INHIBITION OF MONOAMINE OXIDASE BY 3'-METHYL-4-DIMETHYLAMINO AZOBENZENE (3'-Me-DAB) IN RAT LIVER MITOCHONDRIA

Toru EGASHIRA, Toshinori YAMAMOTO and *Yukio KUROIWA

Department of Pharmacology, Medical College of Oita, 1-1506 Idaigaoka, Hazama-cho, Oita 879-56, Japan and *Department of Biochemical Toxicology, School of Pharmaceutical Sciences, Showa University, 1-5-8 Hatanodai, Shinagawa, Tokyo 142, Japan

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Abstract—Effects of 3'-Me-DAB on MAO in rat liver mitochondria, *in vitro*, were investigated. 3'-Me-DAB at a concentration of 1×10^{-5} M inhibits MAO activity about 40%, and this inhibition recovered to the control value after dialysis overnight against 0.001 M phosphate buffer. MAO activity was inhibited in an apparently competitive fashion by 3'-Me-DAB. These results indicate that 3'-Me-DAB binds to mitochondrial MAO with a weak affinity *in vitro*. The K_m value toward benzylamine was 220 μ M using both the mitochondria from the liver of rats fed a basal diet and those from rats ingesting 3'-Me-DAB. The activity of these enzyme preparations did not revert after dialysis to the control values of rats fed a basal diet. The titration experiment of MAO by pargyline suggests that the decrease of MAO activity, *in vitro*, is mainly due to the decrease of active MAO molecules in these mitochondrial preparations from livers of rats ingesting 3'-Me-DAB.

Aminoazo dye carcinogenesis has been reviewed by many authors (1, 2), and 3'-methyl-4-dimethylaminoazobenzene (3'-Me DAB) is known as the most potent carcinogenic aminoazo dye; it induces hepatoma within 3 months of feeding (3).

Recently, many workers have studied the protein binding of aminoazo dye after administration of a single large dose and reported that maximal binding is observed between 2 and 3 days after injection (4, 5). There is also a report that the protein binding of aminoazo dye increases rapidly with time after the initiation of feeding the dye; it reaches a maximal level at 2 and 3 weeks and gradually decreases (4). In a biochemical study of protein binding of aminoazo dye, Hultin (6) observed that dye binds, very

early after injection, more to microsomal proteins than to other cellular fractions. Terayama (7) also studied dye binding in rat liver microsomes and found that azo dye is bound to ribosomal proteins. These data and Gelboin's (8) indicate that azo dyes can also bind well to proteins in the mitochondrial membrane as well as those in the microsomes.

Furthermore, there are also many reports that the decrease of enzyme activity (9, 10) and the change of the patterns of isoenzymes in mitochondria and microsomes (11, 12) occur in proportion to the dose of the dye.

We have observed that the activity of monoamine oxidase (MAO) located in the outer mitochondrial membrane of rats is decreased in proportion to the period of 3'-

Me-DAB feeding (13). This paper describes further details of the mechanism of the inhibition of MAO activity in rat liver mitochondria of animals fed a diet containing 3'-Me-DAB.

MATERIALS AND METHODS

1. Preparation of mitochondria: Male Donryu rats weighing 80–100 g were fed the Oriental diet (Oriental yeast Co., Tokyo, Japan) containing 0.06% 3'-Me-DAB. Matched controls were fed the Oriental diet only. The rats were anesthetized with sodium pentobarbital given i.p., and the livers were quickly removed and homogenized in 10 volumes of 0.25 M sucrose pH 7.0 with a glass homogenizer equipped with a Teflon pestle. The mitochondrial fractions were prepared by the differential centrifugation method described earlier (14). The mitochondria were washed once by resuspension in the 0.25 M sucrose solution and used as the enzyme preparations. All operation were carried out at 4°C.

2. Estimation of protein concentration: The protein content of the enzyme preparation was measured by the method of Lowry et al. (15) with bovine serum albumin as the standard and always adjusted to 2 mg/ml.

3. Assay of monoamine oxidase: MAO activity was measured photometrically at 37°C using benzylamine as substrate by a slight modification of the method of Tabor et al. (16). For the assay, 1.5 ml of a mixture containing 0.3 M phosphate buffer, pH 7.5, 8 mM benzylamine and an appropriate concentration of enzyme preparation were incubated for 40 min at 37°C under aerobic conditions. The reaction was stopped by adding 0.2 ml of 60% perchloric acid. The reaction products were extracted with cyclohexane for 15 sec in a homomixer and centrifuged at 3,000 rpm for 15 min. As a control, the reaction mixture without substrate was incubated in the same way and

then perchloric acid followed by substrate was added and the mixture was centrifuged. The absorbance of the supernatants of the test and control mixtures at 242 nm were read and appearance of benzaldehyde was estimated by subtracting the control value from the test value. MAO activity was expressed as nmoles of benzaldehyde formed/min/mg of protein. The benzaldehyde production was linear between 0.5 to 2.0 mg protein with benzylamine as the substrate and also linear during the incubation time up to 60 min.

