the presence of the indicated ferric catalyst (50 μM). SOD and catalase were added at final concentrations of 50 units and 1300 units respectively. Results are expressed as the ratio of form II/form I and are from two experiments. Values in parentheses are percentage decreases.

Ferric complex	DNA strand cleavage		
	Control	SOD	Catalase
Ferric–EDTA	28.0	22.1 (−21)	0.3 (−99)
Ferric–ATP	9.7	0.5 (−95)	0.4 (−96)
Ferric–histidine	24.0	0.7 (−97)	0.5 (−98)
Ferric–$(NH_4)_2SO_4$	10.3	0.6 (−94)	0.4 (−96)

Effect of anti-oxidants on rifamycin stimulation

The effect of catalase and superoxide dismutase (SOD) on DNA strand cleavage was studied to evaluate the role of H_2O_2 and $O_2^{-\cdot}$ in the reaction pathway. Four different ferric catalysts were used in the presence of 0.4 mM rifamycin. The concentration of the iron was high, 50 μM, and the reaction period was prolonged to 30 min in order to cause nearly complete conversion of form-I DNA into form II, thereby allowing clear evaluation of the various anti-oxidants tested. Catalase produced complete inhibition of the extensive DNA strand cleavage catalysed by the four ferric complexes in the presence of rifamycin (Table 4); catalase was previously shown to inhibit the conversion of form I into form II in the absence of rifamycin as well (Kukiełka and Cederbaum, 1994). SOD was also a powerful inhibitor of DNA strand cleavage catalysed by rifamycin in the presence of ferric–histidine, ferric–ATP and ferric–$(NH_4)_2SO_4$, but was not significantly inhibitory against the ferric–EDTA system.

To evaluate a role for $^{\cdot}OH$ in DNA strand cleavage stimulated by rifamycin, the effect of a variety of competitive $^{\cdot}OH$ scavengers was determined (Table 5). With all four ferric complexes, dimethyl sulphoxide, ethanol and the spin-trapping agent, 5,5-dimethylpyroline-*N*-oxide, produced strong inhibition of rifamycin-stimulated DNA strand cleavage. GSH was also a very effective inhibitor. Several other $^{\cdot}OH$ scavengers found to be more than 85% protective, include mannitol (50 mM) and glycerol (100 mM). In contrast, propyl gallate and butylated hydroxytoluene, used at concentrations that completely prevent

Table 5 Effect of radical-scavenging agents on DNA strand cleavage in the presence of rifamycin SV

DNA strand cleavage by microsomes was assayed in the presence of 0.4 mM rifamycin SV and NADH as reductant, and the indicated additions. Experiments were carried out for 30 min in the presence of the indicated ferric catalyst (50 μM). Results are expressed as the ratio of form II/form I and are from two experiments. ND, Not determined.

Addition	DNA strand cleavage			
	Ferric–EDTA	Ferric–ATP	Ferric–histidine	Ferric–$(NH_4)_2SO_4$
None	28.0	9.7	24.0	10.3
Propyl gallate (10 μM)	21.8	9.4	ND	ND
Butylated hydroxytoluene (10 μM)	33.4	5.8	ND	ND
Dimethyl sulphoxide (50 mM)	0.8	0.4	0.7	0.8
Ethanol (50 mM)	1.8	1.1	1.2	3.1
5,5-Dimethylpyroline-*N*-oxide (30 mM)	1.0	0.9	0.9	1.1
GSH (5 mM)	1.9	0.2	0.5	0.7
Mannitol (50 mM)	1.3	1.1	ND	ND
Glycerol (100 mM)	0.5	0.4	ND	ND

