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14. ABSTRACT

During the current reporting period we have made further progress toward our main purpose of characterizing the ***mechanisms and neuroprotective potential of purines linked to better outcomes in Parkinson's disease.*** We have made significant progress toward the original SA's with the systematic characterization of the astrocytic Nrf2 antioxidant pathway as a potential specifc mechanism of urate neuroprotection, as well as further development of mouse models for characterizing the urate neurobiology in models of PD. The project has also facilitated collaborative advances in in synculein and urate biomarker discovery for PD. The results to date provide a solid foundation on which to build our subsequent experiments of the project. In the past year they have also had a major impact in directly supporting the development of a novel urate-elevating neuroprotective strategy, and its advance to phase 3 clinical efficacy testing in a major randomized clinical trial. Together our findings strengthen the rationale for pursuing purine targets as candidate neuroprotective strategies for PD, and have epidemiological and military, as well as translational significance.

15. SUBJECT TERMS
urate, tri-oxy-purine, synuclein, neuroprotection, neurotoxin, Parkinson's disease

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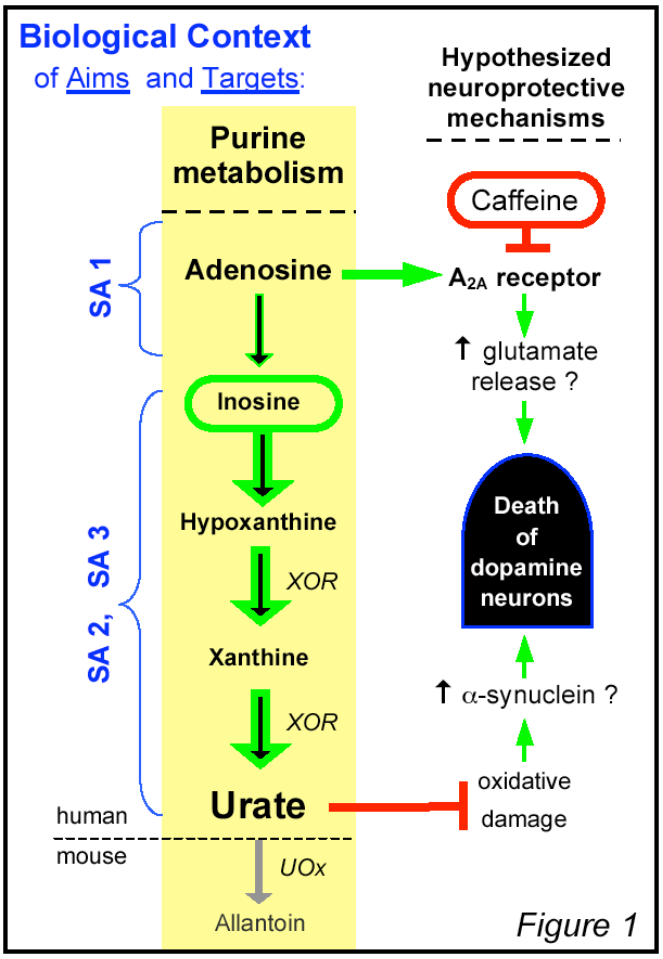
1. Introduction (unchanged from proposal SOW)

The overarching aim of the proposed work is to characterize the ***mechanisms and neuroprotective potential of purines linked to better outcomes in Parkinson's disease*** *(PD)*. We will pursue 3 Specific Aims (SAs) outlined in Section 3 below, and schematized in Figure 1 in the context of purine metabolism and dopaminergic neuron death. **SA1** seeks to determine the effects of the adenosine A_{2A} receptor antagonist caffeine as well as of neuronal A_{2A} receptor knockout (KO) in unilateral toxin models of PD. The potential role of excitotoxic glutatmate release will be investigated. **SA2** will assess the effects of the antioxidant urate (a.k.a. uric acid) on neurotoxicity *in vivo* using complementary pharmacologic and genetic approaches. Inosine, a therapeutically relevant urate precusor, will be tested along with genetic manipulations of urate metabolism, including global KO or conditional KO (cKO) of the *urate oxidase* (*UOx*) or *xanthine oxidoreductase* (*XOR*) genes. **SA3** will explore oxidative and α-synuclein mechanisms of urate protection in a neuronal cell culture models of PD. We propose to systematically pursue the following work on each SA.



SA 1: **Mechanisms of protection by caffeine in toxin models of PD *in vivo***

SA 2: **Neruoprotection by urate in a unilateral toxin model of PD *in vivo*.**

SA 3: **Mechanisms of protection by urate in toxin models of PD in neuronal cultures.**

2. Keywords

1. Parkinson's disease
2. Neurodegeneration
3. Purine
4. Caffeine
5. Adenosine
6. Urate
7. Uric acid
8. Inosine
9. Antioxidant
10. Urate oxidase
11. Gene knockout
12. Murine models
13. Dopamine
14. Synuclein
15. Neurotoxin
16. 6-Hydroxydopamine
17. Neurotherapeutics
18. Translational neuroscience

3. Overall Project Summary:

SA 2: Neruoprotection by urate in a unilateral toxin model of PD in vivo.

Aim 2a: Determine UOx KO phenotype [Yr 1] and superimposed inosine effect in UOx KO [Yr 2].

Aim 2b: Localize the influence of increased urate on neurotoxicity using UOx cKO (Cre/loxP system) mice to elevate urate discretely in dopaminergic neurons [Yr 3] vs astrocytes [Yr 4].

1. ***UOx cKO to elevate urate in mice.*** Despite demonstrated protective effect in 6-OHDA model of PD, *UOx* global KO mice show systemic toxicity of hyperuricemia (Chen et al. 2013) that could confound further mechanistic study of urate in PD animal models. We therefore created *UOx* conditional KO mice based on (tamoxifen) inducible ubiquitously expressed *cre* transgene (*UBC*-cre). (See http://cre.jax.org/introduction.html for summary of inducible Cre/*loxP* methodology.)

1) Baseline urate in *UOx* cKO mice. After tamoxifen injection (100 mg/kg *ip* once daily for 5 days),, *UOx* cKO display moderate and significant elevation of urate in blood, and a trend for an increased urate in the striatum (Fig. 1).

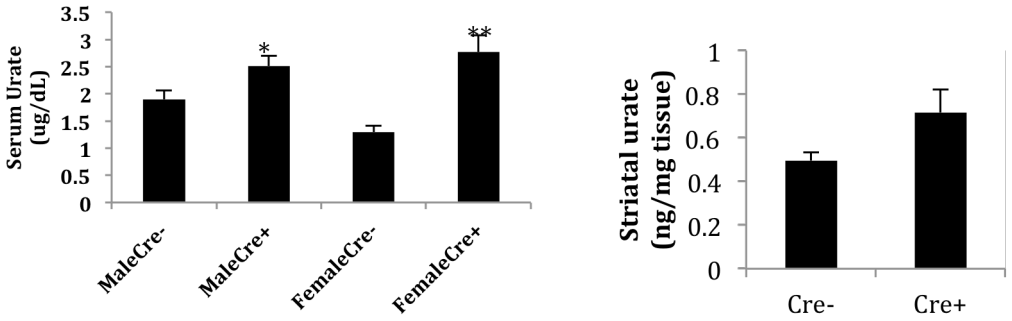


Fig. 1: Elevated serum urate in *UO$_X$* cKO mice. Serum and striatal urate was determined by HPLC. *p<0.05, **p<0.01, vs Cre$^-$. n=18, male, both Cre$^-$ and Cre+; n=15, female, both Cre$^-$ and Cre+**.** Striatal urate, all male, n=10, Cre- and Cre+.

2) *UOx* cKO mice treated with inosine. The moderate urate increase and no obvious toxicity in *UOx* cKO mice make them well suited for testing pharmacological effects of inosine at doses that will further elevate urate, as it does in (*UOx*-deficient) humans. Cre recombination was induced by routine tamoxifen injection, and inosine was added in

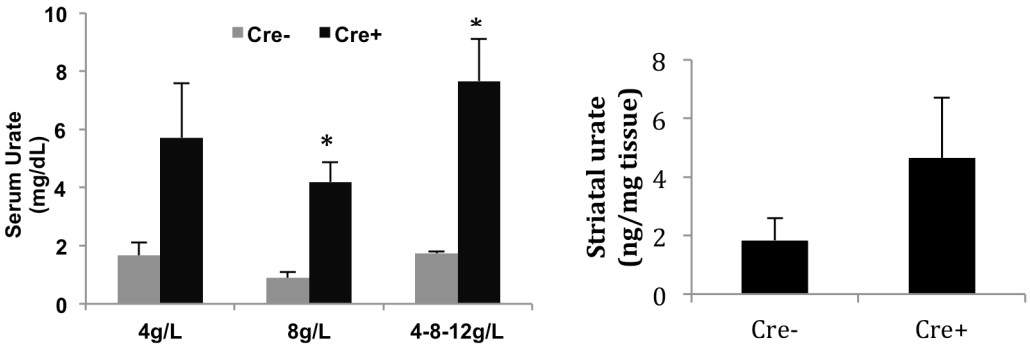


Fig 2: Serum and striatal urate in inosine-treated *UO$_X$* cKO mice. Serum and striatal urate were determined by HPLC. Adult male, n=5 and 4, Cre- and Cre+. *p<0.05, **p<0.01 vs Cre-.

drinking water. As shown in Fig 2, we previously found that in the presence of inosine at 4g/L induced Cre produced a strong trend for increased serum urate after 1 week. The difference between Cre+ and Cre- became statistically very significant at 4 weeks after 8g/L in drinking water. Increasing dose of inosine from 4g (for 3days) to 8 (for 4days), then to 12g/L (for 7 days) caused systemic toxicity and death (data not shown). Inosine did not appear to have effect on serum urate levels in control *UOx*$^{fl/fl}$ *Cre*- mice (data not shown). Mice were sacrificed after being on inosine 8g/L for 5 weeks. There was a trend for increased that did not reach statistical significance in this experiment. Similar modest striatal urate elevation trends short of statistical significance were observed in several other experiments with various approaches to enhancing Cre-mediated recombination/*UOx* disruption via alternative tamoxifen treatment regimens (data not shown).

3) Hybrid *UOx* cKO/KO mice. As above, despite considerable effort we have not been able to demonstrate a consistent or significant CNS urate elevation with the tamoxifen-induced, Cre-mediated recombination/disruption of a homozygous floxed *UOx* gene. We surmise that this may be a technical problem of incomplete gene disruption, with the current cKO method capable of achieving only ~60-80% reduction in *UOx* expression, whereas >80% may be required. (Note that urate elevation is not linearly proportional to *UOx* gene disruption; e.g., with a 50% reduction expression produced in heterozygote constitutive *UOx* KO [*UOx*$^{+/-}$] producing no serum urate elevation at all; Wu et al., 1994.)

In the current reporting period we have invested in an incrementally enhanced recombination strategy that represents a hybrid of a conditional KO (producing a partial ~60-80% reduction in the expression of a floxed *UOx* allele) and a heterozygous constitutive strategy (producing complete disruption in the other globally disrupted *UOx* allele). Thus a hybrid cKO/KO (*UOx*$^{fl/-}$*, cre*) mouse is expected to achieve an 80-90% reduction in *UOx* expression (i.e., 50% reduction in *UOx* expressin at baseline with an additional ~30-40% reduction achieved after tamoxifen induces ~60-80% disruption of the floxed *UOx* allele). We have successfully generated the *UOx*$^{fl/-}$ with or without *cre* mice (offspring of *UOx*$^{-/-}$ x *UOx*$^{fl/fl}$*, cre* crosses) and have are initiating tamoxifen treatment of all offspring, with urate serum and brain urate measurements planned.

2. ***Glut9 cKO to elevate urate in mice.*** During the current reporting period we have pursued another cKO mouse line, which targets *Glut9* a gene encoding the urate transporter Glut9 (a.k.a. SLC2A9), as an alternative approach to urate elevation in mice. *Glut9* is of high translational relevance as it a leading genetic determinant serum urate variance in humans (So & Thorens, 2010), and because its disruption has been found to elevate peripheral levels of urate in homozygous *Glut9* KO mice (Preitner et al., 2009). Furthermore during the current reporting period we have also published evidence polymorphisms in the human *Glut9* gene linked to higher serum urate are also predictive slower clinical decline among patients with early PD (Appendix A.)

We had obtained preliminary evidence that these *Glut9* KO mice also have elevated CNS urate levels. However, despite successful recombination at DNA level in the brain as well as peripheral tissues (tail, liver, kidney), *Glut9*$^{flox/flox}$ *cre*+ mice showed in our hands have shown no significant changes in blood and brain urate as compared to *Glut9*$^{flox/flox}$ *Cre*- litter mate controls. In addition, these mice did not demonstrate urate-elevating response to inosine (data not shown).

SA #3: Protection by urate in cellular models of PD: anti-oxidant and α-synuclein mechanisms.

Our substantial current project period progress in pursuit of the cellular model studies of SA#3 are detailed in and reflected by our major manuscript now under review (resubmitted for minor revisions after favorable reviews in the journal *Neurobiology of Disease*. (Please see Appendix B.)

Final publication of preceding progress demonstrating the neuroprotective effects of the urate precursor inosine (currently in clinical development for PD) also occurred during the project period and is included as Appendix C. Our pursuit of the interplay between urate and α-synuclein mechanisms under this SA has facilitated contributions to biomarker α-synuclein and urate biomarker findings as reported in collaborative studies (Locascio et al. 2015; Schwarzschild et al. 2015, Appendix D).

