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EVIDENCE FOR INVOLVEMENT OF LIVER ALDEHYDE OXIDASE IN REDUCTION OF NITROSAMINES TO THE CORRESPONDING HYDRAZINE

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The present study shows that guinea pig and rabbit liver aldehyde oxidase in the presence of its electron donors such as aldehydes or N-heterocyclic compounds reacts as N-nitrosoreductase with N-nitrosodiphenylamine and other nitrosamines.

KEYWORDS ——— nitrosamine; N-nitrosodiphenylamine; enzymatic reduction; N-nitrosoreductase; liver aldehyde oxidase; reduction product; 1,1-diphenylhydrazine; acetaldehyde diphenylhydrazone

The mechanism for bioactivation of nitrosamines is known to involve α-hydroxylation followed by N-dealkylation of these compounds.[1,2] On the other hand, our recent study[3] demonstrated for the first time the enzymatic reduction of a noncyclic nitrosamine to the corresponding hydrazine by using N-nitrosodiphenylamine and guinea pig liver preparations. Such reductive metabolism of nitrosamines would seem to be another mechanism for bioactivation of these compounds, because many hydrazine derivatives are known to act as direct mutagens.[4] However, the nature of the enzyme responsible for this reductive metabolism remained unknown. Here we describe the first evidence that liver aldehyde oxidase is involved in the reduction of nitrosamines.

The 9,000 x g supernatant of guinea pig liver supplemented with NADPH and FAD, or NADH and FAD reduced N-nitrosodiphenylamine to 1,1-diphenylhydrazine under anaerobic conditions. In addition, the liver preparation supplemented with acetaldehyde exhibited much significant N-nitrosoreductase activity to give acetaldehyde diphenylhydrazone (Table I). This result suggested that acetaldehyde can be utilized not only as a trapping agent for the reduction product, 1,1-diphenylhydrazine, but also as an effective electron donor of the liver N-nitrosoreductase.

When the liver 9,000 x g supernatant was separated into microsomal and cytosolic fractions, the acetaldehyde-linked activity occurred in the latter fraction, but not in the former. When the cytosol was subjected to ammonium sulfate fractionation as described in EXPERIMENTAL, the acetaldehyde-linked activity was associated with a protein which was precipitated when the ammonium sulfate concentration was between 30 and 45% of saturation.

Furthermore, the ability of the precipitate to reduce N-nitrosodiphenylamine was examined in the presence of aldehydes or N-heterocyclic compounds, which are known as electron donors of aldehyde oxidase. The precipitate showed N-nitrosodiphenylamine-metabolizing activity in the presence of these electron donors, but

TABLE I. N-Nitrosoreductase Activity of Guinea Pig Liver 9,000 x g Supernatant

Addition*	N-Nitrosoreductase activity (Reduction product formed)
	nmol/30 min/0.8 g liver
None	0
NADPH	0
NADH	0
NADPH,FAD	45**
NADH,FAD	11**
Acetaldehyde	165***
Acetaldehyde,FAD	200***

Each value represents mean of four experiments.
* NADPH or NADH 2 μmol, acetaldehyde 10 μmol.
** 1,1-Diphenylhydrazine formed was determined.
*** Acetaldehyde diphenylhydrazone formed was determined.

TABLE II. Electron Donor Requirements for Metabolism of N-Nitrosodiphenylamine by the 30-45% Ammonium Sulfate Precipitate from Guinea Pig Liver Cytosol

Addition*	N-Nitrosodiphenylamine disappeared
	nmol/30 min/0.8 g liver
None	24
Acetaldehyde	122
Propionaldehyde	126
Butylaldehyde	127
Benzaldehyde	177
N^1-Methylnicotinamide	118
2-Hydroxypyrimidine	154
NADPH	69
NADH	24

Each value represents mean of four experiments.
* 10 μmol each.

little or none in the presence of NADPH or NADH (Table II). These facts suggested that the aldehyde oxidase in the cytosolic fraction of guinea pig liver functions as N-nitrosoreductase.

The ability of some chemicals to inhibit the aldehyde oxidase and N-nitroso-reductase activities of the 30-45% ammonium sulfate precipitate was comparatively examined. The two activities were similarly susceptible to inhibition by all of these chemicals except allopurinol, an inhibitor of xanthine oxidase (Table III).

