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Allopurinol reduces levels of urate and dopamine but not dopaminergic neurons in a dual pesticide model of Parkinson's disease

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Abstract

Robust epidemiological data link higher levels of the antioxidant urate to a reduced risk of developing Parkinson's disease (PD) and to a slower rate of its progression. Allopurinol, an inhibitor of xanthine oxidoreductase (XOR), blocks the oxidation of xanthine to urate. The present study sought to determine whether lowering levels of urate using allopurinol results in exacerbated neurotoxicity in a dual pesticide mouse model of PD. Although oral allopurinol reduced serum and striatal urate levels 4-fold and 1.3 fold, respectively, it did not alter the multiple motor deficits induced by chronic (7 week) intermittent (biweekly) exposure to intraperitoneal paraquat (PQ) plus maneb (MB). However, striatal dopamine content, which was unaffected after either allopurinol or chronic pesticide exposure alone, was significantly reduced by 22% in mice exposed to the combination. Stereological assessment showed that the numbers of dopaminergic nigral neurons were significantly reduced by 29% and the tyrosine hydroxylase (TH) negative neurons unaffected after PQ+MB treatments. This reduction in TH-positive neurons was not affected by allopurinol treatment. Of note, despite the expectation of exacerbated oxidative damage due to the reduction in urate, protein carbonyl levels, a marker of oxidative damage, were actually reduced in the presence of allopurinol. Overall, allopurinol lowered urate levels but did not exacerbate dopaminergic neuron degeneration, findings suggesting that basal levels of urate in mice do not appreciably protect against oxidative damage and neurotoxicity in the PQ+MB model of PD, and/or that allopurinol produces an antioxidant benefit offsetting its detrimental urate-lowering effect.

Keywords

urate; Parkinson's disease; allopurinol; paraquat; maneb

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1. Introduction

Epidemiological studies have identified both positive and negative risk factors for the incidence of Parkinson's disease (PD). Amongst environmental positive risk factors, pesticide exposures have been linked to an increased risk in developing PD, with Paraquat (PQ) and Maneb (MB) [Costello et al., 2009] among those implicated. Negative risk factors for PD include purine-based compounds, urate and caffeine. In fact, robust epidemiological data have linked higher levels of urate to a reduced risk of developing PD (Weisskopf et al., 2007; Chen et al., 2009) and of its progression (Schwarzschild et al., 2008; Ascherio et al., 2009). Urate accounts for most of the antioxidant capacity in human plasma (Yeum et al., 2004); with its antioxidant properties as powerful as those of ascorbic acid (Ames et al., 1981). Urate has been shown to specifically confer protection in cellular (Jones et al., 2000; Duan et al., 2002; Guerreiro et al., 2009) as well as in animal models of disease such as multiple sclerosis (Scott et al 2002); stroke (Yu et al., 1998) and PD (Wang et al., 2010).

While it has been proposed that higher serum urate levels may be of selective advantage in the evolution of the hominids because of its antioxidant effects; hyperuricemia is associated with multiple diseases in humans and points to the deleterious effects of high concentrations of urate. Current approved pharmacological approaches to lowering urate levels in patients with gout rely on allopurinol to reduce urate production (Bieber et al., 2004; Pea 2005). Allopurinol, an inhibitor of xanthine oxidoreductase (XOR) blocks the successive oxidations of hypoxanthine to xanthine, and xanthine to urate. The enzyme XOR is widely distributed throughout various organs including the liver, heart, lung, brain as well as plasma and can exist in either one of 2 forms, xanthine dehydrogenase (XDH), predominating in healthy tissues or xanthine oxidase (XO) which plays an important role in injured cells and tissues (Harrison 2002). Both forms are interconvertible with one another, with the XO subtype causing reduction of molecular oxygen leading to generation of reactive oxygen species (Berry and Hare, 2004).

The present study sought to determine whether pharmacologically lowering urate levels in mice using chronic allopurinol treatment alters the pesticide-induced neurotoxic phenotype in an environmental toxin model of PD.