4. Key Research Accomplishments:

- Comprehensive demonstration of astrocytic Nrf2 antioxidant pathway as potential mechanism of urate neuroprotection (as reflected in Appendix B).
- Characterization of next generation of genetic probes of urate function has demonstrated urate elevation in controlled (inducible) conditional knockout of the *urate oxidase* (*UOx*) gene.
- Approval for funding by the National Institute for Neurological Disorders and Stroke (NINDS/NIH; May 2015 Council) of a major national clinical trial of urate-elevating inosine treatment for PD based on in part on the results obtained under SA 2 and 3 of the project.

5. Conclusions:

In the current reporting period of the project we have made significant progress toward the original SA's with the systematic characterization of the astrocytic Nrf2 antioxidant pathway as a potential specifc mechanism of urate neuroprotection, as well as further development of mouse models for characterizing the urate neurobiology in models of PD. The project has also facilitated collaborative advances in in synculein and urate biomarker discovery for PD. The results to date provide a solid foundation on which to build our subsequent experiments of the project.

Plans – Studies in the final quarters of the project include characterizing the PD toxin/genetic model phenotype of our newly characterized inducible/conditional (post-natal) knockout (KO) of *UOx*, focusing on the hybrid *UOx* cKO/KO variant mouse model described above (SA 2). This line may also be used for testing oral inosine – to parallel oral inosine treatment in humans – to achieve even greater urate elevations.

We will seek to genetically confirm the role of Nrf2 as an astrocytic mediator of urate's neuroprotective effect using siRNA-mediated knockdown of Nrf2 in primary astrocytes (SA3). Under this SA using the same cellular models of PD will also test protective potential of urate against synuclein -induced or –facilitated neurotoxicity.

In the upcoming final quarters of the project we will also pursue completion and submission of multiple manuscripts covering earlier and recent progress from SA1, 2 and 3.

Significance -- Our characterization of the roles of these purines in mouse models of PD neurodegeneration through this preclinical project remains well positioned to inform and potentially accelerate the initiation of phase III clinical trials of neuroprotective candidates for the disease. Supported by our earlier progress on caffeine and adenosine (SA 1), human studies are under way investigating adenosine-targeted strategies in patients with PD. Caffeine itself (http://clinicaltrials.gov/show/NCT01738178) as well as more specific antagonism of the adenosine A_{2A} receptor (http://clinicaltrials.gov/show/NCT01968031) have entered clinical development in PD trials designed to assess disease-modifying effects. Similarly our own clinical development of inosine as a urate precursor targeted as a candidate neuroprotective strategy has reported results of phase 2 testing (http://clinicaltrials.gov/ct2/show/NCT00833690; The Parkinson Study Group SURE-PD Investigators, 2014) and led us to propose advancing to phase 3 efficacy testing of urate-elevating inosine treatment for disease-modification. With positive NINDS scientific reviews and Council approval this past spring we are expecting a major NIH award in September 2015, with first subjects to be enrolled by mid-2016 in a 60-center randomized placebo-controlled trial. The 5-year project is based in part on the results of the current preclinical DoD/NETPR project and is expected to rigorously test of the hypothesis that treatment with oral inosine dosed to nearly double serum urate to 7-8 mg/dL for two years slows clinical progression in early PD.

In addition to its high translational impact, our exploration of purines in preclinical models of PD has substantial epidemiological and military significance. The mechanistic insights pursued under this project reflect a prototypic interaction between putative environmental protectants (e.g., caffeine, urate) and toxins. As reflected by recent presentations of progress under this DoD award by the PI at the National Neurotrauma Society (July 2015) and his preliminary research proposals, the advances made under this award may be ripe for lateral translation to the field of traumatic brain injury (TBI), a major clinical challenge of military service and civilian life.

6. Publications, Abstracts and Presentations:

- Manuscripts
  - Peer-reviewed
    - Cipriani S, Bakshi R, Schwarzschild MA. (2014) Protection by inosine in a cellular model of Parkinson's disease. *Neuroscience*. Aug 22, 2014;274:242-9.
    - Simon KC, Eberly S, Gao X, Oakes D, Tanner CM, Shoulson I, Fahn S, Schwarzschild MA, Ascherio A; Parkinson Study Group. (2014) Mendelian randomization of serum urate and parkinson disease progression. *Ann Neurol*. Dec 2014;76(6):862-8.
    - Locascio JJ, Eberly S, Liao Z, Liu G, Hoesing AN, Duong K, Trisini-Lipsanopoulos A, Dhima K, Hung AY, Flaherty AW, Schwarzschild MA, Hayes MT, Wills AM, Shivraj Sohur U, Mejia NI, Selkoe DJ, Oakes D, Shoulson I, Dong X, Marek K, Zheng B, Ivinson A, Hyman BT, Growdon JH, Sudarsky LR, Schlossmacher MG, Ravina B, Scherzer CR. (2015) Association between α-synuclein blood transcripts and early, neuroimaging-supported Parkinson's disease. *Brain*. [Published online Jul 28, 2015]
    - Bakshi R, Zhang H, Logan R, Joshi I, Xu Y, Chen X, Schwarzschild MA. (2015) Neuroprotective effects of urate are mediated by augmenting astrocytic glutathione synthesis and release. [*submitted*]

- Abstracts
    - Schwarzschild M, Fitzgerald K, Bakshi R, Macklin E, Scherzer C, Ascherio A. (2015) Association of α-synuclein gene expression with Parkinson's disease is attenuated with higher serum urate in the PPMI cohort. *XXI World Congress on Parkinson's Disease and Related Disorders* (Accepted for presentation; Milan, Dec 2015).

- Presentations (include the following)
    - September 25, 2014 – (Grand Rapids, MI) VanAndel Inst. Parkinson's Symposium: Grand Challenges in Parkinson's Disease. "Non-imaging biomarkers as aides and targets in disease modification trials" *[MA Schwarzschild, Symposium speaker]*
    - November 19, 2014 – (Boston, MA) Statistical Issues in the Analysis of Neurological Studies Symposium. "Slowing Progression of Parkinson's Disease: How do we measure that?" *[MA Schwarzschild, Symposium speaker]*
    - November 24, 2014 – (Bethesda, MD) National Institute for Neurological Disorders and Stroke Society. "Adventures in Translational Neuroscience: Pursuing Convergent Preclinical Clues to Promising Trials for Parkinson's" *[MA Schwarzschild, seminar speaker]*
    - March 8, 2015 – (Ft. Lauderdale, FL) Parkinson Study Group/Cleveland Clinic 3rd annual conference -- Shaping the Management of Parkinson's Disease: A Comprehensive Review of Discoveries and Clinical Trials. "The Most Promising Leads in Neuroprotection" *[MA Schwarzschild, Symposium speaker]*
    - April 14, 2015 – (New York, NY) Columbia University/Target ALS annual meeting. "Preclinical Foundation of Urate-Elevating Therapy for ALS" *[MA Schwarzschild, Invited speaker]*
    - July 1, 2015 – (Santa Fe, NM) National Neurotrauma Society Symposium/ Annual Meeting. "Urate – A Novel Potential Therapy in CNS Injury and Neurodegeneration" *[MA Schwarzschild, Symposium speaker]*

The above together with our previous papers listed in the prior project annual reports total at least 24 project publications directly citing W81XWH-11-1-0150.

7. Inventions, Patents and Licenses: Nothing to report.

8. Reportable Outcomes: See publications/presentations in Sec. 6.

9. Other Achievements:

Funding Applied for Based on the Work Supported by this Award includes:

NIH (NINDS) 1R01NS091493-01A1 (Schwarzschild, PI) 2016-2021 " Pursuing Epidemiological Clues to Neuroprotective Therapy for Parkinson's Disease" [pending].

Michael J. Fox Foundation (LRRK2 Cohort Consortium) "*Purine Biomarkers of LRRK2 PD*" (Schwarzschild, PI) 2014-2016 [2nd year of funding proposed/pending].

Target ALS (Columbia University). "Preclinical Foundation of Urate-Elevating Therapy for ALS" [funded].

DoD Amyotrophic Lateral Sclerosis Therapeutic Development Award program. "Neuroprotective potential of urate in amyotrophic lateral sclerosis" [DoD applic #AL140082; full applicn invited; not funded]

NIH (NINDS) 1U01NS090259-01A1 (Schwarzschild, PI) 2015-2020 – "Phase 3 trial of inosine for Parkinson's disease CCC". [approved; contract pending]

10. References:

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11. Appendices:

Appendix A: Simon KC, Eberly S, Gao X, Oakes D, Tanner CM, Shoulson I, Fahn S, Schwarzschild MA, Ascherio A; Parkinson Study Group. (2014) Mendelian randomization of serum urate and parkinson disease progression. *Ann Neurol*. Dec 2014;76(6):862-8.

Appendix B: Bakshi R, Zhang H, Logan R, Joshi I, Xu Y, Chen X, Schwarzschild MA. (2015) Neuroprotective effects of urate are mediated by augmenting astrocytic glutathione synthesis and release. [*submitted*]

Appendix C: Cipriani S, Bakshi R, Schwarzschild MA. (2014) Protection by inosine in a cellular model of Parkinson's disease. *Neuroscience*. Aug 22, 2014;274:242-9.

Appendix D: Schwarzschild M, Fitzgerald K, Bakshi R, Macklin E, Scherzer C, Ascherio A. (2015) Association of α-synuclein gene expression with Parkinson's disease is attenuated with higher serum urate in the PPMI cohort. *XXI World Congress on Parkinson's Disease and Related Disorders* (Accepted for presentation; Milan, Dec 2015).

RESEARCH ARTICLE

Mendelian Randomization of Serum Urate and Parkinson Disease Progression

Kelly Claire Simon, ScD,[1,2] Shirley Eberly, MS,[3] Xiang Gao, MD, PhD,[1,2] David Oakes, PhD,[3] Caroline M. Tanner, MD, PhD,[4] Ira Shoulson, MD,[5] Stanley Fahn, MD,[6] Michael A. Schwarzschild, MD, PhD,[7] and Alberto Ascherio, MD, DrPH,[1,2,8] on behalf of the Parkinson Study Group

Objective: Higher serum urate concentrations predict more favorable prognosis in individuals with Parkinson disease (PD). The purpose of this study was to test the causality of this association using a Mendelian randomization approach.
Methods: The study was conducted among participants in DATATOP and PRECEPT, 2 randomized trials among patients with early PD. The 808 patients with available DNA were genotyped for 3 *SLC2A9* single nucleotide polymorphisms (SNPs) that identify an allele associated with lower urate concentrations, and for selected SNPs in other genes encoding urate transporters that have modest or no effect on serum urate levels. An *SLC2A9* score was created based on the total number of minor alleles at the 3 *SLC2A9* loci. Primary outcome was disability requiring dopaminergic treatment.
Results: Serum urate concentrations were 0.69mg/dl lower among individuals with $\geq$4 *SLC2A9* minor alleles as compared to those with $\leq$2 ($p = 0.0002$). The hazard ratio (HR) for progression to disability requiring dopaminergic treatment increased with increasing *SLC2A9* score (HR = 1.16, 95% confidence interval [CI] = 1.00–1.35, $p = 0.056$). In a comparative analysis, the HR was 1.27 (95% CI = 1.00–1.61, $p = 0.0497$) for a 0.5mg/dl genetically conferred decrease in serum urate, and 1.05 (95% CI = 1.01–1.10, $p = 0.0133$) for a 0.5mg/dl decrease in measured serum urate. No associations were found between polymorphisms in other genes associated with urate that do not affect serum urate and PD progression.
Interpretation: This Mendelian randomization analysis adds to the evidence of a causal protective effect of high urate levels.

ANN NEUROL 2014;76:862–868

Previous longitudinal investigations have shown that individuals with higher serum urate levels[1–3] or a diet that increases serum urate[4] have a lower risk of developing Parkinson disease (PD). Furthermore, in individuals with early PD, higher urate predicts milder clinical and radiographic progression.[5,6] Urate is a potent antioxidant,[7] and several lines of evidence support a role for oxidative stress in the neurodegenerative process of PD,[8] but whether the inverse association between serum urate and PD progression reflects a neuroprotective effect remains uncertain due to the possibility of unmeasured confounders. Because urate levels are in part heritable (the estimate of between-person variation due to inherited genetic factors ranges from 25 to 70%[9]), we sought to use a Mendelian randomization design[10] to investigate whether genetic polymorphisms that predict serum urate levels predict the rate of clinical progression among individuals with early PD. Although several genes are

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associated with serum urate, and a multiple genes score has been used in a previous study of PD risk,[11] we selected as an instrumental variable for this investigation only the gene for solute carrier family 2 (facilitated glucose transporter), member 9 (*SLC2A9*, also known as *GLUT9*),[12] which explains most of the genetically specified variability in serum urate.[13–19] By using a single gene with a strong effect on serum urate, but no known direct effects in the central nervous system, we minimized the possibility of violating the assumption that there are no genetic effects on PD progression other than those mediated by urate levels.[10] Other genes encoding urate transporters that are known to have modest or no effects on serum urate, but could nevertheless modulate its biological effects, were included in exploratory analyses.

