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SULPHOXIDE REDUCTION BY RAT AND RABBIT TISSUES *IN VITRO*

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Abstract—The reduction of sulindac, sulphinpyrazone and diphenyl sulphoxide to their thioether analogues has been studied *in vitro* using rat and rabbit tissues. Sulindac reduction was about 10-fold higher in homogenates of rat kidney and liver than in other tissues although the tissue differences decreased when dithiothreitol was used as a co-factor. The greatest sulindac reducing activity in rat liver was in the cytosolic fraction whereas reoxidation of the thioether back to the sulphoxide was largely in the microsomal fraction. Studies using NADPH/NADH, acetaldehyde and dithiothreitol as cofactors showed that aldehyde oxidase was the main sulindac reducing system in rat and rabbit liver cytosols but not in renal cytosols where reduction was probably linked to the thioredoxin system, as reported previously. Menadione and hydralazine caused essentially complete inhibition of sulindac reduction by hepatic but not renal cytosol and the inhibition was dependent on preincubation of the enzyme with the inhibitor, which is indicative of aldehyde oxidase activity. Little reduction of sulphinpyrazone or diphenyl sulphoxide was detected with rat or rabbit kidney or renal cytosols, although increased reduction was detected when acetaldehyde was added as a cofactor to rabbit and rat liver cytosols. The data indicate that different enzyme systems are responsible for sulphoxide reduction in the liver and kidney.

Key words: sulphoxide reduction; aldehyde oxidase; thioredoxin; sulindac; sulphinpyrazone; diphenyl sulphoxide

Drugs containing the sulphoxide moiety may either be administered *per se* or formed as a result of sulphoxidation *in vivo* [1–5]. Sulphoxide compounds may be reduced to the corresponding sulphide both *in vitro* [6–9] and *in vivo* [10–14]. Reduction of sulindac and sulphinpyrazone produces active sulphide metabolites which are largely responsible for the anti-inflammatory and anti-platelet activity respectively of the parent drugs [15, 16] (Fig. 1).

Two cytosolic enzymes in mammalian tissues have been shown to be able to effect sulphoxide reduction. Under anaerobic conditions rabbit and guinea pig liver aldehyde oxidase plus electron donors, or a combination of liver aldehyde oxidase and xanthine oxidase + xanthine, catalysed the reduction of sulindac to its sulphide [17–19]. This enzyme system also reduced DPSO† (Fig. 1) and a number of other sulphoxides, e.g. phenothiazine sulphoxide and dibenzyl sulphoxide [18]. The sulphoxide reducing activity of purified guinea pig liver aldehyde oxidase was enhanced by the addition of FAD (six-fold) and methyl viologen (100-fold) [19]. However, these could be replaced by direct electron donors to aldehyde oxidase, such as aldehydes or 2-hydroxypyrimidine [18]. A second sulindac reducing system was reported in rat hepatic and renal cytosolic fractions [20, 21]. Using gel filtration chromatography and purified *Escherichia coli* thioredoxin, the authors demonstrated that the thioredoxin system was involved in the reduction of sulindac. In this system, NADPH served to maintain thioredoxin in its reduced form through thioredoxin reductase, and thioredoxin in turn acted as an electron donor for an unidentified soluble sulphoxide reductase [21]. Other studies on sulphoxide reduction by guinea pig liver have also implicated microsomal NADPH-cytochrome P450 reductase, but its precise role requires further investigation [22].

Although the sulphoxide moiety is present in a large number of xenobiotics, previous studies have concentrated on a small number of substrates and little information is available on the tissue distribution of sulphoxide reduction and the relative contributions of various enzyme systems. This paper describes experiments performed to compare the enzymatic reduction of three sulphoxide-containing drugs by rat and rabbit tissues.

MATERIALS AND METHODS

Chemicals. Sulindac and its sulphide and sulphone metabolites were supplied by Merck, Sharp and Dohme (Hoddesdon, U.K.). Sulphinpyrazone and its sulphide, *p*-hydroxy sulphide, sulphone and *p*-hydroxy sulphone metabolites were gifts from Ciba-Geigy Pharmaceuticals (Horsham, U.K.). Fenbufen was kindly supplied by Lederle Laboratories (Gosport, U.K.). DPSO and sulphide were purchased from Sigma (Poole, U.K.). Nitrendipine was a gift from Bayer (U.K.). All other chemicals and solvents were of Analar or HPLC grade from BDH (Poole, U.K.) or Sigma (Poole, U.K.).

