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Ability to delay neuropathological events associated with astrocytic MAO-B increase in a Parkinsonian mouse model: Implications for early intervention on disease progression

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ABSTRACT

We previously demonstrated that elevation of astrocytic monoamine oxidase B (MAO-B) levels in a doxycycline (dox)-inducible transgenic mouse model following 14 days of dox induction results in several neuropathologic features similar to those observed in the Parkinsonian midbrain (Mallajosyula et al., 2008). These include a specific, selective and progressive loss of dopaminergic neurons of the substantia nigra (SN), selective decreases in mitochondrial complex I (CI) activity and increased oxidative stress. Here, we report that the temporal sequence of events following MAO-B elevation initially involves increased oxidative stress followed by CI inhibition and finally neurodegeneration. Furthermore, dox removal (DR) at days 3 and 5 of MAO-B induction was sufficient to arrest further increases in oxidative stress as well as subsequent neurodegenerative events. In order to assess the contribution of MAO-B-induced oxidative stress to later events, we compared the impact of DR which reverses the MAO-B increase with treatment of animals with the lipophilic antioxidant compound EUK-189. EUK-189 was found to be as effective as DR in halting downstream CI inhibition and also significantly attenuated SN DA cell loss as a result of astrocytic MAO-B induction. This suggests that MAO-B-mediated ROS contributes to neuropathology associated with this model and that antioxidant treatment can arrest further progression of dopaminergic cell death. This has implications for early intervention therapies.

Introduction

Monoamine oxidase B (MAO-B) is found in the brain primarily in non-neuronal cells such as astrocytes and radial glia [20,40,41]. Age-related increases in its levels have been suggested to play a role in neurodegeneration associated with PD [9,10,20,31,33]. This is believed to be as a consequence of increased oxidative stress; substrate oxidation by the enzyme is accompanied stoichiometrically by the reduction of oxygen to H_2O_2 [5,39]. We previously demonstrated that elevations in astrocytic MAO-B levels in an inducible transgenic mouse model results in a selective loss of dopaminergic SN neurons and that severity of this loss was age-dependent [22]. Cell loss was accompanied by increased oxidative stress and selective inhibition of mitochondrial CI activity, all key features of human Parkinson's disease (PD). Reversing MAO-B induction after 14 days was not sufficient to reverse any of the observed effects when examined 2 weeks later. However, what is not clear from our earlier studies is whether reversing the MAO-B increase at earlier time points would be sufficient to prevent subsequent events or the mechanisms involved. We set out in this current set of studies to investigate the exact timing of events and whether reversal of MAO-B induction at earlier time points was capable of halting or delaying the observed neuropathological progression.

Materials and methods

Induction of astrocytic MAO-B levels via dox feeding and impact of dox removal

Dox-inducible astrocytic MAO-B expressing transgenics were generated in the C57Bl/6 background as previously described [22]. Mice were housed according to standard animal care protocols, fed ad libitum, kept on a 12-h light/dark cycle, and maintained in a pathogen-free environment in the Buck Institute Vivarium. Astroglial-specific transgene expression was induced by feeding adult (3–4 months old) males doxycycline at 0.5 g/kg/day provided in pre-mixed Purina chow

Abbreviations: MAO-B, monoamine oxidase B; dox, doxycycline; PD, Parkinson's disease; SN, substantia nigra; CI, mitochondrial complex I; TH, tyrosine hydroxylase; ST, striatum; ROS, reactive oxygen species; DR, dox removal; DA, dopaminergic; NDA, non dopaminergic.

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(Research Diets) for the designated time periods (1, 3, 5, 7, 10, or 14 days) prior to sacrifice for various analyses $n=6$ animals per time point; a subset of 6 animals at time points 3and 5were taken for dox removal studies and sacrificed at day 14 following dox removal at these earlier time points. EUK-189 was given at a dosage of 30 mg/kg/day subcutaneously ($n=6$ per group) daily starting at either days 0 or 3 of initiation of dox treatment through day 14.

Analyses of ROS levels in immuno-isolated striatal dopaminergic synaptosomes

Three hours prior to sacrifice, mice were injected in the tail vein with ~200 μg DCFDA (Calbiochem) diluted in PBS [1]. Dopaminergic and non-dopaminergic synaptosomes were subsequently isolated from striatal tissues using a modified immuno-magnetic protocol as previously described [3,22]. DCF fluorescence was measured in the synaptosomal samples in the absence or presence of 1 mg/ml catalase as previously described [22]. Relative fluorescence was normalized to synaptosomal protein quantified using Bio-Rad reagent.

Analyses of CI activity levels in immuno-isolated striatal dopaminergic synaptosomes

Complex I activities were assayed in isolated dopaminergic and non-dopaminergic synaptosomal fractions as rotenone-sensitive NADH dehydrogenase activity by measuring DCPIP (2,6-dichlorophenolindophenol) reduction in synaptosomal extracts following addition of 200 μM NADH, 200 μM decylubiquinone, 2 mM KCN, and 0.002% DCPIP in the presence and absence of 2 μM rotenone [38]. Values were normalized/protein using BioRad reagent.

