METABOLISM *IN VITRO* OF SULINDAC. SULFOXIDE-REDUCING ENZYME SYSTEMS IN GUINEA PIG LIVER*

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The sulfoxide reduction of sulindac (*cis*-5-fluoro-2-methyl-1-[*p*-(methylsulfinyl)-benzylidenyl]indene-3-acetic acid), an anti-inflammatory agent, was demonstrated *in vitro* by cell-free preparations of guinea pig liver. The sulfoxide reductase activity was located in both fractions of microsomes and $105000 \times g$ supernatant. The microsomal reductase was NADPH- or NADH-dependent, and required the factor present in the soluble fraction for its activity. The factor was heat-labile and non-dialyzable. Furthermore, a partial purification of the microsomal NADPH-cytochrome c reductase resulted in a paralleled increase of the sulfoxide reductase activity. The present observations suggest that the soluble factor described above functions as an electron transfer component coupled with NADPH-cytochrome c reductase.

Keywords—metabolism *in vitro*; sulindac; guinea pig liver; microsomes; $105000 \times g$ supernatant; sulfoxide reductase; NADPH-cytochrome c reductase; soluble factor

Sulindac (*cis*-5-fluoro-2-methyl-1-[*p*-(methylsulfinyl)-benzylidenyl]indene-3-acetic acid) was newly synthesized by Shen *et al.*⁽¹⁾ as one of the nonsteroidal anti-inflammatory agents. Pharmacological experiments showed that the drug has strong analgesic and antipyretic activities in experimental animals⁽²⁾ and in man.⁽³⁻⁵⁾

In regard to the metabolic fate, Hucker *et al.*⁽⁶⁾ and Duggan *et al.*^(7,8) reported that the drug is metabolized by the oxidation and reduction of the sulfoxide group. Furthermore, Duggan *et al.*⁽⁹⁾ suggested that the pharmacological activities of the drug are attributable predominantly, or perhaps exclusively to the reduction product, sulindac sulfide. However, the metabolism *in vitro* of the drug has not yet been studied. The present study was carried out in order to explore the enzyme systems capable of catalyzing the sulfoxide reduction of sulindac in guinea pig liver.

EXPERIMENTAL

Chemicals and Enzymes—Sulindac and sulindac sulfide (5-fluoro-2-methyl-1-[*p*-(methylthio)-benzylidenyl]indene-3-acetic acid) were kindly donated by Merck Sharp and Dohme Research Laboratories. NADPH, NADP, NAD, nicotinamide, glucose 6-phosphate, yeast cytochrome c, yeast glucose 6-phosphate dehydrogenase, yeast alcohol dehydrogenase and bovine pancreas trypsin were purchased from Sigma Chemical Co. Precoated silica gel thin-layer plates (Wakogel F₂₅₄ plates) were obtained from Wako Pure Chemical Industries, Ltd.

Animals—Male guinea pigs weighing 260–280 g were employed in all experiments and fasted overnight prior to use.

Preparation of Tissue Samples—Animals were killed by cervical fracture and exsanguinated. The livers were homogenized with a Teflon-glass

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homogenizer in four volumes of 1.15% KCl. The homogenate was centrifuged at $9000 \times g$ for 20 min, and microsomes were sedimented from the supernatant by centrifugation at $105000 \times g$ for 60 min. The microsomes were washed by re-suspension in a volume of the KCl solution, equivalent to twice the volume of the original liver and re-sedimentation at $105000 \times g$ for 60 min. The unwashed or the washed microsomes were suspended in a volume of 0.1 M phosphate buffer (pH 7.4), equivalent to twice the volume of the original liver. Unless otherwise specified, the unwashed microsomes were used in this experiment.

NADPH-cytochrome c reductase was solubilized from microsomes by trypsin and was partially purified on Sephadex G-100 column chromatography according to the method of Omura and Takesue.¹⁰ The fraction containing the highest enzyme activity was used as an enzyme source. The enzyme preparation was free of cytochrome P-450 and cytochrome b₅.