When the MAO inhibitor pargyline was used, the enzyme was preincubated for 20 min at 25°C with the inhibitor at the concentration indicated in the legend to the corresponding figure before addition of substrate.

4. Titration with inhibitor (14): Various amounts of enzyme were preincubated with pargyline at 37°C for 1 hr in a total volume of 1.35 ml of phosphate buffer, pH 7.5. As a control, the enzyme was preincubated in the same way, but in the absence of an inhibitor. After preincubation, 0.15 ml of substrate was added to estimate MAO activity. The inhibitor concentration used was 5×10^{-6} M pargyline.

5. Determination of Michaelis constants: Studies to determine the K_m values for benzylamine were performed utilizing control rat liver mitochondria and 3'-Me-DAB rat liver mitochondria as enzyme preparations. Six concentrations of substrate, each run in duplicate, were utilized for each determination of K_m . Kinetic constants were determined from graphic treatment of data as described by Lineweaver-Burk.

In investigating the *in vitro* effect of 3'-Me-DAB on MAO activity, the enzyme was preincubated for 20 min at 25°C with 3'-Me-DAB at the concentrations stated in the individual figures before the addition of the substrate.

Fig. 1. Changes of MAO activity in liver mitochondria of rats ingesting 3'-Me-DAB in the diet for several definite periods. MAO activity was assayed using benzylamine as substrate at 37°C for 40 min. The protein concentration of the each of the mitochondrial preparations was adjusted to 2 mg/ml. Each point represents the mean value obtained from triplicate experiments. The mean control value for MAO activity was 10.8 nmole/min/mg of protein.

RESULTS

1. The MAO activity in liver mitochondria of rats ingesting 3'-Me-DAB in the diet: The MAO activity in liver mitochondria of rats maintained on an Oriental diet containing 0.06% 3'-Me-DAB during a 9 week period was determined. As shown in Fig. 1, MAO activity decreased rapidly and was about 55% of the control value after the first week. There was some further decrease in succeeding weeks. The level of MAO activity levelled off at about 30% of the control from the 5th to 9th week.

2. Effect of 3'-Me-DAB on MAO activity, *in vitro*: To determine the mechanism of inhibition of MAO activity by 3'-Me-DAB the effect of various concentrations of 3'-Me-DAB on MAO in rat liver mitochondria, *in vitro*, was studied using benzylamine as substrate. 3'-Me-DAB at a concentration of 1×10^{-5} M inhibited MAO activity about 40%; the inhibition was proportional to the concentration of 3'-Me-DAB, but 3'-Me-DAB had no effect on MAO activity at concentrations below 1×10^{-8} M (Fig. 2).

3. Effects of 3'-Me-DAB on the K_m and

Fig. 2. Effects of 3'-Me-DAB on MAO activity in rat liver mitochondria. After incubation at 25°C for 20 min with the various concentrations of 3'-Me-DAB, MAO activity was determined with benzylamine as substrate at 37°C for 40 min. The mean control value for MAO activity in mitochondria was 11.3 nmole/min/mg of protein. The results are means of triplicate assays.

K_i values: The Michaelis constant was determined from Lineweaver-Burk double reciprocal plots of values obtained photo-metrically. The apparent K_m value for benzylamine was 220 μ M. As shown in

Fig. 3, values of $1/v$ vs $1/[S]$ gave straight lines in the presence and absence of 3'-Me-DAB and these lines crossed at a single point on the ordinate; therefore 3'-Me-DAB is a competitive inhibitor of benzylamine oxidation. The calculated apparent K_i value for 3'-Me-DAB with benzylamine as substrate was $0.35 \mu\text{M}$.

4. Effects of pargyline on MAO in liver of rats ingesting 3'-Me-DAB: Using the mitochondrial preparations from livers of rats ingesting 3'-Me-DAB, the inhibition of MAO activity by various concentration of pargyline was studied (Fig. 4). The protein content of all enzyme preparations were adjusted to 2 mg/ml. Using the mitochondria from the

Fig. 3. Inhibition by 3'-Me-DAB of benzylamine oxidation by rat liver mitochondrial MAO. Lineweaver-Burk plots of the reciprocal of the initial velocity of benzylamine oxidation against the reciprocal of the benzylamine concentration in the presence of 3'-Me-DAB. Abscissa: $1/\text{substrate concentration in mM}$. Ordinate: $1/\text{initial velocity in nmole/min/mg of protein}$. The results are means of triplicate assays. $\circ$ — $\circ$ 5×10^{-7} M 3'-Me-DAB, $\blacksquare$ — $\blacksquare$ 1×10^{-6} M 3'-Me-DAB, $\bullet$ — $\bullet$ no additional 3'-Me-DAB.