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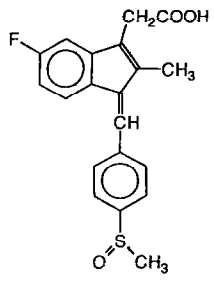
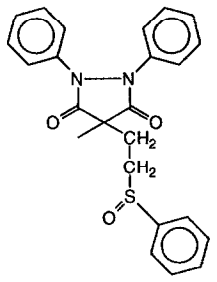
† Abbreviations: DPSO, diphenyl sulphoxide; DTT, dithiothreitol; HPLC, high-performance liquid chromatography.



SULINDAC



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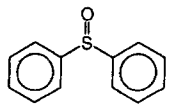

DIPHENYL SULPHOXIDE

Fig. 1. Structures of substrates studied.

Incubation with rat tissue homogenates. Female rats (body weight 250–300 g) were killed by cervical dislocation and the liver, kidneys and lungs were removed and immediately transferred to ice cold plastic tubes. Duodenal mucosa was obtained from about 25 cm of the duodenum/ileum, after flushing the lumen twice with 20 mL 0.1 M phosphate buffer (pH 7.4) and removing the mucosa by scraping with the flat end of a spatula. These tissues were prepared as 33% (w/v) homogenates in 0.1 M sodium phosphate buffer (pH 7.4) at 4° using a Potter glass–Teflon homogenizer. The caecum wall, spleen and the heart were treated in the same way except they were prepared as 25% (w/v) homogenates. The homogenate (1 mL) was mixed with sulindac (100 μg in 1 mL 0.1 M phosphate buffer, pH 7.4; final concentration 0.14 mM) with or without the addition of NADPH plus NADH (final concentration 0.1 mM of each), acetaldehyde (2.5 mM) or dithiothreitol (10 mM) to give a total volume of 2 mL which was incubated for 1 hr at 37°, with shaking, under an atmosphere of oxygen-free nitrogen. The reaction was stopped by the addition of HCl (2 M; 2.0 mL) and then internal standard (25 μg sulphinpyrazone sulphide in 25 μL methanol) was added. The incubates were then extracted and analysed by HPLC as described below.

Incubation with liver sub-cellular fractions. The livers from female rats were removed and homogenized (1 g liver to 3 mL of 1.15% (w/v) KCl) using a Potter glass–Teflon homogenizer. The homogenates were then centrifuged at 10,000 *g* for 20 min. Part of the supernatant was kept in ice for later incubations. Microsomal and cytosolic fractions were obtained by centrifugation of the remaining 10,000 *g* supernatant at 100,000 *g* for 1 hr. The microsomal pellets were re-suspended in 0.1 M sodium phosphate buffer, pH 7.4. All the above procedures were performed at 4°. Liver cytosol was prepared from female rabbits following the same method as described above. The livers were removed after the rabbit was exsanguinated under anaesthesia with thiopentone.

The cytosol or other sub-cellular fractions (0.2 or 0.5 mL) were incubated with the substrates and appropriate cofactors (see results) in 0.1 M sodium phosphate buffer, pH 7.4 (final volume 2.0 mL). Inhibition studies with hydralazine and menadione were performed either by adding the cytosol last to the incubation mixture containing substrate and co-factors or by preincubating the cytosol with the inhibitors in buffer for 30 min at room temperature. In the latter, the reaction was initiated by adding the substrate and cofactor. The mixture was incubated at 37° for 1 hr with shaking either exposed to air or under an atmosphere of oxygen-free nitrogen. All incubations involving hydralazine were performed in 0.1 M phosphate buffer (pH 6.5). The reaction was stopped by the addition of HCl (2 M; 2 mL) followed by internal standard. Samples were extracted and analysed by HPLC as described below.

Incubation with renal cytosol. The kidneys from female rats or rabbits were removed and prepared as 12% (w/v) homogenates in 0.1 M sodium phosphate buffer (pH 7.4) using a Potter glass–Teflon homogenizer. The homogenates were centrifuged at 10,000 *g* for 20 min. The resulting supernatant was centrifuged again at 100,000 *g* for 1 hr to obtain the cytosolic fraction. All centrifugation were performed at 4°. Incubation conditions with the renal cytosol were the same as described above with liver fractions.

