Research Article

In vitro analysis of the activity of human monoamine oxidase type B (hMAOB), treated with the cyanotoxin anatoxin-a: supposed factor of neurodegenerative diseases

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Abstract

In this study, we investigated the hypothesis that an additional source of free radicals may be hydrogen peroxide formed by mono-amino oxidase (MAO) -catalyzed deamination of catecholamines. Also, increased MAO-B activity in the brain has been linked to the development of some neurodegenerative diseases.

The toxicant we used to treat recombinant human MAO-B enzyme is the cyanotoxin anatoxin-a. For anatoxin-a is known that it's an agonist of neuronal acetylcholine receptors with 20 times greater affinity to them compared to the natural neurotransmitter.

In this study, we analyzed the effect of anatoxin at various selected concentrations on the activity of recombinant human MAO-B enzyme. The method we use is to analyze the activity of human MAO-B with the fluorimetric reagent Amplex UltraRed and the substrate tyramine hydrochloride.

The aim of this study is to analyze the effect of anatoxin-a on the hMAO-B enzyme activity and its influence as a factor for the development and progression of neurodegenerative diseases.

Keywords

Introduction

The monoamine oxidase enzyme is known in two isoforms, and inhibitors of their activity are drugs used for psychiatric illness. MAO-B inhibitors are medicines for the treatment of Parkinson's disease (PD), for example, Selegiline. The induction of enzyme activity, free radicals are thought to generate a number of diseases. Significant MAO activity was observed in the brain, where enzymes were detected by specific monoclonal antibodies from Westlund and colleagues in 1985. Although the two isoforms MAO-A and MAO-B are present in the brain, they have different distribution (Westlund et al. 1985), accompanied by higher MAO-B expression measured in indivi-

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dual sections of the human brain at autopsy (Kalaria et al. 1988). In human primates, again MAO-B is the dominant isoform in the brain (Willoughby et al. 1988; Riachi and Harik 1992). In humans, age-related increases in MAO-B expression are observed, as well as in the progression of neurodegenerative diseases (Flower et al. 2002). Immunohistochemical studies have shown that MAO-B is mainly observed in glial cells and serotonergic neurons (Levitt et al. 1982; Westlund et al. 1985; Petzer et al. 2009).

Because of their role in the catabolism of monoamine neurotransmitters in the CNS and peripheral tissues, MAO-A and MAO-B are of considerable pharmacological interest (Youdim et al. 2006). MAO-A preferentially catalyzes the oxidative deamination of serotonin and is irreversibly inhibited by low concentrations of clorgyline. MAO-B preferentially catalyzes the deamination of counterfeit neurotransmitters benzylamine and β-phenylethylamine and is irreversibly inhibited by low concentrations of (R)-deprenyl (Waldmeier 1987; Youdim et al. 2006). Both isoforms catalyze the oxidation of dopamine, epinephrine and norepinephrine (Youdim et al. 2006; Youdim and Bakhle 2006).

A study by Schedin-Weiss et al. (2017) proves the link between increased MAO-B activity and Alzheimer's disease (AD). The study also suggests that MAO-B regulates the pathogenic amyloid β-peptide (Aβ), in neurons by γ-secretase and thus providing a key to understanding the link between the pathogenesis of MAO-B and AD.

MAO-B inhibitors can exert neuroprotective effects by stoichiometrically reducing the products of the reaction – aldehyde and hydrogen peroxide catalyzed by the MAO-B enzyme in the brain (Petzer et al. 2009). On the other hand, the MAO-B enzyme may be associated with **neurodegenerative processes upon exposure to xenobiotic amines** (Petzer et al. 2009).

Anatoxin-a is a secondary amine, in structure similar to the neurotransmitter acetylcholine. Anatoxin-a, also known as very rapid death factor (VFDF), is a secondary, bicyclic amino alkaloid and cyanotoxin with acute neurotoxicity. Symptoms of anatoxin exposure include loss of coordination, muscle fasciculations, convulsions, and death by respiratory paralysis. Its mode of action is through the nicotinic acetylcholine receptor (nAchR), where it mimics the binding of the receptor's natural ligand, acetylcholine. As such, anatoxin-a has been used for medical purposes for the study of diseases characterized by low levels of acetylcholine, such as Parkinson's disease.

