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Cent. Eur. J. Biol. • 4(3) • 2009 • 321–326
DOI: 10.2478/s11535-009-0016-2

Central European Journal of **Biology**

Brain monoamine oxidase A in hyperammonemia is regulated by NMDA receptors

Communication

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Received 21 January 2009; Accepted 24 March 2009

Abstract: Mitochondrial enzyme monoamine oxidase A (MAO-A) generates hydrogen peroxide (H_2O_2) and is up-regulated by Ca^{2+} and presumably by ammonia. We hypothesized that MAO-A may be under the control of NMDA receptors in hyperammonemia. In this work, the *in vivo* effects of single dosing with ammonia and NMDA receptor antagonist MK-801 and the *in vitro* effect of Ca^{2+} on MAO-A activity in isolated rat brain mitochondria were studied employing enzymatic procedure. Intraperitoneal injection of rats with ammonia led to an increase in MAO-A activity in mitochondria indicating excessive H_2O_2 generation. Calcium added to isolated mitochondria stimulated MAO-A activity by as much as 84%. MK-801 prevented the *in vivo* effect of ammonia, implying that MAO-A activation in hyperammonemia is mediated by NMDA receptors. These data support the conclusion that brain mitochondrial MAO-A is regulated by the function of NMDA receptors. The enzyme can contribute to the oxidative stress associated with hyperammonemic conditions such as encephalopathy and Alzheimer's disease. The attenuation of the oxidative stress highlights MAO-A inactivation and NMDA receptor antagonists as sources of novel avenues in the treatment of mental disorders.

Keywords: *Ammonia • Hyperammonemia • Monoamine oxidase • NMDA receptor • Brain mitochondria • Hydrogen peroxide*

1. Introduction

Monoamine oxidase (MAO, EC 1.4.3.4), the enzyme located on the cytoplasmic side of the outer mitochondrial membrane, catalyzes the oxidative deamination of biogenic and xenobiotic amines. The end products of MAO-mediated reactions include several chemical species with neurotoxic potential, such as hydrogen peroxide (H_2O_2), ammonia and aldehydes. The enzyme has important functions in the metabolism of monoamine neurotransmitters in the central nervous system. In the brain, there are two sub-types, MAO-A mainly in neurons [1] and MAO-B in glial cells [2]. MAO-A preferentially oxidizes serotonine, norepinephrine and epinephrine, whereas MAO-B preferentially degrades benzylamine and phenylethylamine. Abnormal production of H_2O_2 by brain MAO isozymes can be critical for pathogenesis of Parkinson's and Alzheimer's diseases and other neurodegenerative disorders.

Selective increase in brain MAO-A activity in humans with hepatic encephalopathy [3] correlates with activation of glutamate NMDA receptors linked to Ca^{2+}-dependent processes and pathology in encephalopathic brains [4]. Excitotoxicity is regarded as a potential mechanism of cell death in acute and chronic neurological diseases as well as under conditions associated with hyperammonemia. It has been shown that acute intoxication with large doses of ammonia result in activation of NMDA receptors in rat brain [5,6]. The mechanisms by which overactivation of NMDA receptors lead to neuronal degeneration and death involve the increase in Ca^{2+} concentration in the postsynaptic neuron [7]. The excessive activation of such receptors by glutamate, agonists, or ammonia results in the active inflow of calcium ions to neurons with simultaneous stimulation of many calcium-dependent

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proteases, massive increases in mitochondrial Ca^{2+} accumulation, impaired function of mitochondria, and generation of active oxygen species [8-11]. Ammonia is a product of metabolic degradation of proteins and other nitrogen-containing compounds. However, excessive ammonia influx or production resulting from the malfunctioning enzyme utilization system can cause hyperammonemia, an abnormal increase in blood ammonia level. When introduced into animals, ammonia increases the calcium content in brain mitochondria [12], causes oxidative stress [13,14] and death [6]. Blocking NMDA receptors prevents ammonia-induced oxidative stress [14].