HPLC analysis of sulindac and its metabolites. Samples were analysed for sulindac and its sulphide by a method similar to that described previously [14]. The incubation mixture containing the internal standard (sulphinpyrazone sulphide; 15 μg) and 2 M HCl were extracted with chlorobutane/1,2-dichloroethane (4:1, v/v; 5 mL) by shaking for 15 min. The upper organic layer was separated by centrifugation (at approximately 4000 *g* for 10 min), removed and shaken with 0.1 M NaOH (400 μL) for 10 min to extract the acidic drug and its metabolites. An aliquot (50–80 μL) of the alkaline solution was injected into a HPLC consisting of a Waters Associates WISP 710B autoinjector, a model M6000A pump, a μBondapak C-18 column (30 cm; 3.9 mm i.d.), a model 450 UV detector set at 254 nm and a model 745 integrator. The mobile phase was 0.2 M ammonium phosphate buffer (pH 3.5) and acetonitrile (53:47, v/v) at a flow rate of 2.2 mL/min. Peak areas were integrated and the ratio of sulindac or its metabolites to that of the internal standard was calculated. Standards of sulindac and its metabolites were extracted and analysed with each batch of samples; the intra-assay coefficient of variation averaged 2.7% in the relevant concentration range studied (5–100 μg per incubate).

HPLC analysis of sulphinpyrazone and its metabolites. The extraction and HPLC analysis was by a method modified from that described previously [13]. Fenbufen was used as the internal standard (5 μg). The incubates were extracted as described

Table 1. Reduction of sulindac by rat tissue homogenates under anaerobic conditions (mean ± SD, N = 6)

Tissues	Activity in homogenates (nmol sulphide metabolite formed/g tissue after 1 hr)			
	Control	+NADH plus NADPH (0.1 mM)	+Acetaldehyde (2.5 mM)	+Dithiothreitol (10 mM)
Liver	107 ± 38	110 ± 34	114 ± 43	170 ± 42**
Kidneys	79 ± 14	123 ± 33*	75 ± 24	216 ± 16**
Lungs	4 ± 4	14 ± 18	5 ± 5	34 ± 33
Duodenum mucosa	7 ± 6	9 ± 6	7 ± 4	40 ± 16*
Caecum wall	1 ± 1	6 ± 6	2 ± 3	64 ± 3**
Spleen	9 ± 5	12 ± 4*	8 ± 4	100 ± 3**
Heart	11 ± 3	13 ± 1	11 ± 3	28 ± 2**

* $P < 0.05$, ** $P < 0.01$ compared to the corresponding control using Student's *t*-test for paired data.

above and the alkaline solution analysed by HPLC using a system similar to that described above but with a Hypersil 3ODS column (15 cm; 4.6 mm i.d.), and a mobile phase of 0.1 M ammonium phosphate buffer (pH 4.1) and methanol (39:61, v/v) at a flow rate of 1.0 mL/min. *p*-Hydroxysulphinpyrazone was not determined due to the presence of interfering peaks. The peak area ratio of sulphinpyrazone or its metabolites to that of the internal standard were calculated by integration. Standards of sulphinpyrazone and its metabolites were extracted and analysed with each batch of samples; the intra-assay coefficient of variation averaged 2.8% in the relevant concentration range studied (2.5–50 μg per incubate).

HPLC analysis of DPSO and its sulphide. After incubation, all samples were placed in an ice-water bath when the internal standard (nitrendipine, 5 μg) was added to each tube. The tubes were then immediately frozen and stored at −20° until analysed. The samples were extracted with chlorobutane:1,2-dichloroethane (4:1, v/v; 5 mL) by shaking for 15 min. The upper organic layer was separated by centrifugation (at approximately 4000 *g* for 10 min), and pipetted into a clean tube. The solvent was evaporated to dryness by passing a constant stream of oxygen-free nitrogen into the tubes heated at 50°. The residue was then redissolved in 400 μL of mobile phase (0.01 M ammonium phosphate buffer: methanol; 34:66, v/v, pH 5.8). An aliquot (80 μL) of the solution was injected into a HPLC system consisting of a Waters Associates WISP 710B injector, a model M6000A pump, a μBondapak C-18 column (30 cm; 3.9 mm i.d.), a model 481 UV detector set at 230 nm and a model 745 integrator. The flow rate was 1.5 mL/min. Peak areas were integrated and the ratio of DPSO or sulphide to that of the internal standard was calculated. This method gave about 100% recovery of DPSO and the internal standard, and about 95% for the sulphide metabolite. All three compounds eluted within 15 min of injection of the sample. Standards and blanks were run with each batch of samples. The intra-assay coefficient of variation averaged 4.5% in the relevant concentration range studied (2.5–50 μg per incubate).