2. Results

2.1. Allopurinol and not PQ+MB significantly attenuates serum and striatal urate levels

Serum urate levels of mice exposed to allopurinol in the drinking water were significantly decreased by approximately 4-fold ($p < 0.0001$) compared to their unexposed water-drinking counterparts. PQ+MB treatment had no effect (Fig 1A). Striatal urate levels were found to be significantly reduced ($p = 0.0024$) in allopurinol treated mice though only by 1.3-fold compared to non-allopurinol treated mice (Fig 1B). Measurement of additional purines included in the purine metabolism pathway such as hypoxanthine and xanthine were consistent with those published by Enrico et al., 1997 with no demonstrated differences after allopurinol treatment. The data values (ng/mg tissue units) for striatal hypoxanthine levels for the groups: Tap water-Saline, Tap-PQ+MB, Allopurinol-Saline and Allopurinol-PQ+MB were 0.04 ± 0.005 ; 0.04 ± 0.004 ; 0.04 ± 0.004 ; 0.04 ± 0.002 , respectively. For the same

groups striatal xanthine levels were 0.10 ± 0.02 ; 0.12 ± 0.03 ; 0.11 ± 0.01 ; 0.08 ± 0.01 . The data values (mg/dL units) for serum hypoxanthine levels for the groups: Tap water-Saline, Tap-PQ+MB, Allopurinol-Saline and Allopurinol-PQ+MB were 0.041 ± 0.003 ; 0.042 ± 0.004 ; 0.04 ± 0.003 ; 0.06 ± 0.02 , respectively. For the same groups serum xanthine levels were 0.0179 ± 0.0014 ; 0.014 ± 0.0006 ; 0.02 ± 0.0010 ; 0.01 ± 0.001 . The lack of a change in the levels of both hypoxanthine and xanthine may be due to the fact that allopurinol increases the conversion of hypoxanthine to inosinic acid and the inhibition of the rate of de novo purine biosynthesis.

2.2. Allopurinol does not potentiate PQ+MB-induced motor dysfunction

The pole test and the beam traversal task were used to detect any motor dysfunction that may reflect toxin-induced dopaminergic deficit. Specifically, MPTP-treated mice have been shown to display slower times in the descent time parameter of the pole test compared to controls, impairments reversed by L-dopa (Matsuura et al, 1997). In addition, Huntington disease knock-in mice displayed significant impairments in the pole test (Hickey et al, 2003), indicating it is a useful test for basal ganglia dysfunction. The beam traversal task is used to specifically assess fine-motor initiation, coordination and postural balance. Hwang et al., (2005) highlighted transgenic mouse models of PD as significantly slower in traversing a narrow, raised beam as well as taking an increased number of steps, compared to wild-type control animals. These test parameters mimic the slower movements and shorter steps observed in PD patients. PQ+MB treated animals exposed to only water demonstrated a significant increase in duration required to descend the pole, reported as descent time (Fig 2A); in addition to increased time required to cross the beam (beam latency) (Fig 2B), as well as the number of steps required to do so (Fig 2C). Allopurinol exposure did not seem to affect either of these measures.

2.3. Allopurinol potentiates striatal DA loss in combination with PQ+MB exposure

Chronic intermittent administration of PQ+MB on its own did not reduce striatal DA content, consistent with prior studies (Thiruchelvam et al., 2000a; 2000b). However, in the presence of urate-lowering allopurinol treatment PQ+MB significantly reduced striatal dopamine by approximately 22% (Fig 3A).

2.4. Allopurinol does not further exacerbate PQ+MB-induced loss of TH+ neuronal cells

By contrast, PQ+MB treatment on its own significantly decreased the number of TH+ neurons in the substantia nigra pars compacta (SNpc) by approximately 28% compared to the corresponding saline-treated group (Fig 3B). Allopurinol treatment did not alter dopaminergic neuron cell counts and did not potentiate pesticide-induced dopaminergic neuron cell loss. Direct assessment of TH- (negative) neurons confirmed the specificity of pesticide-induced neurotoxicity for dopaminergic neurons of the SNpc, as previously shown (Kachroo et al., 2010) [Fig 3C]. Allopurinol, either alone, or in combination with PQ+MB had no effect on TH- (negative) neuron number.

2.5. Allopurinol attenuates protein carbonyl levels in brains of PQ+MB-lesioned mice

Measurement of oxidative damage assessed by the protein carbonyl assay showed a non-significant increase after PQ+MB treatment to levels that were significantly reduced (27%) by allopurinol (Fig 4).

3. Discussion

These findings demonstrate that we can pharmacologically lower levels of urate both in serum and brain (striatum) of mice by chronic oral administration of allopurinol. Our findings are confirmatory with others showing significant attenuation of striatal urate levels after allopurinol treatment (Desole et al., 1995; Enrico et al., 1997; Miele et al., 1995). Under these experimental conditions we showed PQ+MB treatment itself did not directly affect urate levels.