These combined observations certainly argue for examination of the relation between ammonia, NMDA receptors and MAO. Our previous work has produced promising results such as the prevention of ammonia-induced activation of brain MAO A+B with MK-801, an NMDA receptor antagonist [15]. Here, we hypothesize that the NMDA receptor in the brain is associated with regulating the MAO-A activity.

In this study, we examined the *in vivo* effects of ammonium acetate and MK-801, as well as the *in vitro* effect of Ca^{2+} on the activity of MAO-A in the nonsynaptic mitochondria isolated from rat brain.

2. Experimental Procedures

2.1 Animals and treatments

Groups of 3-4-month-old outbred male Wistar rats weighing 250–300 g were used. One group (ammonia) was injected intraperitoneally (i.p.) with ammonium acetate (12 mmol/kg) and killed by decapitation 11 min later. Another group (MK-801) was injected i.p. with MK-801 (1.5 mg/kg) and killed 26 min later. A third group (ammonia + MK-801) was injected with MK-801, 15 min later with ammonium acetate and was killed 11 min after ammonia injection. This procedure led to the increase in brain ammonia level from 0.36 to 4.8 μmol/g tissue [15]. The control group was decapitated 11 min after an injection with saline.

2.2 Materials

ADP, EDTA, EGTA, tyramine, 5-hydroxytryptamine, β-phenylethylamine were bought from Sigma - Aldrich Co. (USA). Sucrose, Tris - buffer, Hepes - buffer, MOPS - buffer and bovine serum albumin were purchased from Serva (Germany), and Ficoll 400 from Pharmacia (Sweden). All other reagents were purchased commercially.

2.3 Preparation of brain mitochondria

The cranium was opened and the forebrain was isolated as soon as possible (usually within 20 s after decapitation) and placed into the cool medium containing 0.25 M sucrose, 1 mM K-EDTA, and 10 mM Tris-HCl, pH 7.4.

All further isolation procedures were performed at 2–4°C. The tissue was fragmented, washed with the medium, and homogenized in a Dounce homogenizer provided with a teflon pestle with a total clearance of 0.1 mm.

The nonsynaptic mitochondria were isolated as described [16]. Usually six up and down strokes of the pestle were performed. The homogenate was then diluted with isolation medium to a final volume of 15 ml and homogenized again with four up and down strokes. The homogenate was centrifuged at 2000 *g* for 3 min; the supernatant was centrifuged again at 2000 *g* for 3 min and the second supernatant was centrifuged at 12000 *g* for 8 min to obtain the crude mitochondrial pellet. The pellet was suspended in 1.5 ml of the 3% Ficoll medium (see below) and layered onto 6.25 ml of the 6% Ficoll medium and centrifuged at 12000 *g* for 30 min. The 6% Ficoll medium contained 6% (w/w) Ficoll, 0.24 M mannitol, 0.06 M sucrose, 0.05 mM K-EDTA and 10 mM Tris–HCl, pH 7.4. The 3% Ficoll medium was the 6% Ficoll medium diluted 1:1 with glass bi-distilled water. The loose, fluffy, white top layer of the pellet was removed. The resulting brown pellet was suspended in isolation medium without EGTA and centrifuged at 12000 *g* for 10 min. The pellet was suspended in the same medium to obtain a protein concentration in a range of 20–25 mg/ ml. The mitochondrial preparation was placed into a special quartz vial tightly mounted in a hole in the lid of a plastic vessel, the latter being filled with fine ice; the vessel was inserted into a plastic foam container. During the experiment, this container with mitochondria was stored in a refrigerator. Time required for the whole isolating procedure of mitochondria was about 70 min.

A quantitative estimate of the purity of the mitochondrial fraction was obtained using marker enzymes. The mitochondria were identified by succinate dehydrogenase (EC 1.3.99.1). The degree of contamination of mitochondria by cytosol was estimated by measuring lactate dehydrogenase activity (EC 1.1.1.27). Acetylcholinesterase (EC 3.1.1.7) was used as a marker for synaptic and other membraneous material; glucose-6-phosphatase was the marker for microsomes; urate oxidase (EC 1.7.3.3) for peroxisomes. The low recovery (less than 1% of the homogenate activity) of lactate dehydrogenase, acetylcholinesterase, urate oxidase and glucose-6-phosphatase, suggest that the mitochondrial fraction was highly pure.