Data analysis and other methods. Standard curves covering the range of amount of substrate used and its metabolites were prepared for each experiment. All standards were treated in the same way as samples except that the homogenates or sub-cellular fractions from individual rats or rabbits were pooled and the internal standard(s) and 2 M HCl (2 mL) were added prior to the standards. Linearity was maintained ($r > 0.997$) between the area ratios and the concentrations of the drugs and their metabolites added (0–100 μg for sulindac; 0–50 μg for sulphinpyrazone and DPSO). Unless otherwise stated, all statistical analyses were performed using two-tailed Student's *t*-test for paired data. A value of $P < 0.05$ was taken as statistically significant. Control samples were prepared in each experiment by boiling the tissues for 30 min before adding to incubation mixtures. Tissues were incubated with increasing concentrations of NADPH/NADH, acetaldehyde, 2-hydroxypyrimidine and DTT to ensure that optimum concentrations of these co-factors were used. Protein assays were carried out by the method according to Lowry *et al.* [23] using bovine serum albumin to prepare the standard curves.

RESULTS

Distribution of sulindac reductase in rat tissues

All tissue homogenates investigated showed detectable reducing activities under anaerobic conditions even without the addition of cofactors (Table 1), with the liver and kidneys being about 10-fold more active than other tissues. The addition of NADPH plus NADH or acetaldehyde stimulated the reaction to a very slight and non-significant extent in tissues other than the spleen and kidneys. With the liver homogenates, neither NADPH plus NADH nor acetaldehyde produced any significant increase in sulindac reduction. The addition of NADPH plus NADH to kidney homogenates produced about a 50% increase over the basal activity, whereas acetaldehyde did not produce any stimulation in the same preparation. DTT increased the reduction of sulindac (2–10-fold) in all the tissues investigated, with the kidney homogenates still showing the highest activity, followed by the liver.

Table 2. Metabolism of sulindac and its sulphide by rat liver fractions *in vitro* (mean ± SD, N = 6 female rats)

Reactions	Liver fractions	Product formed (nmol/mg protein after 1 hr) Aerobic	Anaerobic
Sulphoxide → sulphide	10,000 *g* supernatant	0.26 ± 0.05	0.47 ± 0.25*
	microsomes	0.22 ± 0.31	0.45 ± 0.10*
	100,000 *g* supernatant	1.80 ± 0.23	1.97 ± 0.05*
Sulphide → sulphoxide	10,000 *g* supernatant	0.81 ± 0.03	0.56 ± 0.03**
	microsomes	7.96 ± 2.22	3.24 ± 0.35**
	100,000 *g* supernatant	0.44 ± 0.03	0.40 ± 0.04
Sulphoxide → sulphone	10,000 *g* supernatant	0.08 ± 0.02	0.07 ± 0.03
	microsomes	0.71 ± 0.18	0.32 ± 0.15
	100,000 *g* supernatant	0.04 ± 0.05	0.07 ± 0.05
Sulphide → sulphone	10,000 *g* supernatant	0.08 ± 0.07	0.06 ± 0.04
	microsomes	0.52 ± 0.74	0.14 ± 0.20
	100,000 *g* supernatant	0.03 ± 0.04	0.05 ± 0.04

* $P < 0.05$, ** $P < 0.02$ compared to the corresponding aerobic incubations using Student's *t*-test for paired data.

Metabolism of sulindac and its sulphide by rat liver fractions

The results in Table 2 show that all three subcellular fractions were capable of both oxidizing and reducing sulindac and also oxidising of the sulphide under aerobic and anaerobic conditions. The highest reducing activity was observed with the 100,000 *g* supernatant fraction under anaerobic conditions. The microsomes were extremely active in the oxidation of sulindac sulphide, producing about 20 times more sulindac than the 100,000 *g* supernatant under aerobic conditions. Only a trace amount of the sulphone metabolite was formed from either sulindac or its sulphide analogue in the 10,000 *g* and 100,000 *g* supernatants, whereas the microsomes were about ten times more active in catalysing these reactions, especially under aerobic conditions (Table 2).