livers of rats fed a basal diet, a typical sigmoidal inhibition curve was obtained. The activity was inhibited about 70% with 1×10^{-6} M pargyline. In the mitochondrial preparations from livers of rats ingesting 3'-Me-DAB, the inhibition by pargyline increased proportionally to the period of the 3'-Me-DAB ingestion. MAO activity was inhibited almost completely with 1×10^{-6} M pargyline using the mitochondria from livers of rats ingesting 3'-Me-DAB for 5 weeks.

5. Molecular activity of mitochondrial MAO: The degree of the inhibition of the MAO activity by pargyline was further studied by means of the "titration" technique devised by Ackermann and Potter (17). Pargyline inhibits MAO irreversibly in a ratio of 1 : 1, i.e., the amount of enzyme inhibited is equal to the amount of pargyline added.

Fig. 4. Effects of pargyline on MAO in liver of rats ingesting 3'-Me-DAB. After incubation at 25°C for 20 min with the various concentrations of pargyline, MAO activity was determined with benzylamine as substrate at 37°C for 40 min. The protein concentration of each of the mitochondrial preparations was adjusted to 2 mg/ml. The results are means of triplicate assays. $\bullet$ — $\bullet$: mitochondria from livers of rats fed a basal diet, the control MAO activity was 10.8 nmole/min/mg of protein. $\circ$ — $\circ$: mitochondria from livers of rats ingesting 3'-Me-DAB for 2 weeks, the control MAO activity was 4.5 nmole/min/mg of protein. $\blacksquare$ — $\blacksquare$: mitochondria from livers of rats ingesting 3'-Me-DAB for 5 weeks, the control MAO activity was 3.7 nmole/min/mg of protein.

Fig. 5. Titration of MAO in rat liver mitochondria by pargyline with benzylamine as substrate. Various amounts of mitochondrial preparations were preincubated for 1 hour at 37°C in the presence of the pargyline. At the same time the same amounts of mitochondrial preparations were also preincubated in the absence of pargyline but otherwise in the same way. The MAO activity was then estimated. The protein concentration of each of the mitochondrial preparations was adjusted to 2 mg/ml. Pargyline concentration was 5×10^{-6} M in all cases. ●—●: without pargyline, ○—○ 5×10^{-6} M pargyline. Left: mitochondria from livers of rats fed a basal diet. Middle: mitochondria from livers of rats ingesting 3'-Me-DAB for 2 weeks. Right: mitochondria from livers of rats ingesting 3'-Me-DAB for 5 weeks. The results are means of triplicate assays.

As shown in Fig. 5, inhibitor which titrates the enzyme produces a line that intercepts the x-axis at a point to the right of the origin which corresponds to the amount of enzyme bound by the inhibitor. Figure 5 shows that 0.05 ml of untreated mitochondria was titrated by 7.5 nmoles of pargyline with benzylamine as substrate. However, using the enzyme preparations from livers of rats ingesting 3'-Me-DAB for 2 or 5 weeks, 7.5 nmoles of pargyline titrated 0.08 ml and 0.1 ml of mitochondria, respectively.

6. K_m and V_{max} values of the mitochondrial MAO in liver of rats ingesting 3'-Me-DAB: Values of the Michaelis constants and maximum rate of oxidation of benzylamine were determined from Lineweaver-Burk double reciprocal plots using the enzyme preparations from livers of rats ingesting 3'-Me-DAB for 2 and 5 weeks (Fig. 6).

The V_{max} was decreased in proportion to the time period of 3'-Me-DAB ingestion. However, the K_m values were almost identical, and they were 220 μ M for benzylamine with each of these mitochondrial preparations.

7. Reversibility of the effect of 3'-Me-DAB on MAO: A sample of the suspension of mitochondria was mixed with an equal volume of 1×10^{-5} M 3'-Me-DAB, and as a control, another sample of the suspension was mixed with an equal volume of 0.1 M phosphate buffer. Both mixtures were dialyzed overnight against 0.001 M phosphate buffer, pH 7.0, at 4°C, and their activities were compared at the end of this dialysis. As shown in Table 1, before dialysis, the activity with 3'-Me-DAB was 65.5% of the control value, while after dialysis, the activity was 97.0%.