Assay of Sulindac Reductase Activity—The standard reaction mixture containing microsomes consisted of 0.4 μ mol of sulindac in 40 μ l of methanol, 4 ml of tissue preparation (equivalent to 2 g of liver), 50 μ mol of nicotinamide and an NADPH-generating system (2 μ mol of NADP, 25 μ mol of glucose 6-phosphate and 2 units of glucose 6-phosphate dehydrogenase) or an NADH-generating system (2 μ mol of NAD, 40 μ mol of ethanol and 30 units of alcohol dehydrogenase) in a final volume of 8 ml of 0.1 M phosphate buffer (pH 7.4). Also, the reaction mixture containing the $105000 \times g$ supernatant consisted of 0.4 μ mol of sulindac, 6 ml of tissue preparation (equivalent to 2 g of liver), 50 μ mol of nicotinamide and an NADPH- or NADH-generating system described above in a final volume of 12 ml of the phosphate buffer.

The enzyme was assayed in a Thunberg tube. The body contained a tissue preparation and a cofactor-generating system, and the side arm contained sulindac. The tube was gassed for 5 min with N₂ which had been deoxygenated by passage through a solution of 0.05% sodium 2-

anthraquinone sulfonate, 0.5% sodium dithionite and 0.1 N NaOH, and then evacuated with a water aspirator for 10 min. The reaction was started by mixing the solution of the side arm and body together and continued for 60 min at 37°. In the aerobic experiment, the incubation was carried out in an open vessel. After incubation, the mixture was extracted twice with two volumes of ethyl acetate, and the combined extract was evaporated to dryness *in vacuo*. The residue was then applied to a silica gel plate and developed with CHCl₃-ethyl acetate-acetic acid (16:5:1). The areas corresponding to sulindac (*R_f* 0.31) and sulindac sulfide (*R_f* 0.61) were scraped and eluted separately with 3 ml of 28% ammonia water-methanol (1:49). The amount of each compound was determined spectrophotometrically at 327 nm (the absorption maximum of sulindac) and 344 nm (the absorption maximum of sulindac sulfide), respectively.

Assay of NADPH-Cytochrome c Reductase Activity—The assay was carried out by the method of Omura and Takesue¹⁰ as follows: The reaction mixture contained 0.04 μ mol of cytochrome c, 0.2 μ mol of NADPH and an enzyme preparation in a final volume of 2 ml of 0.1 M phosphate buffer (pH 7.4). When microsomal enzyme activity was assayed, 2 μ mol of KCN was added to the reaction mixture. The absorbance change at 550 nm was recorded on a Hitachi 124 UV spectrophotometer. The reduction of cytochrome c was calculated based on the extinction of reduced cytochrome c as 21.1 mM⁻¹ cm⁻¹.

RESULTS

Intracellular Localization of Sulindac Reductase Activity in Guinea Pig Liver

Table I shows that the reduction of sulindac was catalyzed by enzyme systems present in the microsomal and $105000 \times g$ supernatant fractions, especially under anaerobic conditions. The reductase activity in the former fraction was completely dependent upon the presence of an NADPH- or NADH-generating system, and was stimulated by further addition of FAD. The latter fraction was as active as the $9000 \times g$ supernatant

TABLE I. *Intracellular Localization of Sulindac Reductase Activity in Guinea Pig Liver*

Fraction	Cofactor	Reductase activity (nmol/hr/g liver)	
		Anaerobic	Aerobic
9000 × g Supernatant	None	123 ± 25	19 ± 4
	NADPH	170 ± 6	15 ± 4
	NADH	125 ± 15	22 ± 5
Microsomes	None	2 ± 2	1 ± 2
	NADPH	34 ± 4	1 ± 2
	NADPH, FAD ^{a)}	67 ± 14	—
	NADH	44 ± 4	2 ± 1
	NADH, FAD ^{a)}	62 ± 9	—
105000 × g Supernatant	None	123 ± 24	69 ± 14
	NADPH	159 ± 12	87 ± 20
	NADH	127 ± 18	73 ± 14

Values represent mean ± S.D. of four experiments.

a) FAD was added to the reaction mixture at a concentration of 10^{-5} M.