Subjects and Methods

Study Population

The source population for this study includes participants in 2 randomized clinical trials of PD: the Parkinson Research Examination of CEP-1347 (PRECEPT) and the Deprenyl and Tocopherol Antioxidative Therapy of Parkinsonism (DATATOP) trials. The details of these studies and their participants are described elsewhere.[20,21] We have previously reported an inverse association between serum urate and rate of disease progression in 804 individuals enrolled in PRECEPT and 774 enrolled in DATATOP.[5,6] The population for this study comprises the subset of these individuals from whom DNA was also available. In DATATOP (2-year study with enrollment from September 1987 to November 1988), DNA was collected at the end of the extended follow-up in 1995. DNA was not collected during PRECEPT (a 2-year trial with enrollment from April 2002 to April 2004), but DNA collection began during a follow-up investigation, known as POSTCEPT, in which all the surviving individuals previously enrolled in the original trial at participating sites were invited to participate. Overall, DNA was available for 808 individuals, of whom 63 were excluded from the Mendelian randomization analyses due to lack of serum urate levels or failure in genotyping of *SLC2A9*; furthermore, we excluded 10 patients who reported use of allopurinol at baseline, leaving 735 patients (390 in DATATOP and 345 in PRECEPT). Exploratory analyses of other genes included between 759 and 783 patients, because we excluded only those patients missing the specific single nucleotide polymorphism (SNP) of interest.

SNPs and Genotyping

Numerous SNPs in *SLC2A9* (a urate transporter[22]) have been identified in several genome-wide association studies (GWAS) as the strongest genetic predictors of serum urate levels and gout.[13–19] Because these SNPs are in high linkage disequilibrium (LD),[15] and a single causal variant has not been identified, we selected 3 of the top SNPs for the present study. Specifically, the following SNPs in *SLC2A9* were genotyped: rs6855911, an intronic SNP with minor allele frequency (MAF) of 0.31 (G allele); rs7442295 (intronic, MAF = 0.21 for G allele); and rs16890979 (missense mutation, MAF = 0.22 for T allele; using HapMap data from Utah residents with ancestry from northern and western Europe, abbreviated CEU[23]), for which each minor allele has been associated with a 0.30 to 0.43mg/dl decrease in serum urate in individuals of European descent.[13,18] Because these 3 SNPs are in strong LD (pairwise r^2 range = 0.68–0.76 from Haploview[24] with HapMap CEU data), we used information from these 3 SNPs to create an *SLC2A9* score with values equal to 0 ($\leq$2 minor alleles; ie, preponderance of wild-type alleles), 1 (3 minor alleles and 3 wild-type alleles), and 2 ($\geq$4 minor alleles; ie, preponderance of minor alleles).

Other genes of interest because of their role in the transport of urate include solute carrier family 22, member 12 (*URAT1/SLC22A12)*, which encodes a urate–anion exchanger,[25] adenosine triphosphate (ATP)-binding cassette subfamily G, member 2 (*ABCG2*), and solute carrier family 19 (sodium phosphate), member 3 (*SLC17A3*). All genotyping was performed through the Harvard Partners Center for Genetics and Genomics at the Harvard Partners Genotyping Facility using the OpenAssay SNP Genotyping System (BioTrove, Woburn, MA). Concordance rates for blinded duplicate quality control samples were 100%. Test of Hardy–Weinberg equilibrium revealed no significant deviations (all $p > 0.05$).

Serum Urate and Clinical Outcomes

Serum urate was measured in PRECEPT and DATATOP participants at baseline prior to treatment assignment, as previously reported.[5,6] The outcome evaluated in this study for both DATATOP and PRECEPT participants was the accumulation of disability sufficient to require dopaminergic therapy (this was also the primary outcome of the original studies).[20,21] The mean duration of follow-up until endpoint or study termination was 13.6 months in DATATOP and 13.3 months in PRECEPT.

Statistical Analysis

Initial analyses were conducted separately in DATATOP and PRECEPT. Because all tests of heterogeneity between studies were not significant ($p > 0.05$), data from the 2 trials were pooled, and all models were adjusted for study group and treatment. Differences in serum urate according to genotype were assessed using generalized linear models. Primary analyses to assess the relation between genetic variants and PD progression assumed additive models (per unit increase in score for *SLC2A9*, per allele associations for other genes); secondary analyses used separate indicators for each genetic score category or genotype. Cox proportional hazards models were used to estimate hazard ratios (HRs) and 95% confidence intervals (CIs) for reaching the primary endpoint according to number of minor alleles or genotype. Analyses were adjusted for study, treatment, gender, age, and use of thiazide diuretics at baseline. We assessed potential effect modification by gender and, in

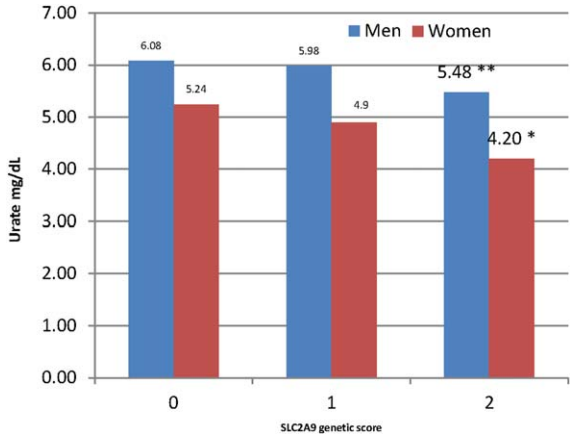

FIGURE 1: Serum urate by *SLC2A9* score. *$p < 0.001$, **$p < 0.01$ for comparison with score = 0 (≤2 minor alleles).

DATATOP, by randomization to α-tocopherol supplementation, which was included in some of the treatment arms in DATATOP. These interactions were assessed by including in the regression models an interaction term that was the cross product of the number of minor alleles of each individual SNP by gender (male/female) or α-tocopherol (yes/no). The association between genetically determined serum urate and PD progression was estimated by 2-stage regression; first, we fitted a generalized linear regression model with serum urate as the dependent variable and the *SLC2A9* score and potential confounders (study, gender, age, and use of thiazide diuretics) as independent variables, then the predicted urate level from the first stage regression was used as a continuous independent variable to determine its association with PD progression in a Cox proportional hazard model, adjusting for potential confounders. Sensitivity analyses were conducted estimating the genetically predicted urate level in a generalized linear model with separate indicators for each *SLC2A9* SNP.

Results

As expected, serum urate concentrations decrease with increasing number of minor *SLC2A9* alleles, with a stronger association in women than in men (Fig 1). In an additive model, the rate of progression to a level of disability requiring dopaminergic treatment increased with the number of the minor alleles associated with lower serum uric acid (UA; HR = 1.16 for each point increase in genetic score, 95% CI = 1.00–1.35, $p = 0.056$). As compared with individuals with ≤2 minor *SLC2A9* alleles, the HR was 1.12 (95% CI = 0.89–1.41) for individuals with 3 minor alleles, and 1.39 (95% CI = 0.98–1.96) for individuals with ≥4 minor alleles. The HR for a genetically determined lower serum urate was higher (HR for 0.5mg/dl lower urate = 1.27) than the corresponding HR for directly measured 0.5mg/dl lower serum urate (HR = 1.05; Fig 2). Results were not materially changed if each *SLC2A9* SNP was used as an independent predictor of serum urate; in this analysis, the HR for 0.5mg/dl genetically predicted lower urate was 1.24 (95% CI = 0.99–1.54).

There was no significant effect modification by either gender or α-tocopherol supplementation (in DATATOP only) on the association between *SLC2A9* score and initiation of dopaminergic therapy (all p for interaction > 0.05). Additionally, there was no evidence of interaction between *SLC2A* score and serum urate.

Overall, polymorphisms in genes other than *SLC2A9* were not significantly associated with serum UA (Table 1) or subsequent initiation of dopaminergic therapy adjusting for age, gender, and treatment (Table 2).

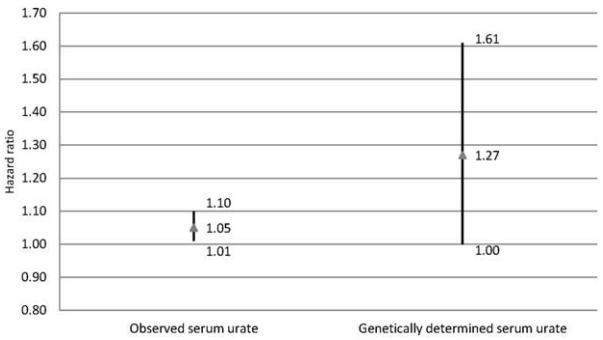

FIGURE 2: Hazard ratios and 95% confidence intervals for initiation of dopaminergic therapy for a 0.5mg/dl observed decrease in serum urate or for genetically conferred decrease in serum urate.

Discussion

In this investigation, we found that among individuals with early PD, SNPs in *SLC2A9* predicted differences in serum urate that are similar to those previously reported in the general population.[13–19] Furthermore, the rate of progression to a level of disability requiring dopaminergic treatment was faster among those patients carrying the *SLC2A9* genotypes associated with lower serum UA. Although the statistical significance was marginal according to conventional levels, these novel results suggest that among participants in DATATOP and PRECEPT the previously reported better prognosis of early PD patients with higher urate levels[5,6] is due to a protective effect of urate itself rather than to confounding by unknown factors.

A limitation of this study is that DNA was collected only several years after the trial completion and was only available for a subset of participants in DATATOP and PRECEPT, so that patients with more rapidly progressive disease may be underrepresented. It is unlikely, however, that this selection would result in a spurious inverse association between the *SLC2A9* SNPs and the rate of PD progression during the trials.

TABLE 1. Serum Urate according to Urate Transport–Related Genotype

SNP	Serum Urate, Mean ± SD	p^a
URAT1-rs11231825, n = 764		
TT	5.5 ± 1.4	0.18
TC	5.3 ± 1.4	
CC	5.3 ± 1.4	
URAT1-rs11602903, n = 780		
AA	5.5 ± 1.3	0.56
AT	5.3 ± 1.4	
TT	5.3 ± 1.4	
URAT1-rs3825016, n = 780		
CC	5.6 ± 1.3	0.36
CT	5.3 ± 1.4	
TT	5.3 ± 1.4	
URAT1-rs3825018, n = 759		
AA	5.3 ± 1.4	0.68
AG	5.4 ± 1.4	
GG	5.4 ± 1.4	
URAT1-rs475688, n = 770		
CC	5.3 ± 1.5	0.95
CT	5.3 ± 1.3	
TT	5.4 ± 1.3	
URAT1-rs476037, n = 763		
AA	6.1 ± 1.0	0.35
AG	5.4 ± 1.4	
GG	5.3 ± 1.4	
URAT1-rs7932775, n = 783		
CC	5.7 ± 1.5	0.86
CT	5.2 ± 1.3	
TT	5.3 ± 1.4	
URAT1-rs893006, n = 761		
AA	5.3 ± 1.4	0.46
AC	5.3 ± 1.4	
CC	5.6 ± 1.4	
ABCG2-rs2231142, n = 779		
GG	5.3 ± 1.4	0.07
GT	5.6 ± 1.5	
TT	4.9 ± 1.1	
SLC17A3-rs1165205, n = 773		
AA	5.3 ± 1.4	0.49
AT	5.3 ± 1.4	
TT	5.4 ± 1.4	

[a]Probability value for trend test, adjusted for study.
SD = standard deviation; SNP = single nucleotide polymorphism.

Furthermore, as in the previous studies including all trial participants, baseline serum urate was inversely related to time to initiation of dopaminergic therapy.

A Mendelian randomization approach has been used to investigate the causality of the association between serum urate and PD risk in 3 previous studies. In the first, conducted among individuals with PD in Italy, Croatia, and Germany, a *SLC2A9* SNP predicting lower serum urate was associated with a younger age at onset of PD.[26] In the second, a case–control study in Spain, individuals in the highest tertile of a genetic score predicting lower serum urate were found to have a 50% higher risk of PD.[11] In the third study, only 1 of 12 genotyped SNPs in *SLC2A9* was associated with a significantly increased PD risk in women, and none in men.[27] In this last study, however, serum urate levels were not available, and it is thus possible that the association between the genotyped SNPs and serum urate in the study population was weaker than expected. An important limitation of these studies is that even for those SNPs with the most robust associations with serum urate, the expected effects on PD risk are small, and power to detect an association is thus modest. Considering these limitations, overall the results of these studies support the hypothesis that higher urate levels reduce PD risk.