Neurodegeneration as assessed by silver staining

Neurodegeneration in dopaminergic SN cells was visualized by silver staining (FD Neurotechnologies, Ellicott City, MD) according to the manufacturer's instructions using proprietary compounds after immunostaining the sections with antibody against tyrosine hydroxylase (TH, Chemicon, 1:500) visualized with Vector Blue alkaline phosphatase (Vector Laboratories). Quantitation was performed via double-blind analyses of TH^+ SN neurons containing punctate silver staining and reported via a relative scale.

SN TH^+ cell counts

Stereological cell counts were performed on immunostained brain sections from brains harvested 2 weeks after induction using antibody against TH (Chemicon, 1:500) followed by biotin-labeled secondary antibody and development using DAB (Vector Laboratories). TH^+ cells were counted stereologically throughout the SNpc [17]. Sections were cut at a 40 μm thickness, and every 4th section was counted using a grid of 100×100 μm. Dissector size used was $35\times35\times12$ μm. Neuronal numbers were verified following $NeuN^+$ (Chemicon, 1:100) immunostaining.

MAO-B activity

MAO-B enzyme activity was measured at days 0, 1, 3, 5, 7, 10, and 14 of dox feeding in cortical homogenates via a radiometric method using ^{14}C-β-phenylethylamine as substrate as previously described [18].

Statistical analysis

All data are expressed as mean ± SD for the number (n) of mice per group. Differences among the means for all experiments described were analyzed using two-way ANOVA with time or treatment as the independent factor. Newman–Keuls post hoc test was employed when differences were observed by analysis of variance testing ($p<0.05$).

Results

Astrocytic MAO-B increase results in subsequent increases in ROS, CI inhibition, and neurodegeneration which could be reversed via dox removal (DR) at days 3–5

Approximately 10–15% of nerve terminals in the striatum (ST) originate from SN dopaminergic neurons [25,26,29]. Enrichment of striatal dopaminergic nerve terminal (synaptosomal) populations therefore allows us to measure the impact of astrocytic MAO-B induction directly within dopaminergic nigrostriatal neurons at various time points on both ROS levels and CI activity [22]. ROS levels were measured in isolated ST dopaminergic versus nondopaminergic synaptosomes 3 h following injection of DCF into the tail vein of MAO-B transgenics following dox induction for 1, 3, 5, 7, 10 or 14 days. ROS levels was found to be elevated approximately 50% in isolated ST dopaminergic synaptosomes by day 1 following MAO-B induction and continued to rise in the first week to a final level of 7-fold control but leveled out on days 7–14 (Fig. 1) in dopaminergic synaptosomes. No increase in ROS was observed in non-dopaminergic synaptosomes. DR at days 3 and 5 was able to halt or delay any further increases in ROS levels beyond what is observed at these time points when measured at day 14.

We next assessed the time course of rotenone-inhibitable CI activity in ST dopaminergic and non-dopaminergic synaptosomes following MAO-B induction (Fig. 2); our previous study demonstrated selective inhibition of enzyme activity following 2 weeks of MAO-B induction [22]. Here we observed loss of CI activity by day 3 of MAO-B induction (~18% decrease); this activity was found to be further reduced on days 5, 7, 10, and 14 respectively following dox induction (to 44% by day 14). No significant change was noted in CI activity in the non-dopaminergic samples. We found that reversal of MAOB induction by DR at 3rd and 5th days prevented further loss of enzyme inhibition beyond what occurred at these time points when examined at day 14.

Stereological SN TH cell counts were performed at days 3, 5, 7, 10 and 14 following dox induction in the absence or presence of dox removal at days 3 and 5 (Fig. 3). Dox removal at these time points

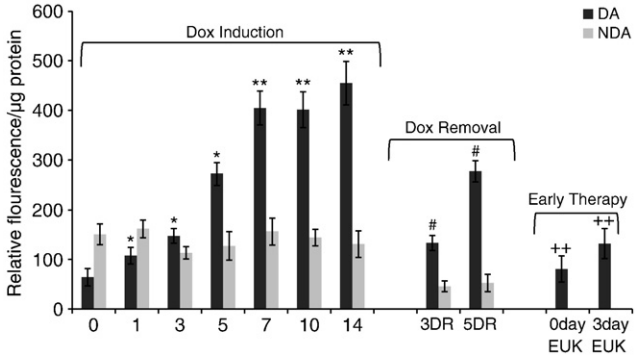

Fig. 1. Elevation in MAO-B results in increased ROS within dopaminergic ST synaptosomes by day 1; increase is halted by dox removal and EUK treatment. ROS levels were estimated in striatal dopaminergic (DA) and non-dopaminergic (NDA) synaptosomes 3 h following tail vein injection of DCFDA at days 0, 1, 3, 5,7, 10 and 14 of dox induction and following dox removal at days 3 and 5; animals were sacrificed for analysis on day 14. EUK189 (30 mg/kg) was administered to animals on days 0 and 3. DCF fluorescence was examined at an excitation wavelength of 488 nm and emission of 512 nm. Data reported are normalized to μg synaptosomal protein. $N=6$ animals per group; F value = 2263, df value = 65 (Prism software). p values: $^{*}p<0.05$, $^{**}p<0.001$, versus day 0 dox, $0.05\geq{}^{\#}p$ versus same day no dox removal (3DR vs. 3 and 5DR vs. 5) where no significant difference was observed, $^{++}p<0.001$ vs. 14 day dox.