TABLE II. *Effect of Washing on Sulindac Reductase Activity of Guinea Pig Liver Microsomes*

Fraction	Reductase activity ^{a)} (nmol/hr/g liver)	
	NADPH	NADH
Unwashed microsomes	66 ± 11	58 ± 6
Washed microsomes	2 ± 2	0
+ washed supernatant fraction ^{b)}	49 ± 3	27 ± 4
+ boiled washed supernatant fraction	5 ± 4	2 ± 2
+ dialyzed washed supernatant fraction ^{c)}	48 ± 1	25 ± 6
+ 105000 × g supernatant (0.1 ml)	37 ± 4	24 ± 7
Washed supernatant fraction ^{d)}	1 ± 1	1 ± 2
105000 × g Supernatant (0.1 ml) ^{d)}	1 ± 2	2 ± 1

Values represent mean ± S.D. of four experiments.

a) The incubation was carried out anaerobically in the presence of 10^{-5} M FAD.

b) Washed supernatant fraction which was obtained from microsomes equivalent to 2g of liver as described in Experimental was used.

c) Washed supernatant fraction was dialyzed for 20 hr at 4° against 300 volumes of 0.05M phosphate buffer (pH 7.4).

d) The supernatant was incubated with 0.4 μmol of sulindac, 50 μmol of nicotinamide, an NADPH- or NADH-generating system, and 0.08 μmol of FAD in a final volume of 8 ml of 0.1M phosphate buffer (pH 7.4).

fraction in the absence of the cofactor-generating system under anaerobic conditions, and the activities in both supernatant fractions were stimulated by addition of an NADPH-generating system. Boiling the tissue preparations prior to incubation abolished the sulindac reductase activities in all the fractions.

Properties of Microsomal Sulindac Reductase

As shown in Table II, the washing of the freshly isolated microsomes with 1.15% KCl resulted in almost complete loss of the NADPH- or NADH-linked sulindac reductase activity in the microsomal fraction. In the presence of the cofactor-generating system, the activity lost by the

washing was partially recovered by the addition of the washed supernatant fraction to the washed microsomes. This supernatant fraction could be replaced by a small amount (0.1 ml) of the $105000 \times g$ supernatant. In this case, the former or the latter fraction by itself did not have any sulindac reductase activity. When heated at 100° for 5 min, the washed supernatant fraction lost the activity completely. However, on dialysis against 0.05 M phosphate buffer (pH 7.4) no alteration of the activity was observed. These facts suggested that the essential factor in this supernatant fraction is a protein-like macromolecular substance.

TABLE III. *Influence of Carbon Monoxide on Sulindac Reduction by Guinea Pig Liver Microsomes*

Gas phase	Reductase activity (nmol/hr/g liver)
Nitrogen	28 ± 6
Carbon monoxide	30 ± 12

Values represent mean $\pm$ S.D. of four experiments.

The reaction mixture consisting of microsomes (equivalent to 2g of liver), an NADPH-generating system and $0.08 \mu\text{mol}$ of FAD in 7.96 ml of 0.1M phosphate buffer (pH 7.4) was placed in a test tube and gassed for 5 min with nitrogen or carbon monoxide which had been deoxygenated by passage through a solution of 0.05% sodium 2-anthraquinone sulfonate, 0.5% sodium dithionite and 0.1N NaOH. Then, to the mixture was immediately added $0.4 \mu\text{mol}$ of sulindac in $40 \mu\text{l}$ of methanol, air space above the mixture was flushed with the gas for an additional 1 min and the tube was sealed up. The mixture was incubated for 60 min at 37° .

TABLE IV. *Effect of Various chemicals on Sulindac Reduction by Guinea Pig Liver Microsomes*

Addition	Concentration	Reductase activity ^{a)} (nmol/hr/g liver)	
		NADPH	NADH
	M	% control	
None (control)		100	100
Monoiodoacetic acid	10^{-3}	9	6
Sodium arsenite	10^{-4}	0	3
Potassium cyanide	10^{-3}	3	3
SKF 525-A	10^{-3}	40	48
Cupric sulfate	10^{-4}	9	37
Cytochrome c	10^{-4}	10	39
Dimethyl sulfoxide	10^{-4}	78	29

Values represent mean of four experiments.

a) The incubation was carried out anaerobically in the presence of 10^{-5} M FAD.