The association between genetically decreased serum UA levels and PD prognosis was somewhat stronger than the comparable association for measured circulating UA, suggesting that the latter may have been attenuated by unmeasured confounding. The lower measurement error and long-term stability of genetically determined changes in serum urate may have contributed to this difference, but it is also possible that the association between serum urate and PD progression is attenuated by unmeasured confounders and thus underestimates the true effect of urate on PD progression. Serum urate is associated with obesity and insulin resistance, which in some investigations have been

TABLE 2. Hazard Ratio for Initiating Dopaminergic Therapy according to Urate Transport–Related Genotype

SNP	Genotypes	Risk Allele	Genotype Frequencies	HR (95% CI)[a]
URAT1-rs11231825	TT/TC/CC	C	89/313/375	1.10 (0.95–1.27)
URAT1-rs11602903	AA/AT/TT	T	81/351/362	1.07 (0.92–1.24)
URAT1-rs3825016	CC/CT/TT	T	78/365/351	1.10 (0.95–1.28)
URAT1-rs3825018	AA/AG/GG	G	355/334/84	0.91 (0.78–1.05)
URAT1-rs475688	CC/CT/TT	T	404/319/61	0.94 (0.81–1.10)
URAT1-rs476037	AA/AG/GG	G	9/145/623	1.03 (0.83–1.29)
URAT1-rs7932775	CC/CT/TT	T	34/258/505	1.05 (0.89–1.25)
URAT1-rs893006	AA/AC/CC	C	358/332/85	0.92 (0.80–1.07)
ABCG2-rs2231142	GG/GT/TT	T	614/169/10	1.04 (0.84–1.29)
SLC17A3-rs1165205	AA/AT/TT	T	221/380/186	0.89 (0.78–1.03)

[a]HR for increasing number of risk alleles, adjusted for gender and age, stratified by a 4-level treatment by study variable.
CI = confidence interval; HR = hazard ratio; SNP = single nucleotide polymorphism.

associated with an increased risk of PD,[28,29] and could be a marker of dysfunctional energy metabolism.[30] In vitro, urate production is stimulated by compounds that lower ATP, including inhibitors of mitochondrial respiration,[30] which have been implicated in the pathogenesis of PD.[31] The observed association between serum urate and PD progression could thus reflect in part the protective effect of urate on neurodegeneration, and in part the adverse effects of the upstream metabolic dysregulation that results in elevated serum urate. Although we did not find a significant interaction between *SLC2A9* genotype and α-tocopherol supplementation or gender, the power for these analyses was modest, and effect modification by these factors therefore cannot be excluded. Whereas among DATATOP and PRECEPT participants serum urate was found to be a stronger prognostic predictor in men than in women,[5,6] it is noteworthy that the results of a recent phase 2 randomized trial in patients with early PD suggested that urate elevation may be more effective in women than in men.[32]

We a priori considered *SLC2A9* the primary gene of interest in relation to serum urate levels, so we did not consider potential joint or synergistic effects of a combination of SNPs recently considered by other authors.[19,33] Although a composite genetic score incorporating several loci could be used,[11,19,33] the contribution of the additional genes to serum urate is small relative to *SLC2A9*. The inclusion of numerous genes with modest effects on serum UA could increase the possibility that at least 1 of these genes affects PD progression via mechanisms other than serum urate, thus violating a key assumption of the Mendelian randomization method.[10] In particular, the second strongest genetic predictor of serum urate levels is the ATP-binding cassette, subfamily G, isoform 2 protein (ABCG2), which has been related to the clearance of neurotoxic polypeptides from the brain[34] and neuroregeneration,[35] and whose expression in brain capillaries is altered in an animal model of PD.[36] We cannot therefore exclude the possibility that variations in *ABCG2* could affect PD progression through mechanisms independent from its effects on urate. The validity of the Mendelian randomization approach in our study is supported by the finding that the genotype used as an instrumental variable (*SLC2A9*) is strongly associated with the exposure of interest (serum urate), and is most likely independent of the factors that confound the association between serum urate and PD progression. The finding that other genes involved in urate transport but without sizable effects on serum urate were not related to PD progression indirectly supports this conclusion. Because urate is also inversely associated with PD risk, one might expect that SNPs in *SLC2A9* that predict lower urate levels should have been found to be associated with PD risk in large GWAS. Therefore, its absence[37] may appear to contradict the hypothesis of a genuine protective effect of urate. However, because of the stringent significance criteria imposed by the large number of tests performed, even large GWAS are underpowered to detect the small effects attributable to single *SLC2A9* SNPs.

In summary, we found that patients in the early stages of PD who carry the variant *SLC2A9* alleles

associated with lower urate levels have a faster rate of disease progression than those homozygous for the wild-type alleles. This finding suggests that the previously reported inverse association between higher urate levels and rate of PD progression is not explained by unmeasured confounders and is thus likely to reflect a genuine neuroprotective effect of urate. Genotypic characterization may be useful in identifying those most likely to respond to urate-elevating interventions. These data raise the possibility that modulation of *SLC2A9* might be an equally or even more effective approach to urate elevation, compared to urate precursor administration,[38] as a candidate strategy for slowing PD progression.

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Authorship

K.C.S.—research project: execution; statistical analysis: design and review; manuscript: writing. S.E.—statistical analysis: design and execution; manuscript: review and critique. X.G.—research project: execution; manuscript: review and critique. D.O.—research project: conception, organization, and execution; statistical analysis: review and critique; manuscript: review and critique. C.M.T.—research project: conception, organization, and execution; manuscript: review and critique. I.S.—research project: conception, organization, and execution; manuscript: review and critique. S.F.—research project: conception, organization, and execution; manuscript: review and critique. M.A.S.—research project: conception, organization, and execution; manuscript: review and critique. A.A.—research project: conception, organization, and execution; statistical analysis: design and review; manuscript: writing, review, and critique.

Potential Conflicts of Interest

S.E.: grants, NIH, Michael J. Fox Foundation, Biogen Idec, Auspex Pharmaceuticals. C.M.T.: consultancy, IMPAX Pharmaceuticals, Abbvie, Pfizer Pharmaceuticals, ADAMAS Pharmaceuticals. I.S.: US Food and Drug Administration, Parkinson's Disease Foundation, Lundbeck A/S, Michael J. Fox Foundation; consultancy, Auspex Pharmaceuticals, AZTherapies, Biogen, Caravel Group, Chelsea Therapeutics, Edison Pharmaceuticals, Genzyme Corporation, Innovation Consulting, Impax, Ipsen, Isis Pharmaceuticals, Knopp, Lundbeck A/S, Medtronic, Neuroglobe, Omeros Corporation, Orphazyme APS, Pick Research Solutions, Prana Biotechnology (consultant, non-executive director), Salamandra, Seneb Biosciences, Sofinnova Venture Partners, Velocity Pharmaceutical Development, Voyager Therapeutics; periodic honoraria, Banner Alzheimer's Institute, Columbia University, JAMA Neurology, National Institute of Neurological Disorders and Stroke, Partners Health Care, University of California Irvine, University of Rochester. S.F.: safety monitoring board, Merz Pharma; grant, Genervon Biotechnology; consultancy, PixarBio; advisory board, Lundbeck Pharma, AstraZeneca Pharmaceuticals, IMPAX Pharmaceuticals.

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Neuroprotective effects of urate are mediated by augmenting astrocytic glutathione synthesis and release.

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Highlights

- Neuroprotection by urate relies on glutathione release from astrocytes.
- Urate induced the Nrf2 antioxidant pathway in astrocytes.
- GSH depletion attenuated urate's neuroprotective effects.

Abstract

Urate has emerged as a promising target for neuroprotection based on epidemiological observations, preclinical models, and early clinical trial results in multiple neurologic diseases, including Parkinson's disease (PD). This study investigates the astrocytic mechanism of urate's neuroprotective effect. Targeted biochemical screens of conditioned medium from urate- versus vehicle-treated astrocytes identified markedly elevated glutathione (GSH) concentrations as a candidate mediator of urate's astrocyte-dependent neuroprotective effects. Urate treatment also induced the nuclear translocation of the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) protein and transcriptional activation of its key target genes in primary astrocytic cultures. Urate's neuroprotective effect was attenuated when GSH was depleted in the conditioned media either by targeting its synthesis or release by astrocytes. Overall, these results implicate GSH as the extracellular astrocytic factor mediating the protective effect of urate in a cellular model of PD. These results also show that urate can employ a novel indirect neuroprotective mechanism via induction of the Nrf2 signaling pathway, a master regulator of the response to oxidative stress, in astrocytes.

Keywords

- Astrocytes\Glutathione\ Neurons\ Nrf2\Urate\

Introduction

Urate (2,6,8-trioxy-purine; a.k.a. uric acid) has been gaining momentum as a promising candidate therapeutic target for people with Parkinson's disease (PD) based on its antioxidant and neuroprotective properties, and on its identification as a predictor of a reduced PD risk and a favorable rate of disease progression (Cipriani et al., 2010). Several groups including ours have documented protective properties of urate in cellular and rodent models of PD and other neurodegenerative diseases (Bakshi et al., 2015). In dopaminergic cell lines, urate blocked cell death and oxidative stress induced by 6-hydroxydopamine (6-OHDA), dopamine or rotenone (Zhu et al., 2012; Jones et al., 2000; Duan et al., 2002). Urate at physiologically relevant concentrations enhanced function and survival of dopaminergic neurons in primary cultures of rat ventral mesencephalon (Guerreiro et al., 2009). Urate also confers protection in various cellular models of neurotoxicity beyond that of PD. Urate protected cultured spinal cord or hippocampal neurons from excitotoxic (glutamate-induced) (Yu et al., 1998; Du et al., 2007) or nitrosative (peroxynitrite-induced) cell death (Scott et al., 2005). Similarly, in intact animals urate protects these neurons from ischemic brain or compressive spinal cord injuries (Yu et al., 1998; Scott et al., 2005).

The neuroprotective effects of urate have also been evaluated *in vivo* in rodent models of PD, and was found to attenuate 6-OHDA toxicity (Gong et al., 2012). Similarly, our group found that mice with a urate oxidase gene (*UOx*) knockout have elevated brain urate levels and are resistant to toxic effects of 6-OHDA on nigral dopaminergic cell counts, striatal dopamine content, and rotational behavior (Chen et al., 2013). Conversely, transgenic over-expression of *UOx* exacerbated these anatomical, neurochemical, and behavioral deficits of the lesioned dopaminergic nigrostriatal pathway.

Although considerable evidence indicates that urate is a powerful direct antioxidant few studies have investigated alternative mechanisms of its protective effect. Previous findings of our group (Cipriani et al., 2012a) and others (Du et al., 2007) have suggested a prominent role of astrocytes in the neuroprotective effects of urate. We demonstrated an essential requirement for astrocytes in order for urate to fully protect dopaminergic cells (Cipriani et al., 2012a) or nigral neurons (Cipriani et al., 2012b). We further determined that in response to urate, astrocytes release a potent neuroprotective factor, which differs from urate because it is insensitive to urate-eliminating incubation with commercial UOx enzyme. In the present study we identify and characterize the astrocytic protective factor and signaling pathways mediating urate's neuroprotective effect on dopaminergic cells.

Materials and Methods

Cell Cultures, Drug Treatment and Conditioned Media Experiments

Astroglial cultures were prepared from the cerebral cortex of 1- or 2- day-old neonatal mice as described previously (Cipriani et al., 2012a). Our astroglial cultures comprised >95% astrocytes, <2% microglial cells, <1% oligodendrocytes, and no detectable neuronal cells. Astrocyte cultures reached confluence after 7–10 days *in vitro*. Urate was dissolved in DMEM as 20X concentrated stocks. Enriched astroglial cultures were treated with 100 μM urate or vehicle. Twenty-four hours later conditioned media (CM) were collected and filtered through a 0.2 μm membrane to remove cellular debris and immediately used for experiments. All reagents for cell culture were obtained from Life Technologies.

The rodent MES 23.5 dopaminergic cell line, which was derived from the fusion of a dopaminergic neuroblastoma and embryonic mesencephalon cells (Crawford et al., 1992), was obtained from Dr. Weidong Le at Baylor College of Medicine (Houston, USA). The cells were cultured as described previously (Cipriani et al., 2012a). MES 23.5 cells were incubated with CM from urate- or vehicle-treated astrocytes for 24h before addition of 200 μM H_2O_2 and incubation for another 24h.

RNA Isolation and Quantitative Real-Time PCR

RNA was extracted from astrocyte cultures by TRIzol (GIBCO/BRL) extraction. RNA quality was determined by spectrophotometry and by visual inspection of electropherograms using the RNA 6000 NanoChip Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies). cDNA synthesis was performed using Superscript$^{@}$ VILO cDNA synthesis kit (Invitrogen). For quantitative gene expression analysis, SYBR green primers and probes (Applied Biosystems) were used. The specificity of each PCR product was confirmed by melting dissociation curve (T_m) analysis. The comparative threshold cycle method was used for quantitative analysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and RPL13 ribosomal RNA were used as RNA loading controls. Equal amplification efficiencies were confirmed for target and reference genes. Primer pairs used for quantitative PCR are listed as follows: mouse GCLM (forward 5'-GCA CAG GTA AAA CCC AAT AG-3', reverse 5'-TTA GCA AAG GCA GTC AAA TC-3'), mouse GCLC (5'-CTA TCT GCC CAA TTG TTA TGG-3', reverse 5'-ACAGGTAGCTATCTATTGAGTC-3'), mouse NQO1 (forward 5'-CCT TTC CAG AAT AAG AAG ACC-3'), reverse 5'-AAT GCT GTA AAC CAG TTG AG-3') and mouse HO-1 (forward 5'- CAA CCC CAC CAA GTT CAA ACA-3', reverse 5'- AGG CGG TCT TAG CCT CTT CTG-3').

Western Blot Analysis

Western blot analysis was performed using lysates from astrocytes. The blots were probed with antibodies against NQO1 (Sigma N5288), GCLC (Abcam ab53179), GCLM (Abcam ab126704) and β-actin (Abcam ab8227) using the dilutions recommended in the product datasheet. Band densities were analyzed with ImageJ software.

Immunofluorescence Microscopy

Primary astrocytes cultures grown on chamber slides were fixed in 4% paraformaldehyde for 20 min at room temperature and then permeabilized for 30 min with 0.1% Tween-20 in phosphate-buffered saline (PBS). After blocking the cells in 3% bovine serum albumin/PBS for 1 h, anti-Nrf2 and anti-glial fibrillary acidic protein (GFAP) antibodies were added at 4°C overnight, followed by incubation with Cy3-conjugated secondary antibody for GFAP and fluorescein isothiocyanate (FITC) conjugated secondary antibody for Nrf2 (Molecular Probes) for another 1 h. Slides were treated with VECTASHIELD mounting medium containing DAPI (4',6-diamidino-2-phenylindole; Vector Laboratories) and mounted. Images were collected with a Nikon A1+/A1R+ confocal microscope and were processed with NIS Element confocal imaging software.