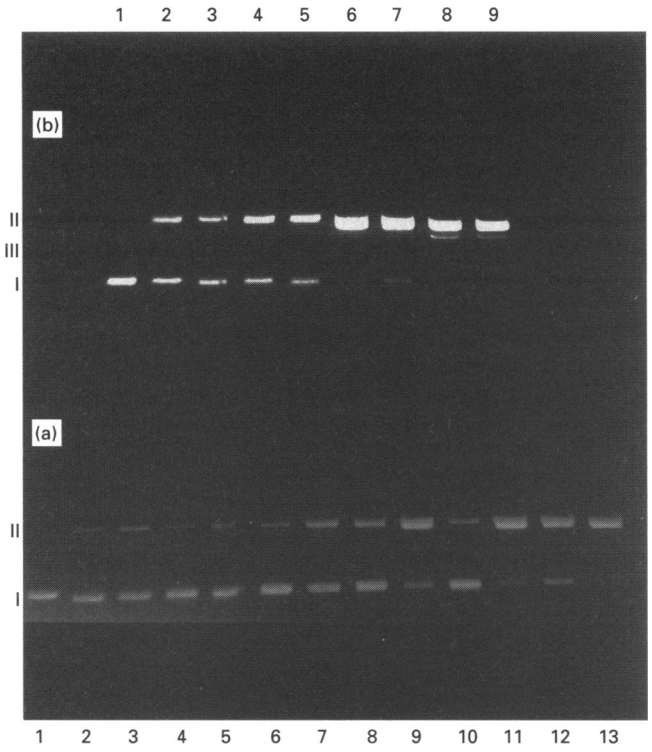

Figure 3 Comparison of the effect of rifamycin SV, paraquat and menadione on NADH- (a) and NADPH- (b) dependent DNA strand cleavage

Rat liver microsomes were incubated with plasmid plus NADH for 15 min (**a**) or plasmid plus NADPH for 5 min (**b**). In (**a**) lane 1, plasmid alone; lanes 2–5, 2 μM desferrioxamine plus no addition (lane 2), rifamycin (lane 3), paraquat (lane 4) or menadione (lane 5); lanes 6–9, 5 μM ferric–ATP plus no addition (lane 6), rifamycin (lane 7), paraquat (lane 8) or menadione (lane 9); lanes 10–13, 50 μM ferric–ATP plus no addition (lane 10), rifamycin (lane 11), paraquat (lane 12) or menadione (lane 13). In (**b**) lane 1, plasmid only; lanes 2–5, 2 μM desferrioxamine plus no addition (lane 2), rifamycin (lane 3), paraquat (lane 4) or menadione (lane 5); lanes 6–9, 50 μM ferric–ATP plus no addition (lane 6), rifamycin (lane 7), paraquat (lane 8) or menadione (lane 9). Final concentrations of rifamycin, paraquat and menadione were 0.4, 0.1 and 0.01 mM respectively.

lipid peroxidation determined as the production of thiobarbituric acid-reactive components, did not or only partially prevented the rifamycin stimulation of microsomal DNA strand cleavage.

Comparison with menadione and paraquat

Menadione and paraquat are widely used redox cycling agents which produce large increases in microsomal production of ROS (Lind and Ernster, 1974; Bus et al., 1976; Bachur et al., 1979; Trush et al., 1981, 1982; Thor et al., 1982; Cadenas et al., 1983; Powis, 1987). These agents are considerably more reactive with NADPH than with NADH as the microsomal reductant (Iyanagi and Yamazaki, 1969; Lind and Ernster, 1974; Bus et al., 1976; Hassan and Fridovich, 1979; Kappus, 1986; Liochev and Fridovich, 1988). Figure 3(a), lanes 2–5, shows that, in the presence of NADH and desferrioxamine, which was added to chelate iron in the microsomes or reaction system, rifamycin, paraquat and menadione had little or a small stimulatory effect on DNA strand cleavage. The three agents produced an increase in form I into form II conversion in the presence of ferric–ATP, especially at the 50 μM iron concentration (Figure 3a, lanes 9–13). Quantification of the gel is shown in Table 6; under these

Table 6 Comparison of the effects of rifamycin SV, paraquat and menadione on NADH-dependent DNA strand cleavage

DNA strand cleavage was determined in the presence of 2 μM desferrioxamine, 5 μM ferric–ATP or 50 μM ferric–ATP with NADH as the reductant. Final concentrations of rifamycin, paraquat and menadione were 0.40, 0.10 and 0.01 mM respectively. Results are expressed as the ratio of form II/form I and are from two experiments. Values in parentheses are percentage changes from the value obtained with no addition.

Reaction conditions	Addition	DNA strand cleavage
Desferrioxamine (2 μM)	None	0.11
	Rifamycin	0.27 (+145)
	Paraquat	0.07 (−36)
	Menadione	0.17 (+55)
Ferric–ATP (5 μM)	None	0.19
	Rifamycin	0.57 (+200)
	Paraquat	0.42 (+121)
	Menadione	1.78 (+837)
Ferric–ATP (50 μM)	None	0.21
	Rifamycin	2.93 (+1295)
	Paraquat	1.43 (+581)
	Menadione	6.20 (+2852)

reaction conditions and at the concentrations utilized, menadione was the most effective stimulator of NADH-dependent DNA strand cleavage, and paraquat was the least effective.