Motor function, as assessed using the pole test and beam traversal task provided a behavioral measure of pesticide toxicity. This was unaffected by allopurinol alone and while PQ+MB treatment worsened motor performance after at least 6 weeks of toxin exposure (12 injections), urate-lowering allopurinol treatment did not affect these deficits. Previously, it has been shown that under certain conditions of *elevating* urate levels (after injections of intraperitoneal (i.p.) administered urate) in rats exposed to 6-hydroxydopamine, behavioral outputs such as locomotion scores and forepaw adjusting step test scores can be improved (Wang et al., 2010). Differences in rodent species, toxin used, extent/direction of urate change, and even our approach of *indirectly* targeting urate levels by inhibiting the enzyme XOR in our model may all contribute to the lack of a hypothesized behavioral effect.

Striatal dopamine content was shown to be unaffected after either allopurinol or chronic pesticide exposure but was significantly reduced in mice exposed to the combination. Based on prior literature, our data are consistent with allopurinol having no direct effect on striatal DA levels (Desole et al., 1995; Miele et al., 1995). The potentiated effect observed in the presence of a toxin may well be a result of allopurinol unmasking a PQ+MB-induced dopamine loss, possibly due to reduced endogenous antioxidant capacity resulting from lower striatal urate levels. In this setting PQ+MB may produce further increases in reactive oxygen species (ROS) and subsequent dopamine oxidation or dopaminergic nerve terminal injury.

In contrast to striatal dopamine levels, nigral dopaminergic cell counts were not reduced by allopurinol in the PQ+MB model of PD. Although such an exacerbation of neurotoxicity had been hypothesized based on the ability of allopurinol to lower levels of the putative antioxidant urate, we did not find a corresponding increase in oxidative damage markers in brain. Interestingly, brain levels of protein carbonyls were actually reduced by allopurinol in brains of mice treated with PQ+MB, suggesting a net antioxidant effect of allopurinol. Thus, at the level of nigral neuron survival, potentially deleterious urate-lowering effects of allopurinol may have been offset by antioxidant benefits. For example, in peripheral tissue, allopurinol or its metabolites can produce significant antioxidant effects on toxin-induced injury (Kitazawa et al., 1991; Knight et al., 2001), possibly PQ+MB via reduced XOR-driven H_2O_2 (as well as urate) generation. Why an antioxidant benefit of allopurinol would

offset a detrimental effect of lower urate on nigral neuron numbers but not on striatal dopamine content is unclear, but may be related to the distinct anatomical and neurochemical nature of these of nigrostriatal neuron features. In any event, an alternative approach to testing urate reduction, such as may be achieved by increasing urate degradation (rather than by decreasing synthesis via allopurinol) could provide a simpler test of urate's role in models of PD. Finally, whether the synergistic toxicity of allopurinol and these pesticides on striatal dopamine levels (and the dissociation of allopurinol effects on nigral and striatal indices of dopaminergic neuron injury) were consistent across animal models of PD should be assessed in complementary, standard toxin (e.g., MPTP and 6-hydroxydopamine) and transgenic (e.g., 3-synuclein) models of the disease.

4. Experimental Procedures

4.1. Drug administration

Two-month-old male C57BL/6NCrl mice were obtained from Charles River Laboratories; Wilmington, MA and housed under a 12:12 hr light:dark cycle. Food and water were provided *ad libitum*. All experiments were performed in accordance with Massachusetts General Hospital and NIH guidelines on the ethical use of animals, with adequate measures taken to minimize pain and discomfort. Prior to the start of the experiment, mice were either continued on water or placed on allopurinol dissolved in water for one month before exposure to intraperitoneal injections of saline or 10 mg/kg PQ (1,1'-dimethyl-4,4'-bipyridinium) dichloride hydrate followed immediately by 30 mg/kg MB (manganese bisethylenedithiocarbamate) in a volume of 10ml/kg body weight. Allopurinol was administered at a dosage of 150mg/L and bottles replaced weekly. PQ and MB both obtained from Sigma were dissolved separately in saline on the day of administration and injected on the opposite sides (lower quadrants) of the animal's abdomen. Mice were treated chronically (twice weekly for 7 weeks) in the following randomly assigned initial groups: Water [saline control (n=8); PQ+MB (n=18)]; Allopurinol [saline control (n=8); PQ+MB (n=18)]. Mortality rates of 33% and 39% in the toxin-treated mice on regular and allopurinol-treated water, respectively, resulted in final group numbers of 12 and 11. Body weights were obtained twice a week prior to injection during the course of the experiment. Drug treatments did not affect body weight (DNS). Mice were continued on allopurinol or water until their sacrifice one week after the last PQ+MB or saline+saline injection.