Fluorescence intensity of Nrf2 in the nuclear and cytoplasmic regions was quantified using ImageJ (http://rsbweb.nih.gov/ij/) as described (Drake et al., 2010). Background-corrected nuclear:cytoplasmic (N/C) ratios were calculated from mean fluorescence intensities measured within a small circular region of interest placed randomly in a region of uniform staining devoid of any punctate structures within the nucleus and the cytoplasm of each cell.

Cell Viability Assays

For evaluation of cell viability, cells were co-stained with propidium iodide (PI)/ annexin V and analyzed using fluorescence activated cell sorting (FACS) as described before (Behbahani et al., 2005). The percentage of apoptotic cells was determined by FACScan Flow Cytometer and CellQuest software (Becton Dickinson).

Screening for Neurotrophic Factors

Screening for GDNF, BDNF and IL6 was conducted using commercial ELISA kits as per manufacturer (Abcam) instructions. Total GSH levels (GSH+GSSG) were measured in lysates and CM from urate- and vehicle-treated astrocytes. Levels of GSH were determined by a simple

in vitro fluorometric detection assay kit as per manufacturer (Abcam ab65322) instructions. All GSH measurements were normalized to total protein levels.

GSH Depletion Assays

MK-571 and BSO were purchased from Sigma and dissolved in DMEM as 100X concentrated stocks. Enriched astroglial cultures were treated with vehicle or urate (100 μM) alone or in combination with BSO (0.25 mM) or MK-571 (50 μM). Twenty-four hours later CM was collected and filtered through a 0.2 μm membrane to remove cellular debris and immediately used for experiments with MES 23.5 cells. To verify the effects of BSO or MK-571, GSH content in the CM was measured. MES 23.5 cells were pretreated with vehicle CM or urate CM, with or without BSO or MK-571, (or were not pretreated) before being exposed to oxidative stressor (H_2O_2) for 24h. The percentage of dead cells was analyzed using the FACS method described above.

Statistical Analysis

Values were expressed as mean ± standard error of the mean (SEM). Differences between groups were examined for statistical significance using one-way ANOVA or two-tailed Student's t-tests, using GraphPad Prism 5 software. A p value less than 0.05 denoted the presence of a statistically significant difference.

Results

Urate induces GSH release from cultured primary astrocytes

First we confirmed the neuroprotective effect of conditioned medium (CM) from urate-treated astrocytes by assessing dopaminergic cell death with a method complementary to those of our previous report (Cipriani et al 2012a). Using a PI/annexin dual staining method to evaluate cell viability we observed complete protection of dopaminergic MES 23.5 cells from oxidative stressor (H_2O_2)-induced cell death after their incubation with CM from astrocytes treated with 100 μM urate (Fig. 1A).

To identify putative inducible factors secreted by urate-treated astrocytes we conducted a targeted screen of their CM for prominent neurotrophic factors known to be released by astrocytes. Using commercial assays we measured levels of glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), interleukin-6 (IL6) and GSH in urate- and vehicle-treated glial CM. No significant difference was found in the levels of BDNF, IL6 and GDNF in urate- versus vehicle-treated CM (Fig. 1B). In contrast, GSH levels were significantly higher in CM from urate- compared to vehicle-treated astrocytes.

Urate induces GSH levels and Nrf2-targeted gene in astrocytes

To determine the mechanism of increased extracellular GSH levels after urate treatment, we examined changes in GSH levels as well as GSH synthesis within primary astrocytes. Urate treatment for 24h significantly increased GSH levels in the astrocytes compared to those in control astrocytes (Fig. 2A). We also examined the protein and transcript levels of two subunits of the γ-glutamyl cysteine ligase (GCL), the rate-limiting enzyme of GSH synthesis. Both the protein and mRNA expression levels of the modifier subunit (GCLM) were significantly induced by urate treatment (Fig 2B and 2C). Because GCL is transcriptionally regulated by nuclear factor (erythroid-derived 2)-like 2 (Nrf2), we also measured the mRNA and protein expression of other key Nrf2-regulated genes including NAD(P)H:quinone oxidoreductase 1 (NQO1) and heme oxygenase-1 (HO-1). Urate treatment for 24h led to a significant increase in mRNA levels of NQO1 in astrocyte cultures (Fig. 2C) and a trend towards an increase in its protein levels (Fig 2B). Transcript levels of HO-1 also appeared increased, though not significantly, with urate treatment (Fig. 2C). The protein levels of HO-1 were not detected by the western blot analysis.

Urate induces nuclear localization of Nrf2 in astrocytes

Activation of the Nrf2 pathway involves translocation of the Nrf2 protein from the cytoplasm into the nucleus where it can transactivate its targets. Nrf2 is known to bind to the antioxidant-response element (ARE) in promoter regions of Nrf2-responsive genes leading to their transcriptional activation in response to oxidative stress or related external stimuli. Here, the subcellular distribution of Nrf2 was studied by immunofluorescence confocal microscopy. We observed significant redistribution of Nrf2 immunoreactivity from its relatively balanced cytoplasmic and nuclear localization in vehicle-treated astrocytes to a predominantly nuclear localization after treatment with urate (Fig. 3). Cultures were also stained with DAPI to visualize nuclei and immunostained with astrocyte-specific anti-GFAP antibody.

GSH depletion attenuates neuroprotection by conditioned medium from urate-treated astrocytes

To determine whether GSH mediates urate's neuroprotective effect we reduced the GSH concentration of CM from urate-treated astrocytes. This was accomplished by two strategies. First, GSH synthesis in urate-treated astrocytes was inhibited by buthionine sulfoximine (BSO), which inhibits GCL, the rate-limiting enzyme of GSH synthesis (Drew et al., 1984). Second, we blocked GSH release from the astrocytes by inhibiting multidrug resistance protein 1 (MRP1) transporter with its competitive inhibitor MK-571 (Hirrlinger et al., 2002). We performed cell viability experiments in MES 23.5 dopaminergic cells treated with CM from astrocytes treated with vehicle or urate in combination with BSO (0.25mM) or MK-571 (50 μM). These drugs were added to the astrocytes concurrently with urate or vehicle 24h prior to the collection of the CM. GSH levels were undetectable in urate or vehicle CM from astrocytes treated with either BSO or MK-571 (Fig. 4A). There was no significant effect of BSO or MK-571 treatment alone on MES 23.5 cell viability in absence of H_2O_2 (Fig. 4B and 4C). Neuroprotection by CM from urate-treated astrocytes was significantly reduced by BSO or MK-571 treatment as depicted by increased cell death compared to urate CM alone (Fig. 4B and 4C), indicating that the presence of GSH in the CM is critical for urate's protective effect.

Discussion

Its well-documented direct antioxidant properties notwithstanding, urate can produce much of its neuroprotective effect indirectly via astrocytes (Cipriani et al., 2012a; Du et al., 2007). They in turn release a potent neuroprotective factor, which differs from urate because incubation of medium conditioned by urate-treated astrocytes with commercially obtained UOx eliminates urate but not the protective influence (Cipriani et al 2012a). Here we identified GSH as a primary candidate for the putative neuroprotective factor that is released from urate-treated astrocytes based on its markedly higher concentration in CM and lysates from urate-treated compared to control astrocytes. The glutathione system is very important for cellular defense and protects against a variety of different reactive oxygen species (ROS). The total GSH content of astroglial cultures measured in the astrocytes and the extracellular media was in the same physiological range as reported previously (Raps et al., 1989, Dringen et al 1999). GSH is a tripeptide that is synthesized by two successive enzymatic reactions. The first, rate-limiting step in GSH biosynthesis is catalyzed by GCL (Lu, 2013). In its catalytically most active form, GCL comprises a catalytic subunit (GCLC) and a modifier subunit (GCLM). Urate significantly increased the transcript and protein levels of the modifier (but not the catalytic) subunit of the enzyme in astrocytes, likely contributing to their increased GSH synthesis given that GCLM increases V_{max} of GCLC and its affinity for its substrates (Franklin et al., 2009). Although the significance of differential induction of GCLM versus GCLC genes is unclear, it is consistent with similarly discordant GCL subunit regulation by other extracellular stimuli (Cai et al., 1995; Cai et al., 1997; Franklin et al., 2009; Moellering et al., 1998).

The GCL genes are part of a broader cellular antioxidant pathway that controls a set of effector genes through a unique cis-acting transcriptional regulatory sequence, termed the antioxidant response element (ARE). Several lines of evidence suggest that Nrf2 is a transcription factor responsible for upregulating ARE-mediated gene expression (Kensler et al., 2007). The Nrf2 pathway has been known to be activated by both oxidative stress as well as antioxidants (Ma, 2013). From our findings, urate appears to be a key activator of Nrf2 signaling and its downstream targets that guard against oxidative stress. Astrocytes are known to interact with surrounding neurons and their neuroprotective properties are well documented (Maragakis et al 2006, Bélanger et al 2009, Sidoryk-Wegrzynowicz et al 2011). Here we demonstrate an unanticipated requirement for a neuroprotective factor released from astrocytes in order for urate to fully protect dopaminergic cells in a cellular model of PD. Interestingly, others have recently reported Nrf2 involvement in urate's neuroprotective effects on dopaminergic cells

(Zhang et al., 2014). They reported that urate protected dopaminergic cell lines in the absence of glial cells but, as noted by the authors, at much (~100-fold) higher concentrations of urate than are needed in the presence of astrocytes (Cipriani et al .,2012a; Zhang et al 2014). They found urate to be protective at concentrations of 200 μM and above, whereas CNS concentrations of endogenous urate are typically 10- to 100-fold lower (Ascherio et al., 2009; Chen et al., 2013). Thus activation of astrocytic Nrf2 signaling by urate and its indirect neuroprotective effects may be more pathophysiologically relevant than are the direct effects of urate on neuronal cells. The importance of astrocytic Nrf2 is in agreement with earlier demonstrations that Nrf2 induction in astrocytes boosts their production of GSH, which in turn can protect glial cells and neighboring neurons (Kraft et al., 2004; Shih et al., 2003). The protective effects of astrocytic Nrf2 against neurodegeneration has been suggested by neuroprotective effects of Nrf2 overexpression in astroglial cells in mouse models of amyotrophic lateral sclerosis (ALS) and PD (Chen et al., 2009; Vargas et al., 2008). Moreover, astrocytes have greater antioxidant potential than neurons (Makar et al., 1994; Raps et al., 1989) and many studies also provide evidence for efflux of GSH from astrocytes as a key factor in neuroprotection (Iwata-Ichikawa et al. 1999, Dringen et al. 2000, Dringen and Hirrlinger 2003). Nrf2/ARE activation in astrocytes leading to increased levels of GSH seems to be a major component of the protection conferred by urate.

In addition to the extensive preclinical data supporting a key role of Nrf2 disruption in PD neurodegeneration, recent epidemiological studies have suggested Nrf2 genetic variants modify PD susceptibility and onset (Todorovic et al 2015, Von Otter et al 2010). From a therapeutic standpoint this astrocytic Nrf2-orchestrated defense system may offer an attractive drug target in several neurodegenerative diseases and other neurological disorders. For example, another small molecule dimethyl fumarate (DMF), which has been found effective and approved for use as a disease-modifying treatment of multiple sclerosis, may confer its cytoprotective effects via activation of the Nrf2 pathway (Scannevin et al., 2012). Urate has been gaining momentum as a promising target or agent for neuroprotection based on accumulating epidemiological observations, laboratory data, and encouraging early clinical (including phase II) trial results for several neurological conditions, most actively for PD (Schwarzschild et al., 2014) and stroke (Chamorro et al., 2014). The present findings implicating a discrete astrocytic antioxidant response signaling cascade in the protective actions of urate, in addition to its established but non-specific direct antioxidant properties, strengthen the biological plausibility of its protective potential and support its further clinical development.

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Figure Legends

Figure 1. Marked elevation in GSH release from astrocytes treated with urate. (A) Conditioned medium (CM) from urate-treated astrocytes protects dopaminergic cells from H_2O_2-induced cell death. Representative graphs of FACS analysis show cell viability using propidium iodide (PI)/Annexin V staining. Percentages of PI+/AnnexinV+ (dead), PI-/AnnexinV+ (apoptotic) and PI−/AnnexinV− (vital) staining are shown for untreated MES 23.5 cells, or those treated with CM from vehicle- or urate (100 μM)-treated astrocytes a day before and during 200μM H_2O_2 treatment for 24h. (B)Targeted screening of several neurotrophic factors in the CM. There was no change detected in levels of BDNF, GDNF and IL6 factors in the urate versus vehicle CM. GSH content was significantly increased in CM from urate- (versus vehicle-) treated astrocyte. *denotes p value < 0.001; (n=4 independent experiments).

Figure 2. Urate induces the Nrf2 pathway in astrocytes.(A) GSH levels are elevated in lysates from 100 μM urate- (versus vehicle-) treated astrocytes. (B) Urate induces protein products of Nrf2-targeted genes as shown by western blots of GCLM, GCLC and NQO-1 proteins in urate-treated astrocytes compared to controls. The approximate molecular weight of the indicated protein in kDa is indicated at Left. The graph represents densitometric analysis of the western blots to semi quantify the protein levels. (C) Quantitative PCR analysis of Nrf2

target genes in urate- (versus vehicle-) treated cells. Mean ± SEM are shown (n = 3). * denotes p value < 0.05.

