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Intramitochondrial formation of oxidized glutathione during the oxidation of benzylamine by monoamine oxidase

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Oxidation of benzylamine by MAO results in an accumulation of GSSG in mouse liver mitochondria. Formation of GSSG is prevented by deprenyl, an inhibitor of MAO-B, but not by catalase. GSSG accumulation also occurs with dopamine as substrate. Oxidation of monoamines by mitochondrial MAO represents a potential oxidant stress for mitochondria.

Mitochondrion; Monoamine oxidase; Oxidative stress; GSSG; H_2O_2; Deprenyl

1. INTRODUCTION

Mitochondrial monoamine oxidase (MAO) is an outer membrane enzyme [1] that produces H_2O_2 which, in turn, oxidizes reduced glutathione (GSH) in a reaction catalyzed by GSH peroxidase (eqs. 1 and 2) [2,3]. The presence of both GSH and GSH peroxidase in mitochondria is well documented [4]. Mitochondrial GSH peroxidase may account for up to 26% of the liver enzyme [5].

$$\text{Monoamine} + O_2 + H_2O \rightarrow \text{Aldehyde} + H_2O_2 + NH_3 \quad (1)$$

$$2\,GSH + H_2O_2 \rightarrow GSSG + 2H_2O \quad (2)$$

Although the oxidation of monoamines by MAO-A and MAO-B has been well studied, very little attention has been given to the question of whether MAO activity influences the organelle where it is localized. Depending on its diffusion or site of generation within the outer membrane, H_2O_2 might oxidize GSH in the cytosol or in the interior of the mitochondrion. In this article, we report a substantial rise in oxidized glutathione (GSSG) upon incubation of mouse liver mitochondria with benzylamine, a well studied MAO-B substrate. In the presence of added catalase to scavenge accessible H_2O_2, the formation of GSSG was only slightly affected. Therefore, a significant amount of H_2O_2 penetrates through the inner membrane. These observations may be particularly relevant to the central nervous system, where the turnover of neurotransmitter monoamines by MAO could lead to an oxidative stress within the mitochondrion.

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2. MATERIALS AND METHODS

Reagents were purchased from Fisher or Sigma and were the highest available grade. Male Swiss-Webster mice (25–30 g) were from Charles River.

Mouse liver mitochondria were prepared at 4°C essentially as described by Clark and Nicklas [6]. The buffer consisted of 0.225 M mannitol, 0.075 M sucrose, 5 mM (*N*-morpholino)propanesulfonic acid (MOPS), and 1 mM EGTA, adjusted to pH 7.4 with NaOH. In brief, livers from 2–3 decapitated mice were minced and rinsed in cold buffer, and then homogenized in a Potter-Elvehjem teflon and glass homogenizer at 750 rpm with 5–6 up-and-down strokes in 10 volumes of buffer. Homogenates were centrifuged in polypropylene tubes in a Sorvall RC 2B centrifuge (SS34 rotor) at 580 × *g* for 10 min. The supernatant was carefully removed and recentrifuged at 8000 × *g* for 10 min. The second supernatant was discarded and the fluffy layer was removed by gentle shaking with the last portion of remaining buffer. The pellet was then dislodged into fresh cold buffer with care to avoid suspending the occasional red spot, consisting of traces of erythrocytes at the bottom of the tube. After gentle homogenization by hand in a total of about 35 ml of buffer in a wide-tolerance smooth glass homogenizer, the sample was centrifuged at 9200 × *g* for 10 min. This step was repeated once. No GSH or GSSG was detected in the supernatant from the last centrifugation.

Mitochondria were suspended in 7 ml of buffer and aliquots (300 μl) were incubated in triplicate for 5 min with gentle shaking at 25°C with 100 μM benzylamine, with and without catalase (300–350 U; 6–7 μg), in a final volume of 500 μl. The reaction was quenched by addition of 50 μl of 4 M perchloric acid and samples were pelleted in a microcentrifuge at 15 000 × *g* for 15–20 min at 4°C. The supernatants were assayed for GSSG. In some experiments, samples were layered over a dibutylphthalate oil layer [7] on top of 4 N perchloric acid and centrifuged for 2 min at 13 000 × *g* to obtain a mitochondria-free supernatant.

GSH was removed with *N*-ethylmaleimide and the samples were analyzed for GSSG by a modification [3] of the enzymatic recycling assay of Tietze [8]. Measurements made with and without sonication of the acidified samples showed that addition of perchloric acid by itself released all of the mitochondrial glutathione. Protein was deter-

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mined according to Lowry et al. [9] with bovine serum albumin as standard.