	Control	Ammonia	MK-801	Ammonia + MK-801
	Enzyme activity, pkat/mg mitochondrial protein			
MAO-A	5.31±0.65 (5)	10.51±0.37* (5)	5.62±0.48 (4)	5.27±0.33 (4)
MAO-B	14.06±0.35 (8)	14.31±0.43 (5)	13.84±0.73 (4)	14.35±0.35 (4)
MAO A+B	16.45±0.48 (8)	25.72±0.45 (5)	17.00±0.29 (4)	17.37±0.67 (4)

Table 1. Activities of monoamine oxidase (MAO) isozymes in brain mitochondria from control rats and rats injected i.p. with ammonia and/or MK-801. Data are presented as the mean ± SEM (number of rats indicated in parentheses) and are given as pkat/mg mitochondrial protein. P values were determined by ANOVA tests for Ammonia, MK-801, and Ammonia + MK-801 groups *versus* control. * P<0.0001.

Intactness of mitochondria was tested by measuring the parameters of oxidative phosphorylation. The mitochondrial preparation was metabolically active and well coupled. With pyruvate plus malate as respiratory substrates, the rates of phosphorylating, controlled and uncoupled respiration were 90±6, 22±4 and 180±10 ng-atoms O/min/mg of mitochondrial protein, and the respiratory control index and ADP/O ratio of 4.1±0.9 and 2.8±0.1, respectively.

2.4 Determination of enzyme activity

MAO activity in intact mitochondria was assayed by the measurement of the rate of H_2O_2 production [15]. 0.5 mM 5-hydroxytryptamine, 0.2 mM β-phenylethylamine, or 0.5 mM tyramine were used as substrates of MAO-A, MAO-B and MAO-A+B isoenzymes, respectively. The reaction was monitored spectrophotometrically (Uvikon 923 spectrophotometer, Kontron Instruments, South Korea) at 240 nm and 25°C.

2.5 Other methods and conditions

The experimental procedures have been approved by the institutional committee and meet the guidelines of the European Union for treatment and use of experimental animals. The terms ammonia and ammonium acetate were used interchangeably. Mitochondrial protein was determined by the Lowry *et al.* [17] method with bovine serum albumin as a standard. Results were expressed as mean ± SEM. Differences among groups were analysed by one-way ANOVA followed by Student's *t*-test to determine statistical significance using the Prizm 4.0 software. Comparison between two experimental groups was based on the two-tailed *t*-test.

3. Results

3.1 Ammonia increases brain MAO-A but not MAO-B activity *in vivo*

Table 1 shows the activities of MAO-A, MAO-B and MAO A+B in brain nonsynaptic mitochondria from control rats and the animals injected i.p. with ammonia and/or MK-801.

The activity of MAO-A increased on an average by 98% after ammonium acetate injection. Blocking NMDA receptors with MK-801 completely prevented the ammonia-induced increase in MAO-A activity, while MK-801 alone did not affect the enzyme. Activity of MAO-B remained unchanged in animals treated with either ammonia, MK-801, or ammonia plus MK-801. MAO A+B activity was changed in parallel with MAO-A activity. Special experiments *in vitro* showed that 1-5 mM ammonium acetate affected neither MAO-A nor MAO-B activities in control mitochondria. Changes in MAO-A activity are novel and those observed here fall exactly within those published previously for MAO-B and MAO A+B activities [15].

