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FORMATION OF N-NITROSODIPHENYLAMINE FROM 1.1-DIPHENYLHYDRAZINE BY RAT LIVER MICROSOMAL PREPARATIONS

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SUMMARY. The present study provides the first evidence for in vitro metabolic conversion of a 1,1-disubstituted hydrazine to the corresponding nitrosamine. The study shows that superoxide radical which is generated by NADPH-cytochrome c reductase is involved in the oxidation of 1,1-diphenylhydrazine to N-nitrosodiphenylamine catalyzed by rat liver microsomes.

Hydrazine derivatives have been widely used in industry and medicine, and some of them have been shown to be carcinogenic and mutagenic (1). As regards their metabolism, Wittkop et al.(2) and Prough et al.(3-6) reported that methylhydrazines can be oxidized by liver microsomes or mixed-function amine oxidase to yield methane and formaldehyde, and that N-aminopiperidine can be converted to dipiperidyltetrazene by the flavoenzyme-catalyzed oxidation. Especially, 1,1-disubstituted hydrazines possess the structural features convertible to the corresponding nitrosamines. However, such metabolic reaction concerning hydrazine derivatives has not been known yet. The present communication describes that 1,1-diphenylhydrazine can be converted to N-nitrosodiphenylamine by rat liver microsomal preparations.

MATERIALS AND METHODS

Preparation of liver microsomes. Male Wistar-strain rats (200-250 g) were used. The liver was homogenized in 4 volumes of 1.15 % KCl, the homogenate was centrifuged for 20 min at 9,000 x g, and microsomes were sedimented from the supernatant by centrifu-

The abbreviation used are : MV, methyl viologen; SOD, superoxide dismutase.

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gation at 105,000 x g for 60 min. The microsomes were washed once with the KCl solution.

Preparation of NADPH-cytochrome c reductase. The enzyme was purified from rat liver microsomes by the method of Omura and Takesue (7).

Analytical procedure. High pressure liquid chromatography (hplc) was performed in a Toyo Soda HLC-803A chromatograph equipped with a UV-8 ultraviolet absorption detector. The instrument was fitted with a 30 cm x 4 mm (I.D.) LS-410K reversed phase column (Toyo Soda). The mobile phase was 0.1 M KH_2PO_4-CH_3CN (1:1). Mass spectrometry was carried out in a Shimadzu 7000 GCMS spectrometer. Ultraviolet (uv) spectra were determined with a Hitachi 320 spectrophotometer. Thin-layer chromatography (tlc) was conducted on silica gel plates (Kieselgel 60 F_{254}, Merck; 0.25 mm thick) developed in petroleum ether-benzene-28 % ammonia water (5:5:1).

Isolation of products. The incubation mixture consisted of 3 µmol of 1,1-diphenylhydrazine, an NADPH-generating system (12 µmol of NADP, 150 µmol of glucose-6-phosphate and 12 units of glucose-6-phosphate dehydrogenase), 3.6 µmol of FAD and liver microsomes (equivalent to 4.8 g of liver) in a final volume of 36 ml of 50 mM Tris-HCl buffer (pH 7.4). The incubation was carried out for 30 min at 37° in an open vessel. After incubation, the mixture was extracted three times with two volumes of ether, and the combined extract was evaporated to dryness in vacuo. When the residue was subjected to preparative tlc, two spots with Rf values of 0.54 and 0.46 were detected as products from the hydrazine. These products, which were designated here as products 1 and 2, respectively, were scraped from the plates and extracted with ether.

RESULTS

When 1,1-diphenylhydrazine was incubated with rat liver microsomes in the presence of NADPH, the formation of products 1 and 2 was observed in tlc (Rf value : 0.54 and 0.46 for products 1 and 2, respectively) and in hplc (Elution time : 16 and 19 min for products 1 and 2, respectively). The mass spectrum of product 1 showed a base peak at m/z 168, and the uv spectrum had an absorption maximum at 295 nm and a shoulder at 235 nm. The product was identified as N-nitrosodiphenylamine by comparing with the authentic sample its mass and uv spectra, and its behavior in tlc and hplc. The mass spectrum of product 2 gave a molecular ion at m/z 169, and the uv spectrum revealed an absorption maximum at 285 nm. These spectra, and the Rf value in tlc and the elution time in hplc of the product were all identical with those of the authentic sample of diphenylamine.

Table I shows the 1,1-diphenylhydrazine-oxidizing activity of rat liver microsomal preparations. Microsomes in the presence of NADPH exhibited the oxidizing activity which was stimulated by FAD or MV. The NADPH-linked activity was markedly inhibited by SOD, but not catalase (data not shown). On the other hand, NADH was effective to a much lesser extent, compared with NADPH. These results suggested that NADPH-cytochrome c reductase (8), which is known as one of superoxide-generating enzymes, is responsible for the oxidation of 1,1-diphenylhydrazine. Therefore, NADPH-cytochrome c reductase was purified from the rat liver microsomes and the ability of the enzyme to oxidize the hydrazine was examined.