Next, the influence of carbon monoxide on microsomal sulindac reductase activity was investigated in the presence of the NADPH-generating system. As shown in Table III, the sulindac reductase activity was not affected by carbon monoxide, suggesting that cytochrome P-450 is not involved in this enzyme reaction.

Effect of Various Chemicals on Sulindac Reductase Activities of Liver Microsomes and 105000 × g Supernatant

Both the NADPH- and NADH-linked activities of the microsomes were almost completely inhibited by monoiodoacetic acid, sodium arsenite and potassium cyanide, and partially inhibited by SKF 525-A and dimethyl sulfoxide. In addition, the NADPH-linked activity was strongly inhibited by cupric sulfate and cytochrome c, and the NADH-linked one was partially inhibited by these chemicals (Table IV).

The NADPH-linked activity of the

TABLE V. *Effect of Various Chemicals on Sulindac Reduction by Guinea Pig Liver 105000 × g Supernatant*

Addition	Concentration	Reductase activity ^{a)} (nmol/hr/g liver)	
		Aerobic	Anaerobic
	M	% control	
None (control)		100	100
Monoiodoacetic acid	10 ⁻³	0	8
Sodium arsenite	10 ⁻⁴	7	11
Potassium cyanide	10 ⁻³	82	112
Dicoumarol	10 ⁻⁴	98	112
Dimethyl sulfoxide	10 ⁻⁴	70	66
Mercaptoethanol	10 ⁻³	45	41
FAD	10 ⁻⁵	87	106
FMN	10 ⁻⁵	83	114
Bovine serum albumin	10 ⁻⁵	92	99

Values represent mean of four experiments.

a) The incubation was carried out in the presence of an NADPH-generating system.

TABLE VI. *Solubilization and Partial Purification of Sulindac Reductase and NADPH-Cytochrome c Reductase of Guinea Pig Liver Microsomes*

Purification step	105000 × g Supernatant (0.1 ml)	Sulindac reductase ^{a)}		NADPH-cytochrome c reductase	
		(nmol/hr/mg protein)	Purification	(nmol/min/mg protein)	Purification
Washed microsomes	–	0.1		228	
	+	1.8		–	
Trypsin extract	–	0.3		693	3.0 X
	+	6.5	3.6 X	–	
Sephadex G-100 fraction	–	0		13700	60.1 X
	+	114.0	63.3 X	–	

Values represent mean of four experiments.

a) The incubation was carried out anaerobically in the presence of an NADPH-generating system.

$105000 \times g$ supernatant was also strongly inhibited by monoiodoacetic acid and sodium arsenite under both aerobic and anaerobic conditions. Dimethyl sulfoxide partially inhibited the enzyme activity, while dicoumarol, which is an inhibitor of DT-diaphorase, and potassium cyanide did not. Also, FAD, FMN and bovine serum albumin have no effect on the enzyme activity (Table V).

Comparison of NADPH-cytochrome c Reductase and Sulindac Reductase of Guinea Pig Liver Microsomes

As described above (Table IV), the inhibition of microsomal sulindac reductase activity by cytochrome c or cupric ion suggested the participation of NADPH-cytochrome c reductase. Therefore, the activities of NADPH-cytochrome c reductase and sulindac reductase of the washed microsomes, trypsin extract and Sephadex G-100 fraction were compared in the presence of NADPH (Table VI).

Each enzyme preparation again exhibited the sulindac reductase activity only in the presence of a small amount of the $105000 \times g$ supernatant. The increase of activity of sulindac reductase by purification was parallel to that of NADPH-cytochrome c reductase in the two-step purification. This result strongly suggested the participation of NADPH-cytochrome c reductase with sulindac reductase.

DISCUSSION

The reduction *in vitro* of some sulfoxide compounds, e.g. di-(*p*-aminophenyl)sulfoxide,¹¹⁾ carbophenothion sulfoxide¹²⁾ and methionine sulfoxide¹³⁾ to the corresponding sulfides has been demonstrated by using rat tissue preparations. However, little is known about the properties of the sulfoxide-reducing enzyme system in animal bodies. In the present study, we showed that the sulfoxide-reducing enzyme activity for sulindac was localized in both microsomal and $105000 \times g$ supernatant fractions of guinea pig liver, although the activity of the former fraction was lower than that of the latter fraction (Table I). Especially, the sulfoxide reduction catalyzed by the washed microsomes required the washed supernatant frac-

tion or a small amount of $105000 \times g$ supernatant in addition to NADPH or NADH (Table II). The factor in the supernatant fraction is heat-labile and non-dialyzable (Table II), and is precipitated by acetone (data not shown). These results suggest that a protein-like factor in the supernatant fraction contributes as an essential component to the microsomal sulfoxide-reducing enzyme systems.