Reduction of sulindac, sulphinpyrazone and DPSO by rat liver cytosol

There was a very marked difference in the reduction of these three substrates by the liver cytosol (Table 3). The reduction of sulindac under anaerobic conditions was increased by 50% over the basal reducing activity with the addition of NADPH plus NADH, whereas both acetaldehyde and DTT produced a 3–4-fold increase. Aerobic incubation of the cytosol with sulindac resulted in reducing activity comparable to that observed under anaerobic conditions; however, the activity was not increased by acetaldehyde. Generally, very low reducing activities were observed towards sulphinpyrazone and DPSO, except that acetaldehyde significantly increased the reduction of DPSO.

Effects of inhibitors on the reduction of sulindac by rat liver cytosol

Sulindac reduction by liver cytosol was completely inhibited by menadione (2×10^{-4} M) and hydralazine (2×10^{-4} M) under anaerobic conditions (Table 4). However, inhibition was achieved only when the cytosol was preincubated with the inhibitor for 30 min and no significant inhibition was observed when the cytosol and inhibitor were added at the same time to the incubation mixture containing sulindac. In order to study further the nature of the inhibition, acetaldehyde was also added to the incubation mixture immediately after sulindac. Once again, preincubation of cytosol with the inhibitor resulted in a significantly lower reducing activity. The activity was inhibited by 81% and 65% with menadione and hydralazine respectively compared to the activity in the presence of acetaldehyde but not the inhibitor. Without preincubation, neither menadione nor hydralazine caused significant inhibition of the reducing activity.

Reduction of sulindac, sulphinpyrazone and DPSO by rat renal cytosol

Little or no reducing activity was detected when sulphinpyrazone or DPSO were incubated with rat renal cytosol (Table 3). Without the addition of cofactors, sulindac was reduced by the cytosol to a slight extent which occurred only under anaerobic conditions. The reducing activity was increased nearly 20-fold following the addition of NADPH and NADH. Much higher activity was observed in the presence of DTT, and similar activities were obtained under both aerobic and anaerobic conditions. This may be related to the general reductant properties of DTT which helped to lower the oxygen tension under aerobic conditions. Unlike the findings with liver cytosol, the addition of acetaldehyde to the incubation mixture did not produce any significant increase in the reduction of sulindac.

Effects of inhibitors on the reduction of sulindac by rat renal cytosol

The sulindac reducing activity in renal cytosol was inhibited by preincubation with menadione (Table

Table 3. The reduction of sulindac, sulphinpyrazone and diphenyl sulphoxide by rat liver and kidney cytosols

Substrate and conditions	Sulphide formed (nmol/mg protein after 1 hr)							
	Control (no cofactor)		+NADPH plus NADH (0.1 mM)		+Acetaldehyde (2.5 mM)		+Dithiothreitol (10 mM)	
	Liver	Kidney	Liver	Kidney	Liver	Kidney	Liver	Kidney
Sulindac								
Anaerobic	2.04 ± 1.39	0.15 ± 0.10	3.09 ± 0.84*	2.89 ± 1.09*	7.59 ± 5.30**	0.09 ± 0.05	7.07 ± 3.23**	30.95 ± 3.12**
Aerobic	1.83 ± 1.46	ND	2.88 ± 1.13*	1.42 ± 0.39*	1.36 ± 1.04	ND	9.02 ± 0.51**	30.05 ± 2.63**
Sulphinpyrazone								
Anaerobic	0.03 ± 0.01	ND	0.01 ± 0.01	<0.01	0.01 ± 0.02	ND	ND	<0.01
Aerobic	<0.01	ND	<0.01	<0.01	<0.01	ND	ND	ND
Diphenyl sulphoxide								
Anaerobic	0.13 ± 0.06	ND	0.05 ± 0.05	ND	3.93 ± 1.44	ND	ND	ND
Aerobic	0.08 ± 0.02	ND	0.03 ± 0.03	ND	0.29 ± 0.15	ND	ND	ND

Results are the mean ± SD for five female rats for liver data and six to eight female rats for kidney data.
* $P < 0.01$, ** $P < 0.001$ compared to the appropriate control using Student's *t*-test for paired data.
ND, not detected.