4.2. Behavioral testing

Behavioral testing involved mice being exposed to the pole test and the beam traversal task (Kachroo et al., 2012). Baseline values for individual mice on these tests were taken prior to, and during, allopurinol treatment but before toxin exposure. No behavioral differences were observed between either time-point. The average of both baselines was used to normalize subsequent assay values and thus reduce their variability. Mice were tested at 4 time-points referred to as Tests 1,2,3 and 4 which correspond to behavioral assessments at 2, 5, 6 and 7 weeks respectively, after the start of the toxin injections.

4.3. Tissue processing

One week after the last injection (week 8), mice were sacrificed by cervical dislocation, decapitated and trunk blood collected. The brains were removed and the rostral and caudal portions separated by an axial cut made across the whole brain at the tail end of the striatum. Both striata as well as a portion of cortex were removed and frozen at -80°C until use. The remaining caudal brain portion was immediately fixed in 4% PFA for 3 days, placed in cryoprotectant and stored at -80°C until use. The striatum was assayed for dopamine and urate by standard reverse phase high performance liquid chromatography with electrochemical detection as routinely performed in our laboratory (Chen et al., 2001; Ascherio et al., 2009). The striatum and cortex were used for purine and protein carbonyl assays, respectively. Fixed brains were cut on a Leica microtome into 30 μm -thick sections and stored for immunolabeling studies in a cryoprotectant consisting of 30% sucrose, 30% ethylene glycol in 0.1 M phosphate buffer. As previously described (Kachroo et al., 2010), sections were chromogenically stained for TH immunoreactivity (IR) followed by counterstaining with Nissl. Tyrosine hydroxylase-positive (TH+) and -negative (TH-) neurons were counted in the region of the SNpc.

4.4. Striatal and serum sample preparation for HPLC purine analysis

Brain samples were weighed and homogenized in 50 mM Phosphoric acid, 0.1 mM EDTA, 50 μM methyl-DOPA (internal standard), and 1 μM DHBA (internal standard). Homogenates were centrifuged at 14,000 rpm for 15 minutes and the supernatant was transferred to a Costar SpinX (#8161) 0.22 μm CA filter tube and centrifuged at 14,000 rpm for 5 minutes. Samples were then stored at -80°C until needed.

Whole blood was collected from the submandibular vein using Goldenrod animal lancets, and centrifuged at 14,000 rpm at 4°C for 15 minutes. Serum was collected and deproteinated by adding 0.4M perchloric acid. The mixture was allowed to incubate on ice for 10 minutes and then centrifuged at 1,400 rpm for 15 minutes. The supernatant was then collected and added to 0.2M potassium phosphate. The resulting solution was added to a Costar SpinX (as above) and centrifuged at 14,000 rpm for 5 minutes. Samples were then stored at -80°C until needed.

4.5. Purine determination in striatal and serum samples

A dual-pump gradient method was used to measure the concentrations of purines in the samples using a Varian Microsorb-MV reverse-phase column (150 x 4.6 mm, C_{18} , 5 μm pore size). Mobile phase A contained 0.52 mM sodium 1-pentanesulfonate and 0.20 M KH_2PO_4 monobasic at pH 3.5 using 85% phosphoric acid (HPLC-Grade, Fisher Scientific, Pittsburgh, PA). Mobile phase B had the same final concentrations as mobile phase A, except for the addition of 10% acetonitrile (v:v). All analyses were performed at a flow rate of 1 mL/min. The method ran at 0% B for 6 minutes and then linearly ramped to 70% B between 6 and 14 minutes. 70% B was maintained until 17.4 minutes, at which point, it returned to 0% B and was allowed to equilibrate until 20 minutes. The sample injection volume was 12 μL . Detection was performed by linking a UV-Vis spectrophotometer upstream of two coulometric cells. The UV-Vis detection was set to a wavelength of 254 nm. The first electrode was set to -100 mV and acted as conditioning cell. The analytical

electrodes 1 and 2 were set at +150 and +450 mV, respectively. Data were collected using CoulArray Data Station 3.0 software (ESA Biosciences) with auto-range gain enabled. A standard curve was analyzed at the beginning of each run to determine the concentrations of purines in the biological samples. Methyl-DOPA and DHBA were used as internal standards to correct for minor variations between samples in the same run.