Similarly, a sample of a suspension of

mitochondrial preparation from rats ingesting 3'-Me-DAB for 5 weeks was mixed with an equal volume of 0.1 M phosphate buffer and

dialyzed overnight against 0.001 M phosphate buffer, pH 7.0. Before dialysis, the activity was 22.8% of the control value, while after dialysis the activity was the same as the value before dialysis.

Fig. 6. K_m and V_{max} values of MAO in liver of rats ingesting 3'-Me-DAB. K_m and V_{max} values were determined from Lineweaver-Burk double reciprocal plots. Abscissa: $1/\text{substrate concentration in mM}$. Ordinate: $1/\text{initial velocity in nmole/min/mg of protein}$. The results are means of triplicate assays. ●—●: mitochondria from livers of rats fed a basal diet. ○—○: mitochondria from livers of rats ingesting 3'-Me-DAB for 2 weeks. ■—■: mitochondria from livers of rats ingesting 3'-Me-DAB for 5 weeks.

DISCUSSION

MAO activity decreased rapidly in the first week on feeding 0.06% 3'-Me-DAB. After that, there was some further decrease which levelled off at about 30%. Previously, Kitagawa et al. (18) reported the change in intracellular pH of rat liver during 3'-Me-DAB feeding and indicated the decrease in the intracellular pH of liver in the early stage of azo dye feeding plays a role in hepatocarcinogenesis. It is of interest that there is a similar pattern between the decrease of MAO activity and drop of the intracellular pH in liver.

This paper described detailed studies on the *in vitro* effect of 3'-Me-DAB on MAO in rat liver mitochondria. 3'-Me-DAB at a concentration of 1×10^{-5} M inhibited MAO activity about 40%, and the inhibition was proportional to the concentration of 3'-Me-DAB. As shown in Fig. 3, MAO activity was inhibited in an apparently competitive fashion by 3'-Me-DAB as determined by Lineweaver-Burk double reciprocal plots. The MAO activity inhibited by 3'-Me-DAB at a concentration of 1×10^{-5} M recovered to the control

Table 1. Reversibility of the effect of 3'-Me-DAB on MAO

	Before dialysis		After dialysis	
	MAO activity*	(%)	MAO activity*	(%)
Control	11.36	100	10.71	100
E+DAB	7.44	65.5	10.38	97.0
DAB-E	2.55	22.5	1.84	17.2

MAO activity was tested before and after dialysis at 37°C for 40 min. Conditions were as described in the text. Control: mitochondria from livers of rats fed a basal diet. E+DAB: control mitochondria+ 1×10^{-5} M 3'-Me-DAB. DAB-E: mitochondrial preparation from livers of rats ingesting 3'-Me-DAB for 5 weeks. *MAO activity was expressed as nmole of benzaldehyde formed/min/mg of protein.

value after dialysis overnight against 0.001 M phosphate buffer. These results show that 3'-Me-DAB binds to mitochondrial MAO *in vitro* with a weak affinity.

The effects of 3'-Me-DAB on MAO activity were studied with rat liver mitochondria while the animals were fed a diet containing 3'-Me-DAB. The K_m value calculated from Lineweaver-Burk plots was 220 μ M using the mitochondria from livers of rats fed a basal diet. However, the K_m value was 220 μ M and did not change using the mitochondrial preparations in livers of rats ingesting 3'-Me-DAB (see Fig. 6). However, the activity of these enzyme preparations did not revert after dialysis to the control values of rats fed a basal diet (see Table 1). From these results, the decrease of MAO activity in rat liver mitochondria by 3'-Me-DAB ingestion may be due to the inhibition of MAO enzyme synthesis or the destruction of the mitochondrial membrane by 3'-Me-DAB itself or metabolites of 3'-Me-DAB. However, it is also possible that N-hydroxylated derivatives of 3'-Me-DAB do not directly bind MAO in the mitochondria, but these bind the newly synthesized MAO since there is evidence that the binding of the dye occurs on the tyrosine, histidine or methionine residues of the protein (19, 20).

Furthermore, to clarify the cause of the decrease of MAO activity in rat liver mitochondria by 3'-Me-DAB ingestion, a titration experiment was performed with pargyline. Pargyline is an irreversible MAO inhibitor that seems likely to inhibit the enzyme by forming a covalently linked inhibitor-flavine adduct in a ratio of 1:1. Here the irreversible inhibition will give a curve parallel to that obtained for the uninhibited enzyme; the intercept on the abscissa shows the amount of enzyme corresponding to the amount of pargyline added (17, 21). The titration experiments of MAO by pargyline

suggest that the decrease of MAO activity is mainly due to the decrease of active MAO molecules in these mitochondrial preparations from livers of rats ingesting 3'-Me-DAB.

We are now using the metabolites of 3'-Me-DAB in further studies on the binding mechanism of the metabolites to the MAO enzyme.

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