The factor in the supernatant fraction is distinguishable from the heat-stable factor described by Terriere and Chan,¹⁴⁾ which stimulates the activities of microsomal aldrine epoxidase and ethylmorphine N-demethylase. The factor seems to resemble the heat-labile factors described by Van Dyke and Wineman¹⁵⁾ on the microsomal dechlorination of 1,1,2-trichloroethane, Nelson *et al.*¹⁶⁾ on the microsomal N-demethylation of aminopyrine and ethylmorphine, or Bloch and coworkers¹⁷⁻¹⁹⁾ on the microsomal epoxidation of squalene.

As shown in Table IV, the inhibition of the microsomal sulfoxide-reducing enzyme activity by cytochrome c suggested the participation of NADPH-cytochrome c reductase. This inhibitor may compete with the sulfoxide-reducing enzyme system for electrons from a cofactor. Similar inhibitory effects of cytochrome c have been observed in other microsomal enzyme systems.²⁰⁻²²⁾ The sulfoxide-reducing enzyme activity was also inhibited by the cupric ion, a known inhibitor of NADPH-cytochrome c reductase. This view was further supported by the finding that in a two-step partial purification, the specific activity of the sulfoxide-reducing enzyme in the presence of both NADPH and $105000 \times g$ supernatant was parallel to that of NADPH-cytochrome c reductase (Table VI).

Thus, the data now presented strongly suggested that at least two components are necessary for the NADPH-linked sulfoxide reduction in guinea pig liver microsomes. As described above, these are NADPH-cytochrome c reductase and a heat-labile soluble factor, although the latter component has not yet been isolated. The sulfoxide-reducing enzyme system reconstituted from the partially purified NADPH-cytochrome c reduc-

tase and the $105000 \times g$ supernatant is as active as the combination of microsomes and the supernatant. In the absence of FAD, the sulfoxide-reducing enzyme activity is about 50% of that of the complete system. Our present results do not allow us to specify how the soluble factor functions in the sulfoxide-reducing enzyme reaction. However, the possibilities are considered that 1)

the $105000 \times g$ supernatant may supply an undescribed electron transfer component coupled with microsomal NADPH-cytochrome c reductase and 2) the supernatant may supply a factor that stimulates the reduction system without functioning as an electron transfer component. In our preliminary experiments, the supernatant did not affect the NADPH-cytochrome c reductase

FIG. 1. *Proposed Enzyme System Responsible for NADPH-linked Sulfoxide Reduction in Guinea Pig Liver Microsomes*

I: Sulindac (cis-5-fluoro-2-methyl-1-[p-(methylsulfinyl)-benzylidenyl]indene-3-acetic acid),
II: Sulindac sulfide (5-fluoro-2-methyl-1-[p-(methylthio)-benzylidenyl]indene-3-acetic acid),
SF: Soluble factor,
FP: NADPH-cytochrome c reductase.

activity, excluding the possibility of mechanism 2). Our current view of the NADPH-linked sulfoxide reduction system in liver microsomes is illustrated as a new electron transfer system across the microsomal membrane (Fig. 1).

This view is supported by the fact that carbon monoxide does not block the NADPH-linked reduction of the sulfoxide group by microsomes (Table III). The nitro group of nitrofurán derivatives^{2,3)} and niridazole^{2,4)} apparently accepts electron directly from NADPH-cytochrome c reductase. On the contrary, the sulfoxide group of sulindac seems to accept electrons indirectly through the soluble factor, because the redox potential of the reductase might be unfavourable for the transfer of electrons to the sulfoxide group. The microsomal NADH-linked enzyme system and the cytoplasmic enzyme system for sulindac reduction are under investigation.

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