Figure 3. Urate induces nuclear translocation of the Nrf2 protein. (A) Astrocytes were incubated with urate (100 μM) or vehicle control for 8h. Cells were immunostained for astrocyte-specific marker GFAP (red) and nuclei were stained with DAPI (blue). Nrf2 was detected using FITC (green) staining. These representative images show a predominant nuclear distribution of the Nrf2 protein after urate treatment, in contrast to the greater proportion of cytoplasmic expression of Nrf2 in vehicle-treated astrocytes. (B) The graph represents the quantification of the nuclear:cytoplasmic (N/C) ratios of Nrf2 staining intensity using ImageJ software. 20-30 cells from 4 independent experiments were counted. * denotes p value < 0.05.

Figure 4. GSH depletion attenuated the protective effects of urate. (A) GSH content was undetectable in CM from astrocytes that were treated with urate as well as either BSO (0.25 mM) or MK-571 (50 μM). (B,C)MES 23.5 cells were exposed to an oxidative stressor (200 μM H_2O_2) after pretreatment with CM. The CM was from vechicle- or urate-treated astrocytes, with the latter also treated with or without BSO (0.25 mM) or MK-571 (50 μM) as indicated.. The protective effect of CM from urate-treated astrocytes against H_2O_2 toxicity (% dead cells) was significantly reduced by astrocyte incubation with BSO (B) or MK-571 (C) (n = 3). * denotes p value < 0.05.

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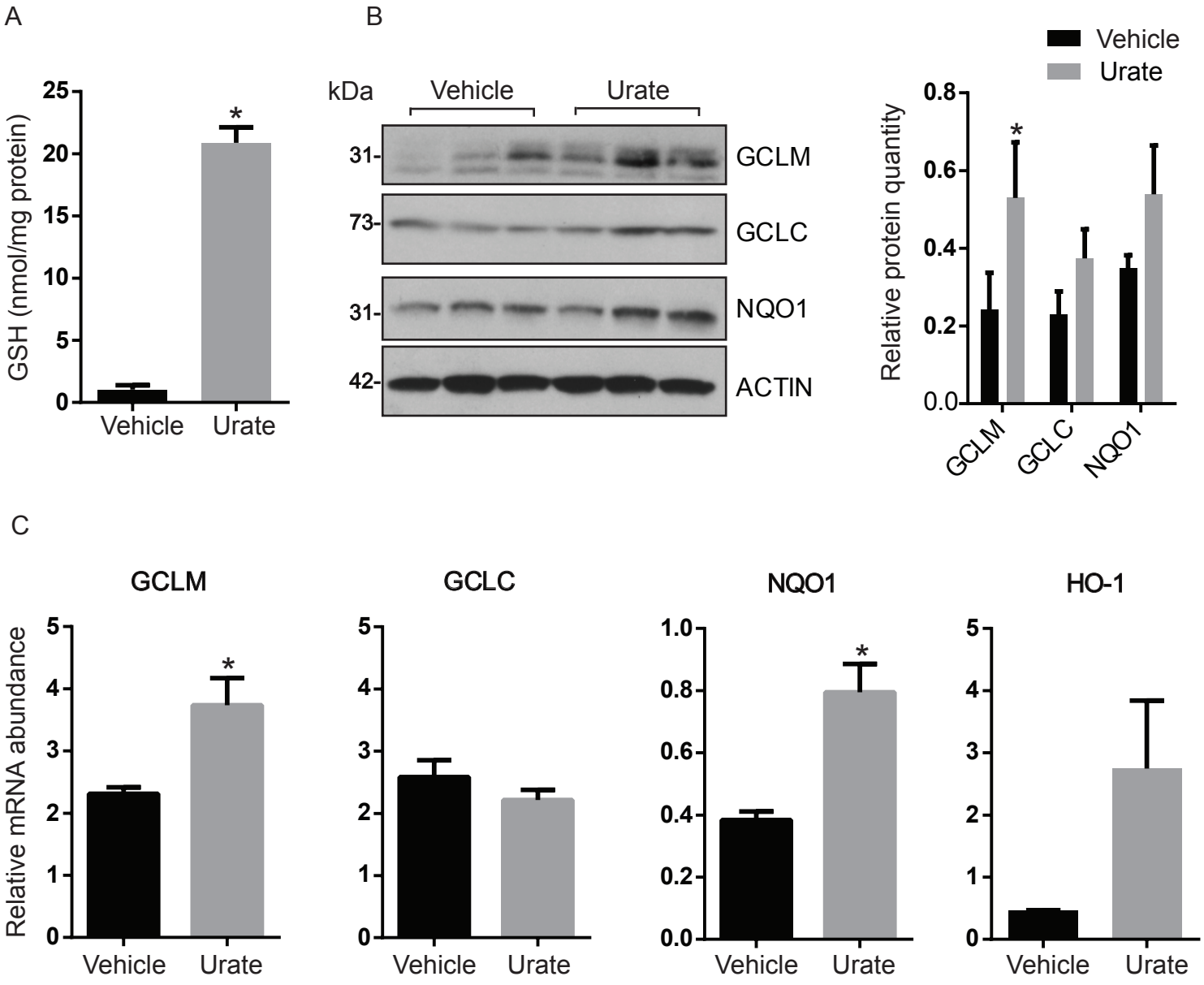
Revised Figure 2



Figure

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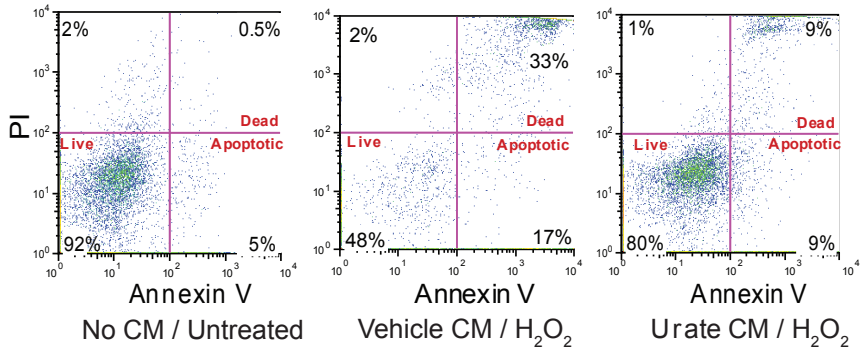
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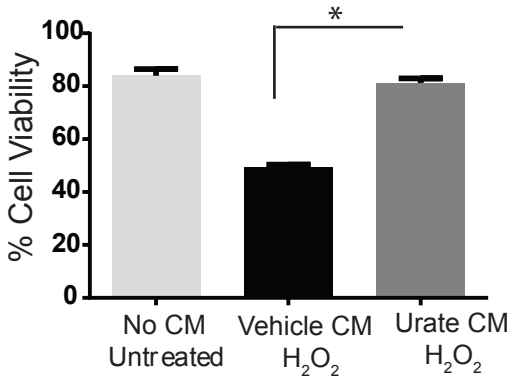
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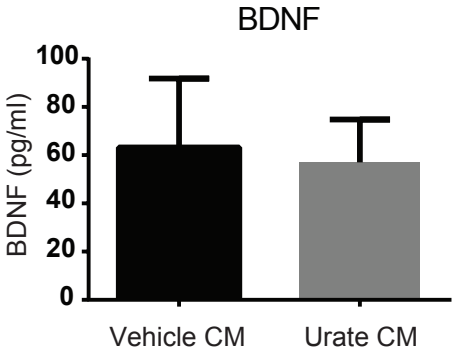
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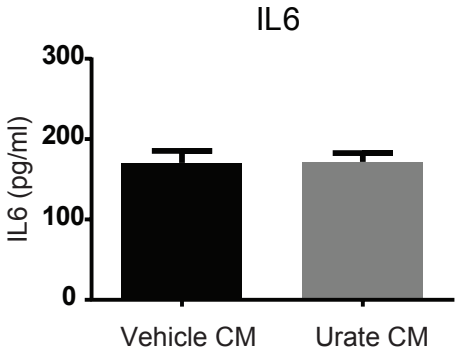
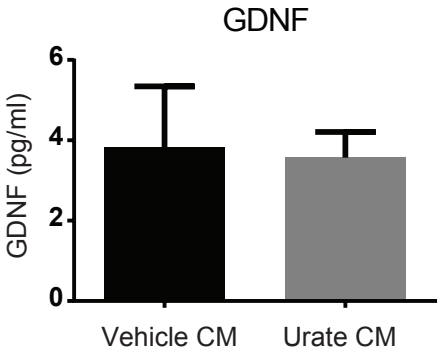
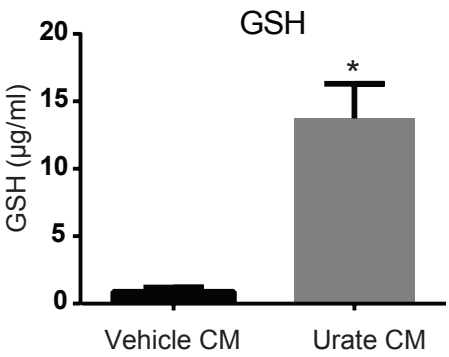
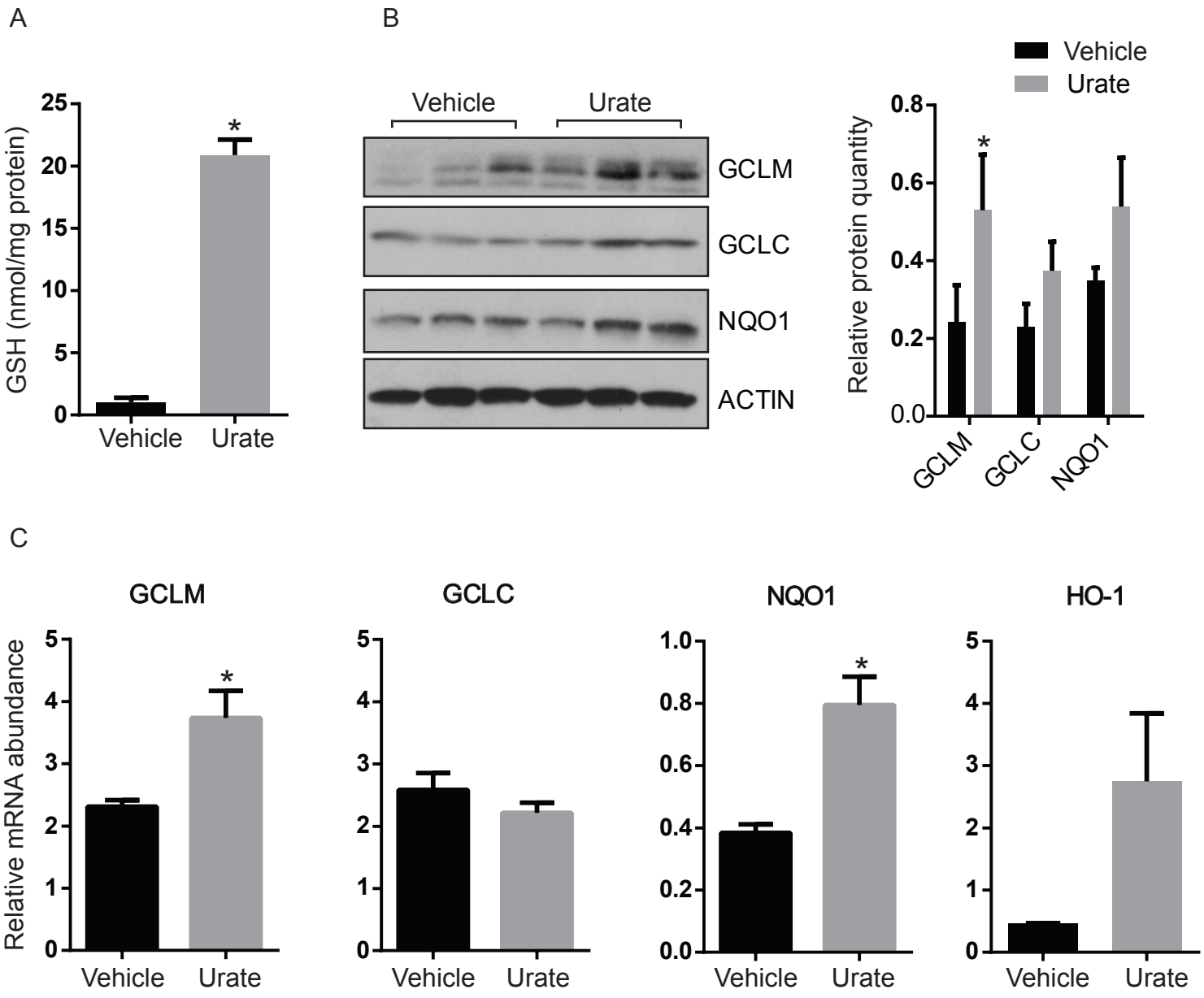
Vehicle CM
Urate CM

