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INVOLVEMENT OF THIOREDOXIN IN SULFOXIDE REDUCTION BY MAMMALIAN TISSUES

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SUMMARY: Sulindac, a sulfoxide with antiinflammatory activity, is reduced to the corresponding sulfide by rat hepatic cytosolic enzymes requiring NADPH for maximal activity. This reaction is inhibited by insulin, L-cystine, glutathione disulfide and 5,5'-dithiobis(2-nitrobenzoic acid), all of which are known to interact with the thioredoxin system comprised of NADPH, thioredoxin reductase and thioredoxin. Sodium arsenite, a known inhibitor of thioredoxin reductase, also inhibited sulindac reduction. Rat hepatic cytosolic fractions from which thioredoxin had been removed by chromatography on Sephadex G-50 showed minimal sulfoxide reductase activity; activity could be restored by addition of purified Escherichia coli thioredoxin or dithiothreitol. These findings are the first demonstration of thioredoxin-dependent sulfoxide reduction by mammalian tissues.

INTRODUCTION

Thioredoxin is a low molecular weight electron transfer protein which, together with NADPH-dependent thioredoxin reductase (EC 1.6.4.5.), was originally identified as a component of the ribonucleotide reductase system of Escherichia coli (1,2). Thioredoxin and thioredoxin reductase are present in a variety of mammalian tissues (3-5). Thioredoxin is involved in disulfide reduction (4,6), in methionine sulfoxide and sulfate reduction by microorganisms (6,7), as a subunit of phage-induced DNA polymerase in E. coli (8) and as a regulatory protein during photosynthesis in spinach chloroplasts (9).

The enzymatic reduction of sulfoxides to sulfides by mammalian tissues has been observed (10-15) but little is known concerning the enzymes involved in this biotransformation. Recent studies have shown that sulindac, (Z)-5-fluoro-2-methyl-1-[p-(methylsulfinyl)benzylidene]indene-3-acetic acid, is

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reduced *in vitro* to the corresponding pharmacologically active sulfide by hepatic tissues (16,17).

Because of the known involvement of thioredoxin in methionine sulfoxide reduction by microorganisms (6,7), we have investigated the possible role of thioredoxin in sulfoxide reduction by rat hepatic tissues. The results of these studies show that thioredoxin is involved in mammalian sulfoxide reduction.

MATERIALS AND METHODS

Male Long-Evans rats (280-320 g) were purchased from Blue Spruce Farms, Altamont, NY. Sulindac and sulindac sulfide were supplied by Merck Sharp and Dohme Research Laboratories, Rahway, NJ. Sephadex G-50 (fine) and G-25 (medium) were obtained from Pharmacia Fine Chemicals, Piscataway, NJ. Insulin (bovine pancreas, crystalline) and other reagents were obtained from Sigma Chemical Co., St. Louis, MO.

Rat hepatic cytosolic fractions were isolated as described previously (16). Rat liver preparations obtained by ammonium sulfate fractionation and gel filtration chromatography were isolated essentially as described by Larson and Larsson (18). Hepatic cytosol (12-13 ml) was adjusted to 40% of saturation by the slow addition (30 min) of solid ammonium sulfate. The mixture was stirred for another 15 min and the resulting precipitate was removed by centrifugation. The supernatant was adjusted to 60% of saturation with ammonium sulfate as described above and the precipitated protein was recovered by centrifugation and dissolved in 3 ml of 10 mM Tris-HCl buffer (pH 7.5). This solution was dialyzed against 10 mM Tris-HCl (pH 7.5) for 3.5 hr with 3 changes of buffer. The dialyzed preparation was centrifuged to remove the precipitated proteins and a 1.5 ml aliquot was applied to both a Sephadex G-25 (1.7 cm x 40 cm) and a G-50 (1.7 cm x 38 cm) column equilibrated with 0.05 M Tris-HCl buffer (pH 7.5). The columns were eluted with the same buffer and 3-5 ml fractions were collected. The flow rates for the Sephadex G-25 and G-50 columns were 20 and 33 ml/hr, respectively. The column effluents were monitored by determining the absorbance at 280 nm and the fractions giving the first peak (void volume) from each chromatogram were pooled and analyzed for their ability to reduce sulindac to sulindac sulfide.

Thioredoxin was prepared from 400 g of *E. coli* cells as described by Holmgren and Reichard (19) with the following modifications. Cells were broken by ultrasonification of 200 ml batches in a Branson S-125 sonicator at a setting of 5 for four 60 sec periods with a 60 sec cooling time between cycles. The supernatant obtained after streptomycin precipitation was adjusted to pH 4.6 with acetic acid rather than adding a fixed volume of acetic acid since it was found that culture growth conditions affected the final pH of the supernatant. The second DEAE-cellulose chromatography step was omitted since it had only a marginal effect on thioredoxin purity. The thioredoxin obtained by this procedure was greater than 90% pure as determined by polyacrylamide gel electrophoresis.