3.2 Calcium increases brain mitochondrial MAO-A activity *in vitro*

The toxic effect of ammonia on the brain is mediated by activation of NMDA receptors [5,6]. This receptor has a calcium channel that is permeable only for extracellular Ca^{2+} and K^+ and for intracellular Na^+. Extracellular glutamate, N-methyl-D-aspartate, other agonists, and ammonia excite this receptor and open the calcium channel. We assumed that the ammonia-induced increase in mitochondrial MAO-A activity might be due to penetration of Ca^{2+} into the neuron through the ionic channel of the NMDA receptor. To examine this hypothesis, we previously assayed the activity of MAO-A in nonsynaptic brain mitochondria of the control rats *in vitro* in the presence of various Ca^{2+} concentrations [18]. MAO-A activity has been shown to increase by 75-80% in the presence of Ca^{2+}, and optimal Ca^{2+} concentration was of approximately 1.3 mM. Cao *et al.* [19] have reported other data about the interaction between Ca^{2+} and MAO-A. In the mouse hippocampal HT-22 cell line incubated with Ca^{2+}, MAO-A activity increased by maximum 20%, with a peak around 0.5–1 mM Ca^{2+}. Here, we re-examined our preliminary results.

A typical dependence of MAO-A activity on calcium concentration is shown in Figure 1. MAO-A activity appeared to be unchanged at < 0.1 mM Ca^{2+}, increased by 12% at 0.2 mM Ca^{2+} and a maximal stimulation was

84% at 1.0-1.2 mM Ca^{2+}. In four other experiments, a maximal stimulation was 65-82%. No further activation of MAO-A was observed at higher calcium concentrations. At 2 mM Ca^{2+} the enzyme activity decreased, but was still higher than that before calcium addition. It has been previously shown that clorgyline, a specific MAO-A inhibitor, eliminates calcium-induced activation of MAO A+B, and pargyline, a specific MAO-B inhibitor, decreases the activity of MAO A+B without changing the pattern of the relationship between the enzyme activity and calcium concentration [18], confirming indirectly that Ca^{2+} affects only MAO-A activity. *In vitro*, 0.1 mM MK-801 did not affect the activity of MAO-A in isolated brain mitochondria (not shown).

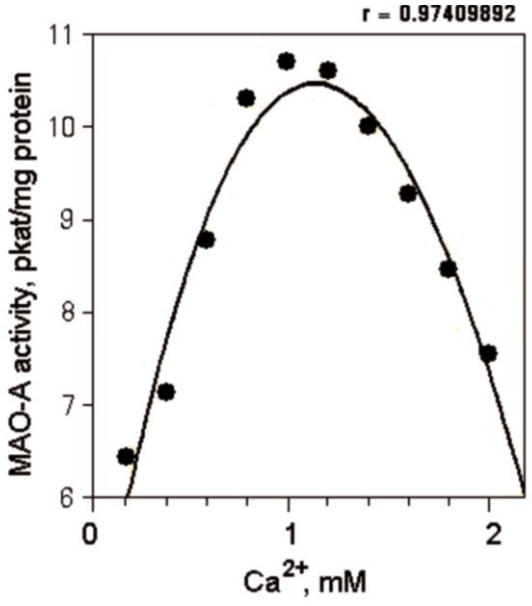

Figure 1. Effect of Ca^{2+} on MAO-A activity in rat brain non-synaptic mitochondria. Mitochondria (0.5 mg protein/ml) were incubated with increasing concentrations of Ca^{2+} indicated on the abscissa and assayed for MAO-A activity. Experimental data (points) represent four independent experiments. Solid line is the 3rd degree polynomial fit graph plotted using CurveExpert 1.3 software. Correlation coefficient is r = 0.97409892 varying from 0.95 to 0.99 in other experiments.

4. Discussion

Acute ammonia intoxication results in oxidative stress as evidenced by acceleration of oxygen radical and H_2O_2 production and by reduction of the activities of antioxidant enzymes in brain nonsynaptic mitochondria [13,15]. The major site of production of reactive oxygen species is considered to be the mitochondrial respiratory chain, including inner membrane-bound complex I, complex III, and complex IV, as well as a matrix enzyme 2-oxoglutarate dehydrogenase [20]. Intracellular H_2O_2 may be also generated by MAO on the outer mitochondrial membrane. However, this source of H_2O_2 has not been clarified in isolated brain mitochondria following injection of rats with large ammonia doses. In this work, we repeated, in part, and further developed our earlier studies [15,18] measuring the activity of MAO-A directly.