4.6. Protein carbonyl assay

Protein carbonyl levels were determined using the OxyBlot Protein Oxidation detection kit from Millipore. A 5mg sample of cortex was removed from frozen brain tissue (kept frozen on dry ice) and added to a tube containing 40 μ l of lysis buffer (RIPA buffer + 50 mM DTT). The sample was hand homogenized, 5 μ l of the resulting homogenate was added to 5 μ l of SDS to a final concentration of 6% SDS. The samples were then derivatized by adding 10 μ l of 1x 2,4-dinitrophenylhydrazine (DNPH) and incubated at room temperature for 15 minutes. The derivatization was halted using 7.5 μ l of Neutralization buffer (aqueous solution of Trometamol and glycerol). The resulting solution was run on a 4% stacking/10% resolving polyacrylamide gel at 90V until the samples had cleared the stacking gel, and then at 110V for 1h. The gel was then transferred to a nitrocellulose membrane. The membrane was then probed with a rabbit anti-DNP (1:150) primary antibody and goat anti-rabbit IgG (1:300) secondary antibody for 1 hour each. The blots were then visualized using horseradish peroxidase.

4.7. Statistical analysis

Optical density measurement for protein carbonyl level assessment was performed using the ImageJ software. All values are expressed as mean $\pm$ SEM. For behavioral tests repeated measures ANOVA (RMANOVA) with *post hoc* analysis was performed. For all other analyses unpaired *t*-tests were performed.

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Highlights

- Pharmacologically lower serum and brain urate levels in mice using allopurinol.
- PQ+MB exposure produced significant motoric deficits not exacerbated by allopurinol.
- Dopamine content was reduced in mice only exposed to allopurinol and pesticides.
- PQ+MB decreased TH-positive nigral neurons not further exacerbated by allopurinol.

Fig 1. Evidence that Allopurinol and not PQ+MB significantly attenuates serum and striatal urate levels in mice

(**A**) Serum urate level, * $p < 0.0001$ vs respective tap water treatment groups; * $p < 0.0001$ (combined allopurinol vs combined tap water treatment groups); unpaired t -test. (**B**) Striatal urate level, * $p = 0.024$ (comparison between combined allopurinol vs combined tap water treatment groups). Groups are: Tap Water [saline control (n=8); PQ+MB (n=12)]; Allopurinol [saline control (n=8); PQ+MB (n=11)]. Combined treatment groups are: Tap Water group (n=20); Allopurinol group [saline control (n=19)].

Fig 2. PQ+MB treatment significantly

(A) increases time to descend the pole. * $p < 0.01$, ** $p < 0.05$, # $p < 0.001$ vs Testing 1; RMANOVA. **(B) increases beam latency.** * $p < 0.01$, ** $p < 0.001$ vs Testing 1; RMANOVA. **(C) increases number of beam steps.** * $p < 0.01$ vs Testing 1; RMANOVA. Data reported as Test– Baseline scores. Water [saline control (n=8); PQ+MB (n=12)]; Allopurinol [saline control (n=8); PQ+MB (n=11)]. Tests 1, 2, 3 and 4 correspond to behavioral assessments performed at 2, 5, 6 and 7 weeks respectively, after the start of the toxin injections.

Fig 3. Allopurinol potentiates striatal DA loss in combination with PQ+MB exposure, but does not further exacerbate loss of total TH+ neuronal cells (bilateral) in the substantia nigra pars compacta

(A) * $p=0.0038$ vs Water-PQ+MB group; unpaired t -test. (B) * $p=0.03$ vs Water-saline group; unpaired t -test. (C) No significant differences within the Tap water group (saline vs PQ+MB); Allopurinol group (saline vs PQ+MB); between Tap water and Allopurinol groups (saline vs saline; PQ+MB vs PQ+MB). Water [saline control ($n=8$); PQ+MB ($n=12$)]; Allopurinol [saline control ($n=8$); PQ+MB ($n=11$)].

Fig 4. Allopurinol attenuates protein carbonyl levels in brains of PQ+MB-lesioned mice

* $p=0.043$ vs Water-PQ+MB; unpaired t -test. Water [saline control ($n=8$); PQ+MB ($n=12$)]; Allopurinol [saline control ($n=8$); PQ+MB ($n=11$)].