Despite its poisonous nature, however, anatoxin and many related man-made analogues have found widespread use in medicine and for pharmacological applications. Since it binds the nicotinic acetylcholine receptor irreversibly, it is an excellent means of studying this receptor, and also the mechanisms of neuromuscular action. Modified analogues are being used in order to further elucidate the receptor sub-types, and this research may lead to the development of new drugs which have none of the toxicity associated with anatoxin itself, but which act merely as acetylcholine replacement candidates (Edwards 1999).

In the course of this development, we want to demonstrate the neurotoxic effect of anatoxin-a in the brain, and in particular its stimulation effect on MAO-B activity.

Materials and methods (experimental part)

Analysis of the monoamine oxidase activity of recombinant human MAO-B was performed using a fluorometric method using an Amplex UltraRed reagent (Bautista-Aguilera et al. 2014). Tyramine hydrochloride is used as the substrate.

The Amplex Red Hydrogen Peroxide / Peroxidase Assay Kit provides the opportunity for sensitive analysis using the Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) reagent for the detection of hydrogen peroxide or peroxidase activity. Amplex Red reagent, Horseradish Peroxidase (HRP), is used to detect hydrogen peroxide in biological samples or generated by enzyme-coupled reactions.

In the presence of peroxidase, the Amplex Red reagent reacts 1 : 1 stoichiometrically with hydrogen peroxide, producing a red phosphorescent oxidation product, resorufin (resirufin). Due to the high extinction coefficient $(58,000 \pm 5,000 \text{ cm-1M-1})$, the analysis can be performed fluorometrically or spectrophotometrically. The reaction is used to detect small amounts of hydrogen peroxide (in the order of 10 pM, in a volume of 100 μl).

Solutions are prepared according to the manufacturer's instructions. Amplex Red stock solution (10 mM) was prepared by dissolving the Amplex Red reagent in dimethyl sulfoxide (DMSO). The reaction buffer consisting of 0.05 M sodium phosphate (pH = 7.4) was obtained from a concentrated solution provided in the manufacturer's kit diluted with distilled water.

Working solutions of anatoxin-a, reagents and human recombinant enzyme MAO-B (hMAOB) were prepared in reaction buffer according to the manufacturer's instructions. Pure MAO-B working solution in reaction buffer, MAO-B working solution containing hydrogen peroxide were used as controls. Anatoxin concentrations are increasing: 0.05 μM, 0.1 μM, 0.2 μM, 0.25 μM, 0.5 μM, 1 μM, 5 μM, 10 μM, 20 μM, 50 μM. Anatoxin fumarate standard, completely soluble in the reaction buffer used, was used. Substances together with hMAOB were embedded in a 96-well plate, and then the plate was incubated for 30 min (dark, at 37 °C).
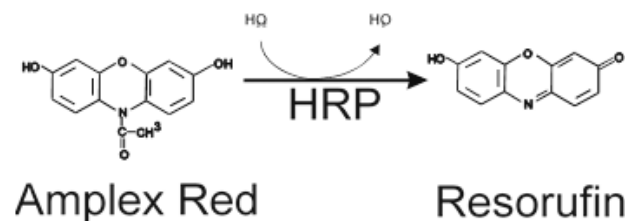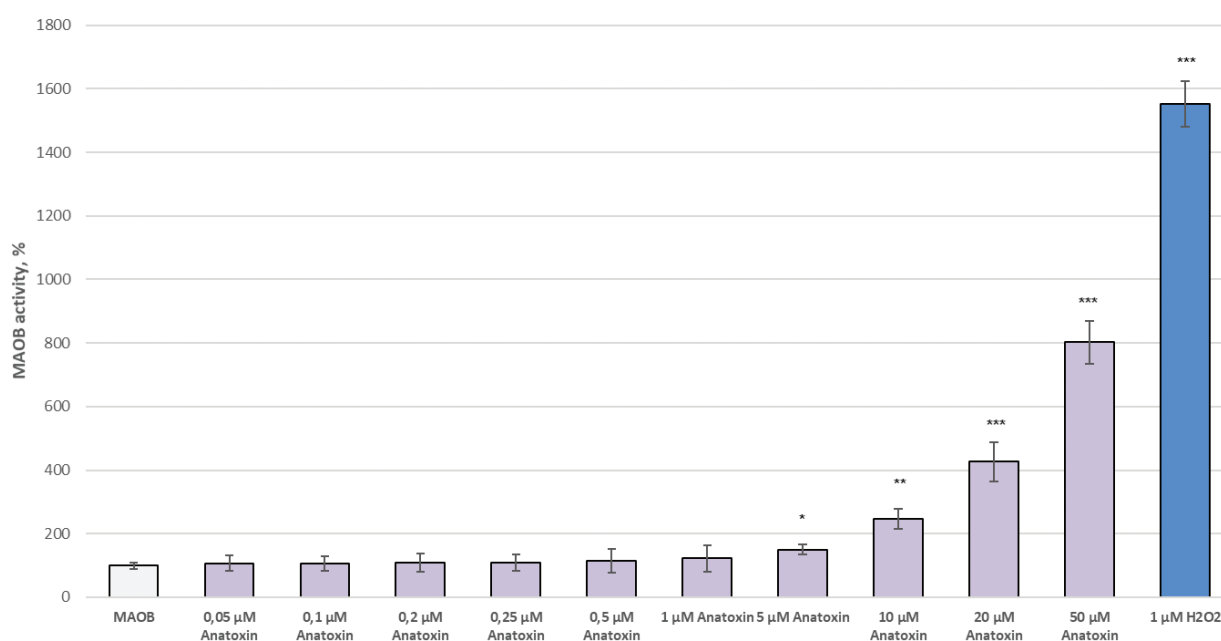