Table I. Oxidation of 1,1-diphenylhydrazine to N-nitrosodiphenylamine by rat liver microsomes and NADPH-cytochrome c reductase

Addition	N-Nitrosodiphenylamine formed	
	Microsomes (nmol/30 min/mg protein)	NADPH-cytochrome c reductase (nmol/30 min/unit)
None	0	0
NADPH	3.4 ± 0.3	27 ± 10
NADH	0.3 ± 0.1	——
NADPH,FAD*	6.0 ± 0.2	67 ± 16
NADPH,MV*	13.2 ± 1.5	422 ± 25
NADH,FAD*	1.7 ± 0.1	——
NADPH,SOD**	0.7 ± 0.2	0
NADPH,FAD*,SOD**	0.9 ± 0.1	0
NADPH,MV*,SOD**	0.4 ± 0.1	0

Each value represents mean ± S.D. of four experiments.
The incubation mixture consisted of 0.5 μmol of 1,1-diphenylhydrazine, 2 μmol of NADPH or NADH, and microsomes (2 mg protein) or purified NADPH-cytochrome c reductase (0.1 unit) in a final volume of 2.5 ml of 50 mM Tris-HCl buffer (pH 7.4). The incubation was carried out for 30 min at 37° in an open vessel. The mixture, after adding 0.4 μmol of biphenyl as an internal standard, was extracted three times with two volumes of ether and the combined extract was evaporated to dryness in vacuo. The residue was subjected to hplc and N-nitrosodiphenylamine formed was determined from its peak area. *0.25 μmol, **50 units

As a result, the enzyme supplemented with NADPH, like the microsomes, exhibited the oxidizing activity which was stimulated by FAD or MV, whereas completely inhibited by SOD (Table I).

To confirm the involvement of superoxide radical in such hydrazine oxidation, we examined whether milk xanthine oxidase (9-12), which is another superoxide-generating enzyme, shows the 1,1-diphenylhydrazine-oxidizing activity. As shown in Table II, the enzyme supplemented with hypoxanthine, like NADPH-cytochrome c reductase described above, exhibited the oxidizing activity which was stimulated by MV, whereas inhibited by SOD.

From these facts, we concluded that superoxide radical which is generated by NADPH-cytochrome c reductase is involved in the oxidation of 1,1-diphenylhydrazine to N-nitrosodiphenylamine catalyzed by rat liver microsomes.

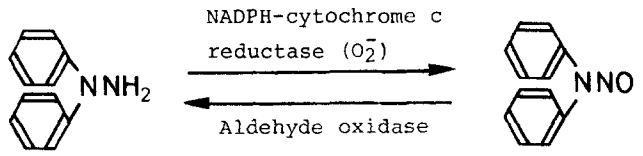
DISCUSSION

As described above, the present study provided the first evidence for in vitro metabolic conversion of a 1,1-disubstituted

Table II. Formation of N-nitrosodiphenylamine from 1,1-diphenylhydrazine in a milk xanthine oxidase-hypoxanthine system

System	N-Nitrosodiphenylamine formed (nmol/30 min/unit)
Complete	25 ± 3
plus MV*	40 ± 6
plus SOD**	0
minus hypoxanthine	0

Each value represents mean ± S.D. of four experiments. The incubation mixture consisted of 0.5 μmol of 1,1-diphenylhydrazine, 2 μmol of hypoxanthine and milk xanthine oxidase (1 unit) in a final volume of 2.5 ml of 50 mM Tris-HCl buffer (pH 7.4). The mixture was incubated for 30 min at 37° in an open vessel. The determination of N-nitrosodiphenylamine formed was performed as described in the legend of Table I.
*0.25 μmol, **50 units



1,1-Diphenylhydrazine N-Nitrosodiphenylamine

Fig. 1. Metabolic relationship between 1,1-diphenylhydrazine and N-nitrosodiphenylamine.

hydrazine to the corresponding nitrosamine. In a preliminary experiment, furthermore, we examined whether N-aminomorpholine can be enzymatically converted to the corresponding nitrosamine, using the gas chromatograph equipped with a flame thermonic detector. As a result, a significant formation of N-nitrosomorpholine, which was markedly inhibited by superoxide dismutase, was observed when the cyclic hydrazine, rat liver NADPH-cytochrome c reductase, NADPH and methyl viologen were incubated for 10 min at 37^{o} under aerobic conditions.

Thus, NADPH-cytochrome c reductase appears to play a prominent role in the oxidation of 1,1-disubstituted hydrazines to the corresponding nitrosamines catalyzed by liver microsomes. However, the present study does not exclude the possible involvement of cytochrome P-450 in such hydrazine oxidation, for the oxidation of 1,1-diphenylhydrazine catalyzed by liver microsomes could be slightly inhibited by carbon monooxide (data not shown).

Previously, we showed that nitrosamines such as N-nitrosodiphenylamine and N-nitrosomorpholine can be reduced to the corresponding hydrazine derivatives by liver aldehyde oxidase in the presence of its electron donors (13,14). These and the present results indicate the possible metabolic interconversion between hydrazine derivatives and the corresponding nitrosamines (Fig. 1).

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