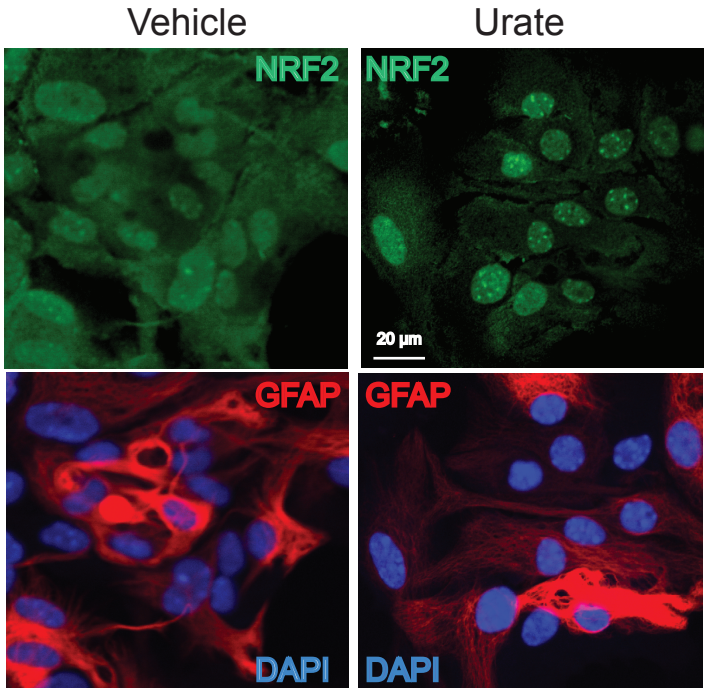

IL6
IL6 (pg/ml)
300
200
100
0
Vehicle CM
Urate CM


GDNF
GDNF (pg/ml)
6
4
2
0
Vehicle CM
Urate CM

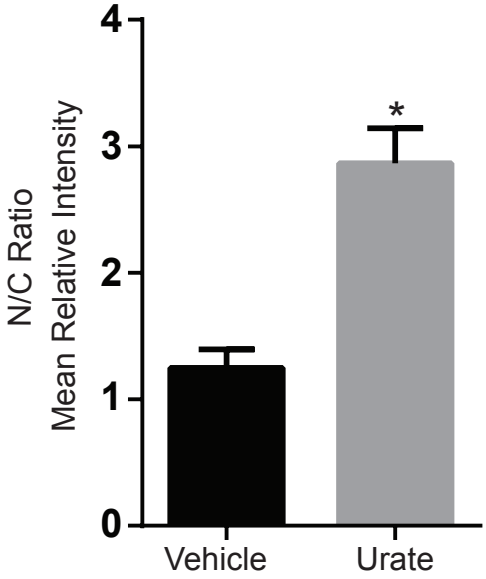

GSH
GSH (µg/ml)
*
20
15
10
5
0
Vehicle CM
Urate CM

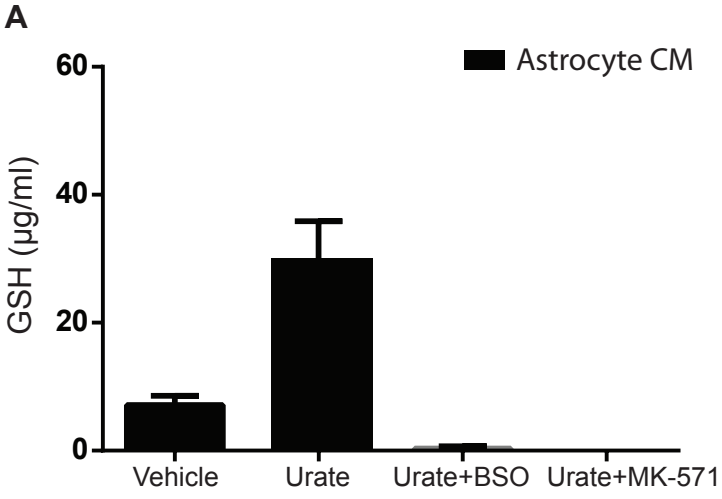

A
GSH (nmol/mg protein)
25
20
15
10
5
0
*
Vehicle
Urate
B
Vehicle
Urate
GCLM
GCLC
NQO1
ACTIN
Vehicle
Urate
Relative protein quantity
0.8
0.6
0.4
0.2
0.0
*
GCLM
GCLC
NQO1
C
GCLM
GCLC
NQO1
HO-1
Relative mRNA abundance
5
4
3
2
1
0
1.0
0.8
0.6
0.4
0.2
0.0
*
Vehicle
Urate

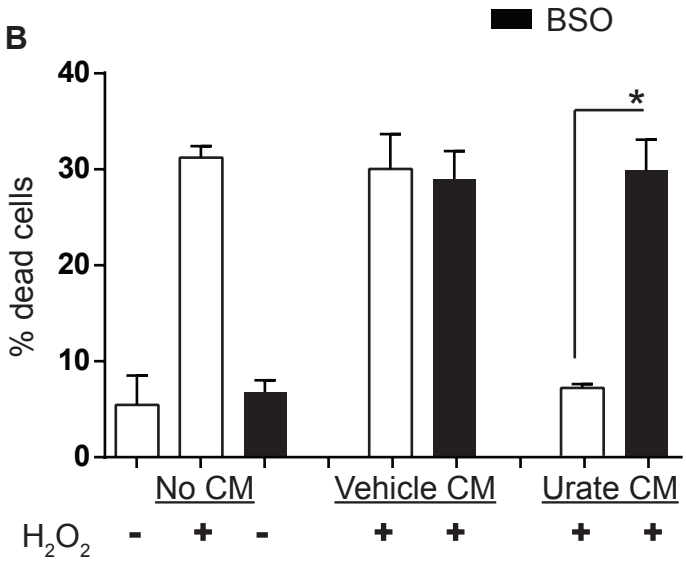
A

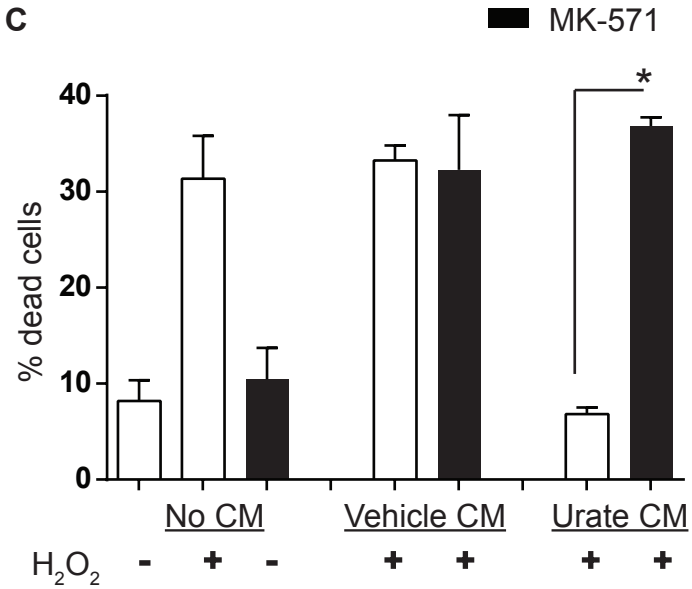

Vehicle
Urate
NRF2
NRF2
20 µm
GFAP
GFAP
DAPI
DAPI

B


N/C Ratio
Mean Relative Intensity
4
3
2
1
0
*
Vehicle
Urate


A
Astrocyte CM
GSH (µg/ml)
60
40
20
0
Vehicle
Urate
Urate+BSO
Urate+MK-571


B
BSO
% dead cells
40
30
20
10
0
*
No CM
Vehicle CM
Urate CM
H2O2 - + - + + + +


C
MK-571
% dead cells
40
30
20
10
0
*
No CM
Vehicle CM
Urate CM
H2O2 - + - + + + +

Neuroscience 274 (2014) 242–249

PROTECTION BY INOSINE IN A CELLULAR MODEL OF PARKINSON'S DISEASE

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Abstract—Inosine (hypoxanthine 9-beta-D-ribofuranoside), a purine nucleoside with multiple intracellular roles, also serves as an extracellular modulatory signal. On neurons, it can produce anti-inflammatory and trophic effects that confer protection against toxic influences *in vivo* and *in vitro*. The protective effects of inosine treatment might also be mediated by its metabolite urate. Urate in fact possesses potent antioxidant properties and has been reported to be protective in preclinical Parkinson's disease (PD) studies and to be an inverse risk factor for both the development and progression of PD. In this study we assessed whether inosine might protect rodent MES 23.5 dopaminergic cell line from oxidative stress in a cellular model of PD, and whether its effects could be attributed to urate. MES 23.5 cells cultured alone or in presence of enriched murine astroglial cultures MES 23.5–astrocytes co-cultures were pretreated with inosine (0.1–100 μM) for 24 h before addition of the oxidative stress inducer H_2O_2 (200 μM). Twenty-four hours later, cell viability was quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay or immunocytochemistry in pure and MES 23.5–astrocytes co-cultures, respectively. H_2O_2-toxic effect on dopaminergic cells was reduced when they were cultured with astrocytes, but not when they were cultured alone. Moreover, in MES 23.5–astrocytes co-cultures, indicators of free radical generation and oxidative damage, evaluated by nitrite (NO_2^-) release and protein carbonyl content, respectively, were attenuated. Conditioned medium experiments indicated that the protective effect of inosine relies on the release of a protective factor from inosine-stimulated astrocytes. Purine levels were measured in the cellular extract and conditioned medium using high-performance liquid chromatography (HPLC) method. Urate concentration was not significantly increased by inosine treatment however there was a significant increase in levels of other purine metabolites, such as adenosine, hypoxanthine and xanthine. In particular, in MES 23.5–astrocytes co-cultures, inosine medium content was reduced by 99% and hypoxanthine increased by 127-fold. Taken together these data raise the possibility that inosine might have a protective effect in PD that is independent of any effects mediated through its metabolite urate.

Key words: MES 23.5 cells, astrocytes, urate, HPLC, cell viability, oxidative stress.

INTRODUCTION

Inosine is a purine shown to have trophic protective effects on neurons and astrocytes subjected to hypoxia or glucose-oxygen deprivation (Haun et al., 1996) and to induce axonal growth following neuronal insult *in vivo* and *in vitro* (Zurn and Do, 1988; Benowitz et al., 1998; Petrausch et al., 2000; Chen et al., 2002; Wu et al., 2003). Moreover, inosine showed anti-inflammatory effects in the central nervous system (CNS) and periphery (Jin et al., 1997; Hasko et al., 2000; Gomez and Sitkovsky, 2003; Shen et al., 2005; Rahimian et al., 2010). Some (Toncev, 2006; Markowitz et al., 2009) but not all (Gonsette et al., 2010) clinical studies have suggested a possible antioxidant protective effect of inosine in multiple sclerosis patients (Markowitz et al., 2009). In these trials inosine consistently elevated serum urate, which was proposed to mediate any protective effect of inosine (Markowitz et al., 2009; Spitsin et al., 2010).

Oxidative stress is thought to be a key pathophysiological mechanism in Parkinson's disease (PD) leading to cellular impairment and death (Ross and Smith, 2007). Urate – a major antioxidant circulating in the human body – has emerged as an inverse risk factor for PD. Clinical and population studies have found the urate level in serum or CSF to correlate with a reduced risk of developing PD in healthy individuals and with a reduced risk of clinical progression among PD patients (Weisskopf et al., 2007; Schwarzschild et al., 2008; Ascherio et al., 2009). Moreover, in cellular and animal models of PD, urate elevation has been shown to reduce oxidative stress and toxicant-induced loss of dopaminergic neurons (Wang et al., 2010; Cipriani et al., 2012a,b; Gong et al., 2012; Zhu et al., 2012; Chen et al., 2013). Although inosine can elevate urate concentration in the periphery in animals and humans, little is known about its effect on the urate level in the CNS (Ceballos et al., 1994; Scott et al., 2002; Rahimian et al., 2010; Spitsin et al., 2010). A cellular study indicated that inosine added to cortical astroglial (but not neuronal) cultures increases urate concentration in the medium (Ceballos et al., 1994).

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Abbreviations: CNS, central nervous system; DHBA, 3,4-dihydroxybenzylamine; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; HPLC, high-performance liquid chromatography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PD, Parkinson's disease; SDS, sodium dodecyl sulfate.

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0306-4522/

In the present study we characterized a protective effect of inosine on oxidative stress-induced dopaminergic cell death in a cellular model of PD and investigated whether urate elevation might mediate the effect.

EXPERIMENTAL PROCEDURES

Animals

C57BL/6 mice were employed to obtain astroglial cultures. All experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals with approval from the animal subjects review board of the Massachusetts General Hospital.

MES 23.5 cell line

The rodent MES 23.5 dopaminergic cell line (Crawford et al., 1992) was obtained from Dr. Weidong Le at the Baylor College of Medicine (Houston, USA). MES 23.5 cells were cultured on polyornithine-coated T75 flasks (Corning Co, Corning, NY) in culture medium; Dulbecco modified Eagle medium (DMEM, Invitrogen/Gibco), added with Sato components (Sigma Immunochemicals), and supplemented with 2% newborn calf serum (Invitrogen), 1% fibroblast growth factor (Invitrogen), penicillin 100 U ml^{-1} and streptomycin 100 μg mL^{-1} (Sigma), at 37 °C in a 95% air–5% carbon dioxide, humidified incubator. Culture medium was changed every 2 days. At confluence, MES 23.5 cells were either sub-cultured new T-75 flasks or used for experiments. For experiments, MES 23.5 cells were seeded at a density of 600 cells per mm^2 onto polyornithine-coated plates or flasks (according to the assay, see below) in culture medium. Twenty-four hours later, it was changed to DMEM serum-free medium. At this time, increasing concentrations of inosine (0–100 μM) were added to the cultures for 24 h and again during toxicant treatment. 200 μM H_2O_2 wasadded to the cultures for 24 h and then cells were used for assays.