Figure 1. Preparation of the final fluorescent product resorfin (Held 2003).

After incubation time, the reaction is initiated by adding, to each well of the 96-well plate, a 50 µl Mix Solution containing solutions of Amplex Red reagent, HRP, and tyramine as an enzyme substrate in the reaction buffer. As the reaction was continuous to monitor the kinetics, fluorescence was read every 30 min (0, 30, 60, 90, 120 and 150 min, respectively) in the dark, shaking the reaction mixture and at a constant temperature of 37 °C. Fluorometric reading was performed on a Synergy 2 Microplate Reader at two wavelengths (570 nm and 690 nm).

Results and discussion

Administered alone, anatoxin-a induced the activity of human recombinant hMAOB enzyme (hMAOB) statistically significant at the highest concentrations (up to 1 µM).

these metabolites could induced ROS production, which could destroy the nerve cells.

Conclusion

From the results of the experiment, it can be concluded that at a concentration above 1 µM anatoxin-α in vitro, increased MAO-B enzyme activity is detected. It can be concluded that this is due to increased ROS production and conditions of increased oxidative stress. This is also observed with age-related increase in MAO-B activity, as well as with the progression of neurodegenerative diseases.

Anatoxin as a xenobiotic exhibits properties of a pathogen causing in vitro increased MAO-B activity. Subsequent experiments may be considered one of the causes

Figure 2. Results from hMAOB activity, treated with anatoxin-a. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs control (pure hMAOB).

At the lowest concentrations (0,05 µM; 0,1 µM; 0,2 µM; 0,25 µM; 0,5 µM; 1 µM), anatoxin didn't influence, statistically significant, the hMAOB enzyme.

Concentration 5 µM anatoxin, increased the MAO-B activity, statistically significant, with 50%; 10 µM anatoxin – with 146%; 20 µM – with 326% and 50 µM – with 702%, compared to the control (pure hMAOB).

H_2O_2 (at concentration 1 µM) increased the activity of hMAOB with 1452%, compared to the pure hMAOB enzyme. Anatoxin revealed lower enzyme induction, compared to the H_2O_2. This shows the validity of the method used.

Garrick and Sloley (1980), found that dopamine was primarily an MAO-B substrate in human to dihydroxyphenylacetic acid (DOPAC), N-acetyldopamine and N-acetyloctopamine, gamma-glutamyl dopamine. Some of

of the development of neurodegenerative diseases. At this stage, we have reason to believe anatoxin-a may be a factor in the development or progression of Alzheimer disease (AD) and Parkinson's disease (PD).

Acknowledgements

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