Unless otherwise indicated, incubation mixtures contained 150 µmoles of Tris-HCl buffer (pH 7.4), 0.2 µmoles of NADPH, 0.26 µmoles of sulindac and 4-6 mg of protein in a total volume of 2.0 ml. The reaction mixtures were incubated in air for 30 min at 37° with shaking. The reaction was stopped

Table 1: Effect of Substrates for Thioredoxin-dependent Enzyme Systems on Sulindac Reduction by Rat Hepatic Cytosol

Compound	Sulindac Reduction (% of control)
Insulin, 2.08 mg/ml	12.8 ± 5.4 (3)
Glutathione disulfide	2.8 ± 0.6 (3)
L-Cystine	8.5, 7.6 (2)
5,5'-dithiobis(2-nitro benzoic acid)	2.1, 10.1 (2)
Sodium arsenite	0.6, 4.3 (2)

Incubation mixtures were prepared, incubated and analyzed as described in MATERIALS AND METHODS. Inhibitors were added to a final concentration of 1 mM except as noted. Values in parentheses indicate the number of experiments; the data are shown as individual values or mean ± S.D. Control activity was 52.0 ± 8.8 pmol sulindac sulfide/min/mg protein (n=3).

by placing the reaction tubes in ice. Sulindac sulfide was measured by a combined isotope dilution-HPLC method (16). Protein was measured by the Coomassie Brilliant Blue method (20).

RESULTS

Several known substrates for thioredoxin-dependent enzyme systems were tested for their ability to inhibit sulindac reduction (Table 1). The disulfides, insulin, glutathione disulfide, L-cystine and 5,5'-dithiobis(2-nitrobenzoic acid), were effective inhibitors of sulindac reduction in hepatic tissue. Sodium arsenite also inhibited sulindac reduction.

Further evidence for the involvement of thioredoxin was obtained by partially purifying the enzymes involved as described by Larson and Larsson (18). The ammonium sulfate fraction reduced sulindac in the presence of NADPH and most of this activity was retained in the void volume fraction of the Sephadex G-25 column; in contrast, the void volume fraction of the Sephadex G-50 column contained relatively little activity (Table 2). The activity of the Sephadex G-50 fractions could be restored by adding purified *E. coli* thioredoxin (Fig. 1). Finally, the activity of the Sephadex G-50 column fractions could also be restored by addition of dithiothreitol; *E. coli* thioredoxin itself did not reduce sulindac (data not shown).

Table 2: Sulindac Reduction by Partially Purified Rat Hepatic Cytosol

Fraction	Sulindac Reduction (% of ammonium sulfate fraction)
Ammonium sulfate, 40-60% saturation	100
Void volume of Sephadex G-25	83.3 ± 8.5
Void volume of Sephadex G-50	5.8 ± 7.3

Fractions were isolated, incubated and analyzed as described in MATERIALS AND METHODS. Values are shown as mean ± S.D. for 3 experiments. Total activity in the ammonium sulfate fraction was 0.88 ± 0.53 nmol sulindac sulfide/min.

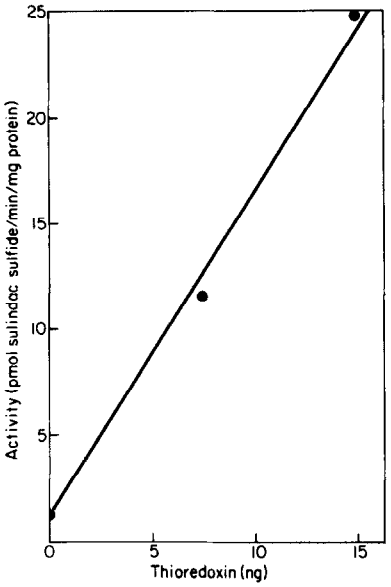

Figure 1. Dependence of sulindac reduction by rat hepatic tissue on thioredoxin. Rat hepatic cytosolic fractions, from which thioredoxin had been removed by chromatography on Sephadex G-50, were incubated in the presence of sulindac, NADPH and purified E. coli thioredoxin; sulindac sulfide was measured as described in MATERIALS AND METHODS. Values represent the mean of two separate experiments.

DISCUSSION

These results show that thioredoxin is involved in sulfoxide reduction by mammalian tissues. Insulin, glutathione disulfide, L-cystine and 5,5'-dithiobis(2-nitrobenzoic acid), which are known to be reduced by thioredoxin (4,6), inhibited the reduction of sulindac. Arsenite, which has been shown to inhibit thioredoxin reductase (4), also inhibited sulindac reduction.

Larson and Larsson (18) showed that thioredoxin was present in ammonium sulfate and Sephadex G-25 fractions of rat liver but could be removed by chromatography on Sephadex G-50. This is the expected result since thioredoxin has a molecular weight of about 12,000 daltons. In the present study it was found that ammonium sulfate and Sephadex G-25 fractions fortified with NADPH catalyzed the reduction of sulindac but that this activity was lost by chromatography on Sephadex G-50. However, the activity of the Sephadex G-50 fractions could be restored by adding purified E. coli thioredoxin. These results also confirm the earlier observation that E. coli thioredoxin cross-reacts with mammalian thioredoxin reductase (4).

Furthermore, the activity of the Sephadex G-50 column fractions could be restored by addition of dithiothreitol which has been shown to support the activities of several thioredoxin-dependent enzyme systems (18,21,22); dithiothreitol was also found to substitute for NADPH in sulindac reduction by rat liver (16).

Although the current study does not clearly establish the presence of a sulfoxide reductase such as that present in E. coli (23), it was observed that thioredoxin did not directly reduce sulindac; it is assumed that thioredoxin serves to maintain a sulfoxide reductase in a reduced form. This is consistent with the observation that dithiothreitol could restore the activity of the Sephadex G-50 column fractions from which thioredoxin had been removed.

These studies present the first evidence for the involvement of the thioredoxin system in sulfoxide reduction by mammalian tissues. The results are also significant in that they demonstrate the interaction of a xenobiotic compound with the physiologically important thioredoxin system; the possible pharmacological and toxicological implications of this interaction warrant additional investigation.

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