5). In the presence of NADPH plus NADH, inhibition was significantly greater ($P < 0.05$) with preincubation compared with addition at the start of the incubation, although considerable inhibition was achieved even without preincubation. Menadione also inhibited the activity significantly in the presence of DTT both with or without preincubation. In contrast, a comparatively low level of inhibition was observed using hydralazine even with preincubation (Table 5). The inhibition was minimal (6%) without preincubation and no inhibition was observed when DTT was also added to the incubation.

Reduction of sulindac, sulphinpyrazone and DPSO by rabbit liver and renal cytosol

The extent of reduction of these three substrates by rabbit liver cytosol under anaerobic conditions is shown in Table 6. Sulindac was reduced to a far greater extent than sulphinpyrazone and DPSO especially in the presence of cofactors. Sulindac reduction was greatly increased by the addition of NADPH plus NADH to a level slightly lower than that observed in rat liver cytosol (Table 3). However, the most striking results were the large stimulatory effects of acetaldehyde and DTT on the rabbit cytosolic sulindac reductase activity. The activity was increased by 79- and 54-fold, respectively, over the control incubations; such effects were much higher than those observed with rat liver cytosol (Table 3). The reduction of sulphinpyrazone was also significantly increased by acetaldehyde.

The reduction of sulindac by rabbit renal cytosol was greatly enhanced by the addition of NADPH plus NADH or DTT, whereas acetaldehyde had no significant effect on the enzyme activity (Table 6). Sulphinpyrazone and DPSO were again very poor substrates, and only sulphinpyrazone was reduced slightly in the presence of DTT.

DISCUSSION

Previous studies have demonstrated the involvement of rabbit and guinea pig liver aldehyde oxidase [18, 19] and the rat thioredoxin system [20, 21] in the reduction of sulphoxide xenobiotics. The use of acetaldehyde and DTT as direct electron donors and stimulants to the aldehyde oxidase and thioredoxin system respectively provided useful information on the relative contribution of these enzyme systems in various tissues. The enzymes responsible for the reduction of sulindac were widely distributed in rat tissues, with the liver and kidneys being the most active sites under anaerobic conditions. Addition of DTT resulted in extensive stimulation in all tissue homogenates investigated. This mirrors closely the wide distribution of the thioredoxin system in rat [24]. It also implies that the as yet unidentified terminal sulindac reductase coupled to the thioredoxin system [21] is also an enzyme showing a wide tissue distribution. The general lack of stimulation by acetaldehyde may be explained by the fact that the distribution of aldehyde oxidase in rat is mainly confined to the liver and small intestine [25]. Unexpectedly, acetaldehyde did not stimulate sulindac reduction in simple homogenates of rat liver but did in cytosol preparations. Possible reasons for

Table 4. Effect of inhibitors (menadione and hydralazine) on the reduction of sulindac by rat liver cytosol under anaerobic conditions

Incubation conditions					
NADPH and NADH (0.1 mM)	Acetaldehyde (2.5 mM)	Menadione (M) or hydralazine (H) (mM)	Preincubation with inhibitor	Sulphide formed (nmol/mg protein after 1 hr)	% Inhibition
−	−	−	−	1.61 ± 0.77	
+	−	−	−	2.15 ± 0.27	
−	+	−	−	7.00 ± 1.00	
−	−	M (0.005)	+	0.24 ± 0.21*	85
−	−	M (0.01)	+	0.04 ± 0.06**	98
−	−	M (0.02)	+	0.00 ± 0.00**	100
−	−	M (0.005)	−	1.64 ± 0.79†	0
−	−	M (0.01)	−	1.94 ± 0.56†	0
−	−	M (0.02)	−	2.20 ± 0.82†	0
−	+	M (0.01)	+	1.33 ± 0.48*	81
−	+	M (0.01)	−	5.08 ± 2.06†	27
−	−	−	−	0.63 ± 0.46	
+	−	−	−	1.10 ± 0.32	
−	+	−	−	8.90 ± 0.98	
−	−	H (0.01)	+	0.05 ± 0.06*	92
−	−	H (0.02)	+	0.01 ± 0.02*	98
−	−	H (0.01)	−	0.62 ± 0.42†	2
−	−	H (0.02)	−	0.58 ± 0.28†	8
−	+	H (0.02)	+	3.10 ± 2.62*	65
−	+	H (0.02)	−	8.57 ± 0.50†	4

The results are the mean ± SD for six female rats with different animals used for the menadione and hydralazine studies.