When NADPH was used as the microsomal reductant, there was some conversion of form-I DNA into form II by rifamycin, paraquat and menadione in the presence of 2 μM desferrioxamine (Figure 3b, lanes 2–5). Extensive conversion of form I into form II was found with NADPH in the presence of 50 μM ferric–ATP (lane 6); both paraquat and menadione potentiated this conversion by causing formation of linear DNA (form III; lanes 8 and 9). Rifamycin was not effective in producing linear DNA (lane 7).

DISCUSSION

ROS are produced when the antibiotic rifamycin SV is incubated with metals and oxygen (Kono, 1982; Kono and Sugiura, 1982; Quinlan and Gutteridge, 1987, 1991; Saez et al., 1991), and the anti-bacterial activity of this agent may involve, in part, production of ROS. Rifamycin SV was previously shown to stimulate microsomal production of $O_2^{-\cdot}$ and H_2O_2, especially with NADH as the microsomal reductant (Kukielka and Cederbaum, 1992). Since $O_2^{-\cdot}$ and H_2O_2 can, in the presence of metal catalysts, generate $^{\cdot}OH$ via Fenton- or Haber–Weiss-types of reaction, the ability of rifamycin SV to stimulate microsomal production of $^{\cdot}OH$ was determined. Conversion of supercoiled DNA into the open circular form has been demonstrated in a variety of systems that generate ROS, mainly $^{\cdot}OH$-like species (Braun and Fridovich, 1981; Muindi et al., 1985; Myers et al., 1987; Berlin and Haseltine, 1988; Schneider et al., 1988, 1989; Sinha et al., 1988; Aruoma et al., 1989; Rumyantseva et al., 1989), and this system appears to be very sensitive for the detection of $^{\cdot}OH$ produced by rat liver microsomes (Kukielka and Cederbaum, 1994). Rifamycin SV was found to stimulate NADH-catalysed microsomal DNA strand cleavage in a time- and concentration-dependent manner. The stimulation by rifamycin requires a metal catalyst such as iron, as little stimulation occurs (a) in the absence of added iron [microsomes contain a small pool of tightly bound iron (Minotti, 1989), which may be responsible for the small stimulation by rifamycin in the absence of added iron], or (b) in the presence of desferrioxamine, which chelates iron

usually producing a non-reactive form. Several different ferric complexes are effective at interacting with rifamycin SV to promote DNA strand cleavage; ferric–EDTA, as expected, is the most reactive ferric complex, although other iron complexes such as ferric–histidine and ferric–$(NH_4)_2SO_4$ also display considerable reactivity. The requirement for an iron catalyst rules out a direct role for rifamycin and its metabolites, e.g. rifamycin semiquinone, in DNA strand cleavage.

The oxidant responsible for DNA strand cleavage and for the enhanced activity found in the presence of rifamycin SV appears to be $^{\cdot}OH$ in view of the requirement for a metal catalyst, the potent inhibition by SOD and catalase, and the almost complete protection by GSH and a variety of typical $^{\cdot}OH$ scavengers. Although microsomes in the presence of NADH and most iron complexes undergo lipid peroxidation, the failure of potent antioxidants such as propyl gallate and butylated hydroxytoluene, at concentrations that almost completely abolish peroxidation, to prevent rifamycin-stimulated DNA strand cleavage indicates little or no role for a peroxidative event in the overall reaction. Moreover, ferric–EDTA, which was the most effective ferric catalyst in the absence and presence of rifamycin, generally inhibits microsomal lipid peroxidation (Morehouse and Aust, 1988; Puntarulo and Cederbaum, 1988). One difference between ferric–EDTA and three other ferric complexes in catalysing the rifamycin stimulation of DNA strand cleavage is that SOD fails to inhibit the ferric–EDTA-catalysed reaction, whereas it completely inhibits the rifamycin stimulation found with ferric–ATP, ferric–histidine and ferric–$(NH_4)_2SO_4$. Ferric–EDTA can be directly reduced by microsomal reductases such as NADPH–cytochrome *P*-450 reductase and NADH–cytochrome b_5 reductase (Morehouse and Aust, 1988; Morehouse et al., 1984), hence the inability of SOD to inhibit is probably due to the lack of a requirement for $O_2^{-\cdot}$ to reduce ferric–EDTA. Reduction of ferric–ATP and other ferric complexes by these reductases is inefficient (Jansson and Schenkman, 1977; Hirokata et al., 1978; Morehouse et al., 1984; Morehouse and Aust, 1988; Vegh et al., 1988) the strong inhibition of DNA strand cleavage by SOD indicates that $O_2^{-\cdot}$, largely generated from cytochrome *P*-450 and not the reductases, plays an important role in the reduction of these ferric complexes, hence SOD is a powerful inhibitor of $^{\cdot}OH$ production. It would appear that the stimulation of NADH-dependent production of $O_2^{-\cdot}$ by rifamycin SV (Kukielka and Cederbaum, 1992) plays a dual role in the overall ability of rifamycin SV to stimulate DNA strand cleavage: one role is to reduce ferric complexes such as ferric–ATP, ferric–histidine and ferric–$(NH_4)_2SO_4$ and the second is to provide the H_2O_2 which serves as the precursor of the ultimate oxidant, $^{\cdot}OH$. The poor stimulation by rifamycin SV of NADPH-dependent DNA strand cleavage probably reflects the elevated activity found with this cofactor compared with NADH, which, in turn, is probably due to the higher rates of production of $O_2^{-\cdot}$ and H_2O_2 by microsomes with NADPH than with NADH. The greater effectiveness with NADH may also be due to interaction of rifamycin SV (in contrast with paraquat) with NADH–cytochrome b_5 reductase or cytochrome b_5.