TABLE III. Effect of Various Chemicals on N-Nitrosoreductase and Aldehyde Oxidase Activities of the 30-45% Ammonium Sulfate Precipitate from Guinea Pig Liver Cytosol

Addition	Concentration	N-Nitrosoreductase activity* (Reduction product formed)	Aldehyde oxidase activity
	M	% of control	
None (control)	—	100	100
Menadione	5×10^{-5}	35	0
Potassium cyanide	1×10^{-3}	0	0
p-Chloromercuri-benzoic acid	1×10^{-3}	0	0
Amidol	2×10^{-4}	0	0
Sodium arsenite	1×10^{-4}	0	18
Chlorpromazine	2×10^{-4}	42	19
Dicumarol	1×10^{-4}	68	48
Antimycin A	1×10^{-4}	53	56
Tiron	2×10^{-4}	69	67
Allopurinol	1×10^{-3}	92	95

Each value represents mean of four experiments.

* In this experiment, 10 μmol of acetaldehyde was used as an electron donor and acetaldehyde diphenylhydrazone formed was determined.

TABLE IV. N-Nitrosoreductase Activity of Rabbit Liver Aldehyde Oxidase

Addition*	N-Nitrosoreductase activity** (Reduction product formed)
	nmol/30 min/mg protein
None	0
Acetaldehyde	37
Acetaldehyde,FAD	56

Each value represents mean of four experiments.

* Acetaldehyde 10 μmol.

** Acetaldehyde diphenylhydrazone formed was determined.

Next, the ammonium sulfate precipitate was chromatographed on a DEAE-cellulose (DE-52) column. After adsorption of the protein, elution was carried out with 10 mM phosphate buffer (pH 7.4), followed by a gradient of increasing NaCl concentration up to 0.2 M concentration. As a result, the elution peak of aldehyde oxidase free of xanthine oxidase was observed at the 0.125 M concentration of NaCl. This peak position was identical with that of N-nitrosoreductase.

Furthermore, purified rabbit liver aldehyde oxidase in the presence of acetaldehyde also exhibited a significant N-nitrosoreductase activity towards N-nitrosodiphenylamine as shown in Table IV.

From these results, we concluded that guinea pig and rabbit liver aldehyde oxidase can catalyze the reduction of N-nitrosodiphenylamine. Our preliminary study showed that cyclic nitrosamines such as N-nitrosomorpholine, N-nitrosopiperidine and N-nitrosopyrrolidine can also be reduced to the corresponding hydrazines by liver aldehyde oxidase supplemented with its electron donors.

EXPERIMENTAL

Male guinea pigs (350-400 g) were killed by decapitation and their livers were removed. The tissue was homogenized in 4 volumes of 1.15% KCl, the homogenate was centrifuged for 20 min at 9,000 x g, and the supernatant fraction was centrifuged for 60 min at 105,000 x g. The 105,000 x g supernatant (cytosol) was subjected to ammonium sulfate fractionation, and protein which were precipitated between 0 and 30%, 30 and 45%, and 45 and 60% ammonium sulfate saturation were collected. Rabbit liver aldehyde oxidase was prepared according to the purification method of Rajagopalan et al.[5]

In the assay of N-nitrosoreductase activity, a typical incubation mixture consisted of 0.5 μmol of N-nitrosodiphenylamine, 0.6 μmol of FAD, an electron donor and enzyme preparation in a final volume of 6 ml of 0.1 M K,Na-phosphate buffer (pH 7.4). When purified rabbit liver aldehyde oxidase was used, 7.5 mg of bovine serum albumin and 72 μg of catalase were added to the above mixture. Anaerobic incubation was performed using a Thunberg tube. The tube was gassed for 5 min with deoxygenated nitrogen, evacuated with an aspirator for 10 min and again gassed with nitrogen. The reaction was started by mixing the solution of the side arm and the body together, and continued for 30 min at 37°C. After incubation, the reduction product or the substrate was determined by HPLC as follows: HPLC was carried out by using a Toyo Soda HLC-803 HPLC apparatus equipped with a reversed phase LS-410K column. The mobile phase was 0.1 M KH_2PO_4-CH_3CN (1 : 1) and the flow rate was 1 ml/min. The retention time was 16 min for N-nitrosodiphenylamine, 13 min for 1,1-diphenylhydrazine or 34 min for acetaldehyde diphenylhydrazone. The aldehyde oxidase activity was assayed according to the method of Felsted et al.,[6] measuring the increase in optical density at 300 nm which accompanies the oxidation of N^1-methylnicotinamide to the 2- and 4-pyridones.

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