* $P < 0.05$, ** $P < 0.01$ compared with appropriate controls by Student's *t*-test for paired data.

† $P < 0.05$ compared to + preincubation.

Table 5. Effect of inhibitors (menadione and hydralazine) on the reduction of sulindac by rat renal cytosol under anaerobic conditions

Incubation conditions					
NADPH and NADH (0.1 mM)	DTT (10 mM)	Menadione (M) or hydralazine (H) (0.2 mM)	Preincubation with inhibitor	Sulphide formed (nmol/mg protein after 1 hr)	% Inhibition
−	−	−	−	0.10 ± 0.04	
+	−	−	−	1.73 ± 0.39	
−	+	−	−	10.48 ± 1.39	
+	−	M	+	0.07 ± 0.03**	96
+	−	M	−	0.73 ± 0.35*†	58
−	+	M	+	2.93 ± 0.41**	72
−	+	M	−	3.24 ± 0.37**	69
−	−	−	−	0.06 ± 0.03	
+	−	−	−	1.58 ± 0.23	
−	+	−	−	11.18 ± 0.52	
+	−	H	+	1.21 ± 0.17*	23
+	−	H	−	1.49 ± 0.22	6
−	+	H	+	10.91 ± 1.20	2
−	+	H	−	11.89 ± 0.40	0

The results are the mean ± SD for four female rats with different animals used for the menadione and hydralazine studies.

* $P < 0.05$, ** $P < 0.01$ compared with appropriate controls by Student's *t*-test for paired data.

† $P < 0.05$ compared to + preincubation.

this include the presence of other enzymes in the homogenate, such as mitochondrial aldehyde dehydrogenase, which may have rapidly removed the acetaldehyde and the presence of microsomal oxygenases which may have re-oxidized the sulphide.

Ratnayake *et al.* [26] reported that sulindac was not reduced by washed rat liver microsomes alone or in the presence of a small amount of 105,000 *g* supernatant fraction. Our studies showed that unwashed microsomes possess significant reducing

Table 6. Reduction of sulindac, sulphinpyrazone and diphenyl sulphoxide by rabbit liver and kidney cytosols under anaerobic conditions

Substrate	Tissue	Sulphide formed (nmol/mg protein after 1 hr)			
		Control no cofactor	+NADPH plus NADH (0.1 mM)	+Acetaldehyde (2.5 mM)	+Dithiothreitol (10 mM)
Sulindac	Liver	0.25 ± 0.16	2.10 ± 0.47*	19.74 ± 0.75***	13.51 ± 6.09*
	Kidney	0.66 ± 0.35	9.82 ± 5.57**	0.77 ± 0.14	22.73 ± 7.94**
Sulphinpyrazone	Liver	0.01 ± 0.01	0.01 ± 0.01	2.72 ± 0.49***	0.04 ± 0.04
	Kidney	ND	ND	<0.01	0.02 ± 0.01
Diphenyl sulphoxide	Liver	0.76 ± 0.22	0.25 ± 0.20	0.77 ± 0.33	0.46 ± 0.08
	Kidney	ND	ND	ND	ND

Results are the mean ± SD for four rabbits.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to the appropriate control using Student's *t*-test for paired data.
ND, not detected.

activity towards sulindac (Table 2), which was also reported for guinea pig liver microsomes [22]. The amount of sulphide detected at the end of the incubation represents an equilibrium between the reduction of the sulphoxide and the re-oxidation of the sulphide. Considering the high oxidizing activity towards the sulphide detected in microsomes, the significant amount of sulphide formed at the end of the incubation indicates that this fraction may play a significant role in sulphoxide reduction. Recent studies on the sub-cellular localization of aldehyde oxidase [27, 28] have shown that the enzyme is also present in the liver microsomes of some mammalian species. Although no such findings in rat have been reported, this may explain the high microsomal activity observed in the present study.

In rat liver fractions, the highest sulindac reductase activity was observed in the cytosol under anaerobic conditions. Such activity was significantly increased by the addition of acetaldehyde and DTT, indicating that both the aldehyde oxidase and thioredoxin systems are present. However, the results obtained with the aldehyde oxidase inhibitors menadione and hydralazine suggested that the aldehyde oxidase system plays a dominant role under anaerobic conditions, since the basal reducing activity was completely inhibited following preincubation with these two compounds.