The fact that ferric–DTPA is not effectively reduced by $O_2^{-\cdot}$ (Buettner et al., 1978; Cohen and Sinet, 1982) probably explains why the stimulation of DNA strand cleavage by rifamycin is poor with this iron complex relative to the others. Rifamycin stimulation of H_2O_2 production (via $O_2^{-\cdot}$ dismutation) probably accounts for the striking DNA strand cleavage that occurs in the presence of ferric–EDTA.

Rates of $^{\cdot}OH$ production and DNA strand cleavage by microsomes are higher with NADPH than with NADH as the microsomal reductant. Rifamycin is not effective in further augmenting the high NADPH-dependent rates, although paraquat and menadione are, generating linear DNA from supercoiled DNA. Paraquat is much less effective than menadione in promoting NADH-dependent microsomal production of ROS (Iyanagi and Yamazaki, 1969; Hassan and Fridovich, 1979; Kappus, 1986; Liochev and Fridovich, 1988) and in catalysing DNA strand cleavage. Rifamycin, in contrast with menadione and paraquat, is more effective at catalysing NADH-dependent microsomal DNA strand cleavage than at catalysing the NADPH-dependent reaction. However, menadione appears to be the most reactive of the three redox cycling agents with both microsomal reductants.

In summary, these results indicate that rifamycin SV can increase microsomal production of ROS which are capable of causing DNA strand cleavage. Rifamycin stimulation requires an iron catalyst and is probably the result of the increased production of $O_2^{-\cdot}$ and H_2O_2 as a consequence of rifamycin interaction with the microsomes. In contrast with other typical redox cycling agents, the stimulation by rifamycin is much more prominent with NADH than with NADPH as the microsomal reductant. Pallanza et al. (1967) and Furesz et al. (1967) have shown that an oral derivative of rifamycin is concentrated in the liver and slowly eliminated. Hepatic concentrations after administration of oral doses ranging from 2 to 10 mg/kg body weight of rifampicin to mice, guinea pigs and human volunteers ranged from 22 to 67 μg/g of liver or approx. 0.03–0.10 mM. As shown in Table 1, these concentrations of rifamycin SV produced two- or three-fold increases in NADH-dependent microsomal production of $^{\cdot}OH$. It is possible that interaction of rifamycin, NADH and iron to generate $O_2^{-\cdot}$ may play a role in some of the hepatotoxic side effects associated with the use of the antibacterial antibiotic when used alone or in combination with other drugs in the treatment of tuberculosis (LeSobre et al., 1969; Hollins and Simmons, 1970; Lees et al., 1971a,b). These interactions may also play a role in the cytocidal activity of rifamycin SV.

These studies were supported by USPHS grant AA-03312 from the National Institute on Alcohol Abuse and Alcoholism. We thank Ms. Pilar Visco Cenizal for typing the manuscript.

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Received 3 August 1994/31 October 1994; accepted 17 November 1994