3. RESULTS

Incubation of mitochondria with benzylamine resulted in a substantial rise in GSSG (Table I). GSSG was not detected in the incubation medium when the mitochondria were removed just prior to assay by centrifugation through a dibutylphthalate oil layer. Formation of GSSG was not prevented by addition of catalase to the medium (Table I). The mean inhibition of the GSSG rise by catalase was only 15.5 ± 7.9% (SEM, $P<0.05$, paired t-test, $n=6$ experiments). In two additional experiments, the amount of added catalase was increased 5-fold without significant change in effect.

Benzylamine is a preferred MAO-B substrate. Table II shows results of two experiments with the selective MAO-B inhibitor, deprenyl, and the selective MAO-A inhibitor, chlorgyline. Deprenyl fully prevented the rise in GSSG, whereas clorgyline did not. In separate experiments, inhibition of MAO-A by clorgyline was verified by HPLC assay of the oxidation of the MAO-A substrate, serotonin (not shown). Therefore, the rise in mitochondrial GSSG during incubation with benzylamine can be ascribed to the H_2O_2 generated by MAO-B.

Additional experiments were carried out with dopamine (100 μM) as substrate. Incubation as in Table I in the presence of catalase (10 μg/ml) resulted in a rise of 21.2 ± 8.2 ng GSSG/mg protein compared to corresponding controls ($P<0.02$, paired t-test, $n=4$ experiments in triplicate).

4. DISCUSSION

Incubation of mouse liver mitochondria with benzylamine (an MAO-B substrate) gives rise to markedly increased levels of GSSG. The GSSG is restricted to the mitochondria and is not found in the suspending medium. The latter results are in accord with observations of Olafsdottir and Reed [7] that GSSG generated on incubation of mitochondria with t-butylhydroperoxide is not exported. The profound inhibitory effect of deprenyl, a selective MAO-B inhibitor, is in keeping with the enzyme isoform preference of benzylamine. Clorgyline, a selective inhibitor of MAO-A, was not effective in preventing the intramitochondrial accumulation of GSSG (Table II).

Table I

Effect of benzylamine, with and without catalase, on GSSG levels

	GSSG (ng/mg protein)	
	Without catalase	With catalase
Control	16.9 ± 3.2	17.1 ± 3.3
Benzylamine	48.9 ± 6.3[a]	42.0 ± 5.3[a]

Mitochondria were incubated with and without benzylamine (100 μM) and catalase (10–12 μg/ml) for 5 min at 25°C. Results are the mean and SEM from 6 experiments, each performed in triplicate.
[a]$P<0.005$ compared to the corresponding control (paired t-test).

Table II

Effect of deprenyl and clorgyline on the rise in GSSG evoked by benzylamine.

	GSSG (ng/mg protein)	
	Experiment 1	Experiment 2
Control	21.5 ± 3.4	14.3 ± 2.8
Benzylamine	40.2 ± 3.4[a]	43.5 ± 3.4[a]
Clorgyline + benzylamine	39.6 ± 3.2	36.9 ± 1.9
Deprenyl + benzylamine	16.0 ± 4.2[b]	5.5 ± 3.2[b]

Mitochondria were added to tubes containing 20 μM MAO inhibitor and 100 μM benzylamine (final concentrations) and incubated as in Table I. Results are the mean and SEM of triplicates.
[a]$P<0.01$ compared to control; [b]$P<0.01$ compared to benzylamine alone

Since liver mitochondria are contaminated with peroxisomes, the inability of added catalase to prevent GSSG accumulation does not mean that all of the generated H_2O_2 was coupled to the oxidation of GSH within the mitochondria. It is likely that peroxisomal catalase acted as a sink for H_2O_2 in the incubation medium. Nonetheless, it is clear that MAO activity represents a source of an oxidant stress for mitochondria in vitro, and that the generated H_2O_2 cannot be removed by added catalase. The inaccessible nature of the H_2O_2 raises the possibility that the active site of MAO-B responsible for mitochondrial GSSG accumulation may face the inner mitochondrial membrane. In intact perfused liver, a portion of the GSSG generated by benzylamine metabolism effluxes from the organ [2,10], indicating that some of the generated H_2O_2 is scavenged by GSH peroxidase in the cytosol and, perhaps, that mitochondrial GSSG reaches the cytosol in the living state.

We also observed elevated GSSG after incubation of liver mitochondria with dopamine. In brain, dopamine exists in high local concentration at dopaminergic synapses and serves as a natural substrate for MAO-A and MAO-B in neurons and glia. Elevations in GSSG occur in vivo in mouse striatum when dopamine turnover by MAO-A or MAO-B is increased by injection of reserpine or haloperidol [11,12]. It seems likely that both mitochondrial and cytosolic GSSG are increased under these circumstances. A recent report has described the loss of GSH during incubation of brain mitochondria with benzylamine and other MAO substrates [13].

The current study shows that MAO activity evokes a rise in GSSG in isolated mitochondria in vitro. Therefore, increased MAO activity may serve as a source of an oxidant stress for mitochondria in vivo.

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