There are no published data on the effect of ammonia injection on MAO-A activity in the isolated brain mitochondria. Previously we observed an increase in MAO A+B activity in brain non-synaptic mitochondria following injection of rats with ammonia [15,18]. Because the activity of MAO-B remained unchanged, we concluded that MAO-A activity increased selectively in hyperammonemia. In the current study, we have shown the first direct evidence that MAO-A activity in isolated brain mitochondria increases in hyperammonemia.

Data similar to ours, but obtained using different hyperammonemia models and different tissues are available. For instance, a 50% increase in MAO-A activity was observed in the brain tissue of rats with chronic hyperammonemia caused by portacaval shunting, while MAO-B activity did not change [21]. Similar findings were obtained in autopsied brain frontocortical (50% increase in MAO-A activity) and cerebellar (increase by 145%) tissues from cirrhotic patients with hepatic encephalopathy [3] and reverse transcriptase-polymerase chain reaction indicated an increase in the level of *MAO-A* gene expression (by 155%). A normal level of MAO-A activity was reported in the brain cortex of mice with congenital hyperammonemia [22], while the activity of MAO-A in the various brain sections was decreased in acute hyperammonemia induced by ammonium chloride injection (4 mmol/kg) [23]. Some disagreements between these and our data may be due to differences in experimental conditions: in the animal species (rat, mouse, or man), model of hyperammonemia (acute, chronic congenital, chronic after portacaval shunt or chronic in cirrhosis), toxic agent (ammonium acetate or ammonium chloride), its dose (12 or 4 mmol/kg), test tissue (brain tissue, brain sections, or forebrain nonsynaptic mitochondria).

H_2O_2 as well as ammonia are products of the MAO reaction. Although it is hard to imagine that ammonia can activate the enzyme, this was really the case. Intraperitoneal injection of rats with ammonia caused great elevation of brain ammonia level [15] and an increase in MAO-A activity in isolated brain non-synaptic mitochondria (Table 1). Ammonia appeared to increase the direct enzyme reaction as evidenced by increase in the H_2O_2 production velocity. Thus, this brain

isoenzyme can contribute to excess H_2O_2 production in acute and chronic ammonia intoxication *via* an allosteric mechanism and by the regulation of MAO-A gene expression, respectively.

Very little published data are available on the interaction between Ca^{2+} and MAO in nonsynaptic brain mitochondria. Previously, we studied the *in vitro* effect of Ca^{2+} on MAO activity in brain mitochondria and found that Ca^{2+} induced the elevation of activity of MAO-A but had no effect on MAO-B [18]. This supports the hypothesis that the NMDA receptor is involved in activation of MAO-A *in vivo*. However, optimal concentrations (1.3 mM) and maximal effects (75-80%) of Ca^{2+} differed from those observed with other objects. For example, in the mouse hippocampal HT-22 cell line these figures were 0.5-1 mM and 20%, respectively [19]. Egashira *et al.* [24], using $CaNa_2EDTA$ as Ca^{2+} source in *in vitro* experiments, found that MAO-A activity was enhanced by about 40% with 0.1 μM $CaNa_2EDTA$ in monkey brain mitochondria, but unaltered in rat brain mitochondria. Therefore, we re-examined and confirmed our previous results (Figure 1). The kinetics of MAO-A activity in the HT-22 cell line in the presence of Ca^{2+} revealed a decrease in K_m, indicating that Ca^{2+} facilitates the enzymatic reaction. V_{max} remained unchanged, suggesting that Ca^{2+} was acting *via* an allosteric mechanism [19].

In summary, we have shown that ammonia administration *in vivo* leads to the increase in brain mitochondrial MAO-A activity indicating excessive H_2O_2 generation. MK-801 prevents ammonia-induced changes of enzyme activity suggesting that they are mediated by activation of NMDA receptors. *In vitro*, calcium stimulates MAO-A activity in brain nonsynaptic mitochondria with maximum effect at 1.0-1.2 mM. These data suggest that induction of MAO-A activity *in vivo* by both ammonia and calcium is regulated by NMDA receptors.

Acknowledgements

This work was not supported by any funds but author's salaries. The authors declare that there are no competing interests.

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