It is interesting to note that previous studies on guinea pig liver aldehyde oxidase functioning as sulphoxide reductase have all been performed under anaerobic conditions [17–19]. In contrast Anders *et al.* [20] used aerobic conditions when they demonstrated that rat liver cytosol lost 94% of its sulindac reducing activity after the removal of thioredoxin using gel filtration chromatography and which suggested a dominant role for the thioredoxin system. The activity of the aldehyde oxidase system in rat liver cytosol (Table 3) (as indicated by the increase with acetaldehyde) was very low under aerobic conditions. This suggests that the functioning of aldehyde oxidase as a sulphoxide reductase may be severely limited under aerobic conditions and that the thioredoxin system is dominant under these conditions as reported by Anders *et al.* [20]. Further evidence is required to prove this, but the inhibitory effects of oxygen on the reduction of sulphoxides have been previously reported in mouse preparations [29] which indicates the presence of both anaerobic and aerobic systems in this species.

The reduction of sulindac by rat renal cytosol was dependent on NADPH plus NADH. These co-factors could be replaced by DTT in the incubation, which resulted in much higher reducing activities. This implies the involvement of the thioredoxin system. Furthermore, it indicates that only the thioredoxin system is important in the renal cytosol since addition of acetaldehyde did not have any effect on the reducing activity even under anaerobic conditions (Table 3).

Menadione is a classic inhibitor of rabbit and guinea pig aldehyde oxidase probably by interacting at the FAD site of the active centre of the enzyme [30]. Hydralazine is a potent inhibitor of aldehyde oxidase in oxidative reactions [31]. Both inhibitors exhibit a progressive inhibition with time, since when a second aliquot of enzyme is added after the initial inhibition is established, the enzyme activity proceeds at the expected rate and then declines gradually. This indicates that these compounds interact either with a reduced form of the enzyme or the enzyme–substrate complex [30, 31]. In the present study the reducing activity of the rat liver cytosol was only affected when the enzyme was preincubated with these two inhibitors prior to addition of the substrate. This indicates that the inhibition was due to 'turnover' of the enzyme or the formation of an inhibitory product during preincubation. However, the inhibition was reversible since addition of acetaldehyde partially restored the reducing activity. In rat kidney cytosol, pre-incubation with menadione showed some inhibition but hydralazine had comparatively little inhibitory effect on the sulindac reductase. This further supports the view that aldehyde oxidase is not involved in sulindac reduction in the rat kidney, and that menadione may have been inhibiting another enzyme system.

The reduction of sulindac by rabbit liver and

kidney cytosols showed similar results to those in rats. The reducing activity of liver cytosol was increased by both acetaldehyde and DTT, while that of kidney cytosol was increased by DTT only. However, the stimulatory effects of these electron donors were much greater than those observed in the rat. This may be related to the higher concentration of aldehyde oxidase and possibly thioredoxin, in rabbit tissues [32].

In contrast to sulindac, sulphinpyrazone and DPSO were generally very poor substrates for both the liver and renal cytosolic sulphoxide reductase. Nevertheless, under anaerobic conditions, acetaldehyde increased the reduction of sulphinpyrazone by rabbit liver cytosol and the reduction of DPSO by rat liver cytosol. The reason for this is not clear, but may be related to the volume of the active site of aldehyde oxidase as well as the presence of suitable functional groups in the substrate molecule for binding to the active site. On the other hand, surprisingly DTT has no effect on the reduction of these two substrates by the cytosolic enzymes. It appears therefore that aldehyde oxidase may have a broader substrate specificity towards sulphoxides than the reductase coupled to the thioredoxin system. Sulindac has a methyl group attached to the sulphoxide, while sulphinpyrazone and DPSO are both phenyl sulphoxides. Fukazawa *et al.* [33] reported the purification of two sulphoxide reductases from rat kidney cytosol which predominantly reduced methyl sulphoxides. Results from the present study showed that in rat and rabbit tissues, sulindac was a much better substrate than the two phenyl sulphoxides. Further investigations are required to establish this trend, which may have important implications on the design of newer sulphoxide-containing drugs.

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