Enriched astroglial cultures

Astroglial cultures were prepared from the brains of 1- or 2-day-old neonatal mice as previously described (Cipriani et al., 2012b). Briefly, cerebral cortices were digested with 0.25% trypsin for 15 min at 37° C. The suspension was pelleted and re-suspended in culture medium (DMEM, fetal bovine serum (FBS) 10%, penicillin 100 U ml^{-1} and streptomycin 100 μg ml^{-1} to which 0.02% deoxyribonuclease I was added). Cells were plated at a density of 1800 cells per mm^2 on poly-L-lysine (100 μg ml^{-1})/DMEM/F12-coated flasks and cultured at 37 °C in humidified 5% CO_2 and 95% air for 7–10 days until reaching confluence.

In order to remove non-astroglial cells, flasks were agitated at 200 rpm for 20 min in an orbital shaker and treated with 10 μM cytosine arabinoside (Ara-C) dissolved in cultured medium for 3 days. After the treatment, astrocytes were subjected to mild trypsinization (0.1% for 1 min) and then sub-plated (120 cells per mm^2) onto poly-L-lysine (100 μg ml^{-1})/DMEM/F12-coated plates or flasks (according to the assay, see below) in DMEM plus 10% FBS for assays. Astroglial cultures comprised >95% astrocytes, <2% microglial cells and <1% oligodendrocytes; no neuronal cells were detected (Cipriani et al., 2012b).

MES 23.5–astrocytes co-cultures

MES 23.5 cells were cultured on a layer of enriched astroglial cultures prepared as described above. Briefly, astrocytes were allowed to grow for 48 h on poly-L-lysine (100 μg ml^{-1})/DMEM/F12-coated plates or flasks (according to the assay, see below) in DMEM plus 10% FBS. Then, MES 23.5 cells were seeded on top at a concentration of 600 cells per mm^2 in MES 23.5 culture medium. An astrocyte:MES 23.5 cell ratio of 1:5 was chosen on the basis of our previous observations (Cipriani et al., 2012b), which indicated this proportion of astrocytes as sufficiently low to avoid a direct effect of astrocytes on dopaminergic cell survival. Twenty-four hours later, medium was changed to DMEM serum-free medium and subjected to treatments. Inosine was added to the cultures 24 h before and during 200 μM H_2O_2 treatment. In our previous study this H_2O_2 concentration was shown to have no effect on astrocyte viability (Cipriani et al., 2012b). At the end of treatment, MES 23.5 cells were easily detached from astrocytes and dissociated by gently pipetting up and down the medium before processing for biochemical assays.

Conditioned media experiments

Enriched-astrocyte cultures were grown on poly-L-lysine (100 μg ml^{-1})/DMEM/F12-coated 6 well-plates in DMEM plus 10% FBS. Astrocytes were allowed to grow for three days and then the medium was changed to MES 23.5 culture medium in order to reproduce co-culture conditions. The day after, medium was changed to DMEM containing 100 μM inosine or vehicle. Twenty-four hours later, conditioned medium was collected and filtered through a 0.2 μM membrane to remove cellular debris. MES 23.5 cells were treated with increasing concentrations of conditioned medium 24 h before and during H_2O_2 treatment.

Drugs

Inosine was dissolved in DMEM as 20× concentrated stocks. H_2O_2 was dissolved in PBS (0.1 M, pH 7.4) as 100× concentrated stocks. Drugs were obtained from Sigma.

Cell viability and toxicity assessments

In MES 23.5 cultures, cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma). This assay is based on the conversion of the yellow tetrazolium salt MTT by mitochondrial dehydrogenase of live cells to the purple formazan (Hansen et al., 1989). Briefly, MES 23.5 cells were cultured in polyornithine-coated 96-well plates (600 cells per mm^2) and grown for at least 24 h. Then, the medium was changed to DMEM serum-free medium

for 24 h before H_2O_2 was added. In order to assess inosine protection, increasing concentrations of drug (0–100 μM) were loaded 24 h before and again during toxicant treatment. After washes, 100 μl of MTT solution (0.5 mg ml^{-1} in DMEM) was added for 3 h at 37 °C. Then, MES 23.5 cells were lysed with 10 μl/well of acidic isopropanol (0.01 M HCl in absolute isopropanol) to extract formazan that was measured spectrophotometrically at 490 nm with a Labsystems iEMS Analyzer microplate reader.

In MES 23.5–astrocytes co-cultures, surviving MES 23.5 cells were quantified by immunocytochemistry (Lotharius et al., 2005; Dumitriu et al., 2011; Cipriani et al., 2012b). MES 23.5 were grown on top of astrocytes in 96-well plates as described above. Increasing concentrations of drug (0–100 μM) were loaded 24 h before and again during toxicant treatment. After washing in PBS, cultures were fixed with 4% (wt/vol) paraformaldehyde for 1 h at room temperature. Then, cells were incubated with an Alexa 488-conjugated antibody specific for neuronal cells, Milli-Mark FluoroPan Neuronal Marker, (Millipore; 1:200, overnight at 4 °C). Fluorescence was read at 535 nm by using a microplate reader.

High-performance liquid chromatography (HPLC)

To determine purine content in cells and medium samples, MES 23.5, MES 23.5–astrocytes co-cultures or enriched astroglial cultures were prepared as described above and cultured in T75 flasks. Purine content was determined using our previously described HPLC-based analytical methods (Burdett et al., 2013). Briefly, cell medium was collected and added with 30% vol/vol of a buffer containing 150 mM phosphoric acid, 0.2 mM EDTA, and 1 μM 3,4-dihydroxybenzylamine (DHBA; used as internal standard). Cells were collected after washing in ice-cold PBS and purines were extracted in the same buffer used for medium. Samples were then filtered through a 0.2-μm Nylon microcentrifuge filter (Spin-X, Corning) at 4 °C. Samples were maintained at 4 °C and injected using an ESA Biosciences (Chelmsford, MA) autosampler, and chromatographed by a multichannel electrochemical/UV HPLC system with effluent from the above column passing through a UV–VIS detector (ESA model 528) set at 254 nm and then over a series of electrodes set at −100 mV, +250 mV and +450 mV. To generate a gradient two mobile phases were used. Mobile phase A consisted of 0.2 M potassium phosphate and 0.5 mM sodium 1-pentanesulfonate; mobile phase B consisted of the same plus 10% (vol/vol) acetonitrile. Mobile phase B increased linearly from 0% to 70% between 6th and 14th min of the run.

Nitrite (NO_2^-) release

MES 23.5–astrocytes co-cultures were grown on a 96-well plate as described above. After treatments, nitrite release (NO_2^-), an indicator of free radical generation, was quantified in cell medium by the Griess assay. An azo dye is produced in the presence of nitrite by the Griess reaction and colorimetrically detected. Briefly, 100 μl of supernatant collected from treated cultures were added to 100 μl of Griess reagent (Sigma) and absorbance was read at 540 nm with a microplate reader. Blanks were prepared by adding medium containing toxicants and/or protectants to the Griess solution.

Protein carbonyl protein assay

MES 23.5–astrocytes co-cultures were grown in 6-well plates as described above. After treatments, cultures were washed with ice-cold PBS and oxidized proteins were detected in MES 23.5 cells, using the Oxyblot assay kit (Chemicon). MES 23.5 cells were detached from astrocytes in ice-cold PBS, spun to form a pellet at 4 °C and resuspended in ice-cold RIPA buffer containing 50 mM DTT. Cells were allowed to lyse on ice for 15′. For the assay, 20 μg of protein were derivatized in 10 μL of 2,4-dinitrophenylhydrazine (DNPH). After derivatization samples were subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel (10% [wt/vol] acrylamide, 0.1% [wt/vol] SDS) and transferred electrophoretically onto 0.2 μ nitrocellulose membranes. Membranes were loaded with an antibody specific to dinitrophenylhydrazone moiety of the proteins and reaction visualized by chemiluminescence.

Protein detection

After treatment, cells were washed in ice-cold PBS, collected and resuspended in ice-cold Ripa buffer. Cells were incubated on ice for 15', followed by sonication for complete lysis. Proteins were quantified in 4 μl of each sample using Bio-Rad Protein Assay reagent (Biorad Laboratories) and measured at 600 nm with a microplate reader.

Statistical analysis

Statistical analysis was performed by GraphPad Prism version 4.00 (GraphPad Software Inc.). Unpaired Student's *t*-test was used when two group samples were compared. ANOVA analysis followed by Newman–Keuls was used when more than two group samples were compared. Values were expressed as mean ± SEM. Differences with a $P < 0.05$ were considered significant and indicated in figures by symbols explained in legends.

RESULTS

Astrocytes mediated protective effect of inosine on dopaminergic cells

Previously we showed that urate protected a dopaminergic cell line (MES 23.5) against oxidative stress when cells were cultured with astrocytes (Cipriani et al., 2012b). To assess whether inosine protected the dopaminergic cell line in a similar way we tested inosine on MES 23.5 cells cultured alone or with cortical astrocytes (MES 23.5–astrocytes co-cultures) treated with 200 μM H_2O_2.

Inosine on its own had no effect on MES 23.5 viability (one-way ANOVA, $P > 0.05$) (Fig. 1A), and showed only a trend toward modest protection with increasing

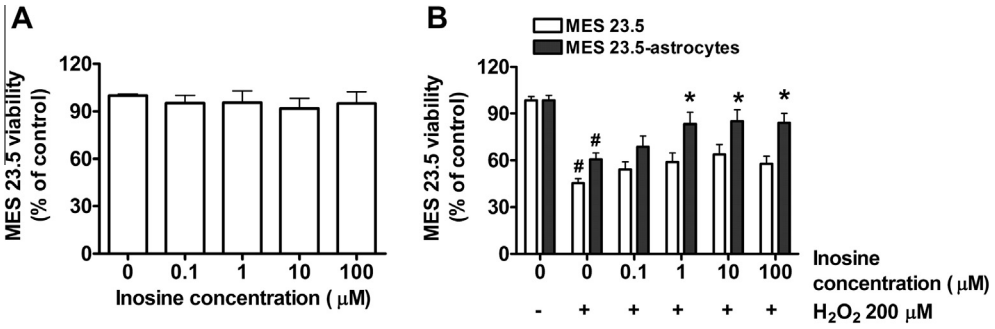

Fig. 1. Astrocytes potentiated the protective effect of inosine on 200 μM H_2O_2-treated MES 23.5 cells. (A) Viability of MES 23.5 cells treated for 24 h with increasing concentrations of inosine (0–100 μM). (B) Effect of inosine treatment (0–100 μM) at 24 h of toxic treatment with 200 μM H_2O_2 on viability of MES 23.5 cells cultured alone (white bars) or in the presence of astrocytes (gray bars). Cultures were treated with inosine 24 h before and during toxic treatment. Data represent mean ± SEM of values from four experiments, each of which yielded a mean of triplicate determinations for each condition. One-way ANOVA analysis ($P < 0.001$) followed by Newman–Keuls multiple comparison test ($^{\#}P < 0.01$ vs respective '0 inosine/ $-H_2O_2$' value; $^{*}P < 0.05$ vs '0 inosine/ $+H_2O_2$' value).

concentrations from 0.1 to 100 μM against H_2O_2 toxicity (one-way ANOVA, $P > 0.05$) in pure MES 23.5 cultures. However, in the presence of a relatively low density of astrocytes (plated at a density of 120 cells per mm^2), MES 23.5 cell viability significantly increased in comparison to inosine-untreated cells ($P < 0.05$; Fig. 1B).

Inosine decreased toxicant-induced oxidative stress

To determine whether protection was associated with reduced oxidative stress and protein damage, we measured the effect of inosine on oxidative stress markers in H_2O_2-treated co-cultures of MES 23.5 cells and astrocytes. At 24 h, inosine decreased the level of NO_2^- (nitrite), an indicator of free radical generation, from 2-fold to 1.4-fold of the control value in cell medium ($P = 0.00139$, Fig. 2A). Moreover, at 3 h inosine decreased protein oxidation, measured as protein carbonyl content in MES 23.5 cells (after removal from astrocytes), from 4.6- to 2.7-fold of control value ($P = 0.002$) (Fig. 2B).

Protection mediated by astrocytes does not require their physical contact with dopaminergic cells

We previously observed that astrocytes mediate urate's protective effect through the release of protective factor(s). To assess if astrocytes mediated inosine's protective effect in the same fashion, MES 23.5 cells were treated with increasing percentages of medium collected from untreated or inosine-treated astrocytes. Medium from untreated astrocytes did not show a statistically significant effect on H_2O_2-treated MES 23.5 viability at any given concentration ($P > 0.05$). On the other hand, conditioned medium from astrocytes treated for 24 h with 100 μM inosine improved MES 23.5 viability in a concentration-dependent manner ($P < 0.001$). This observation was confirmed by a two-way ANOVA analysis that showed significant effect of conditioned medium ($F_{1,151} = 46.28$, $P < 0.0001$) and conditioned medium percentage ($F_{1,151} = 7.31$, $P < 0.0001$) and significant interaction between these two factors ($F_{1,151} = 3.59$, $P = 0.0079$; Fig. 3).

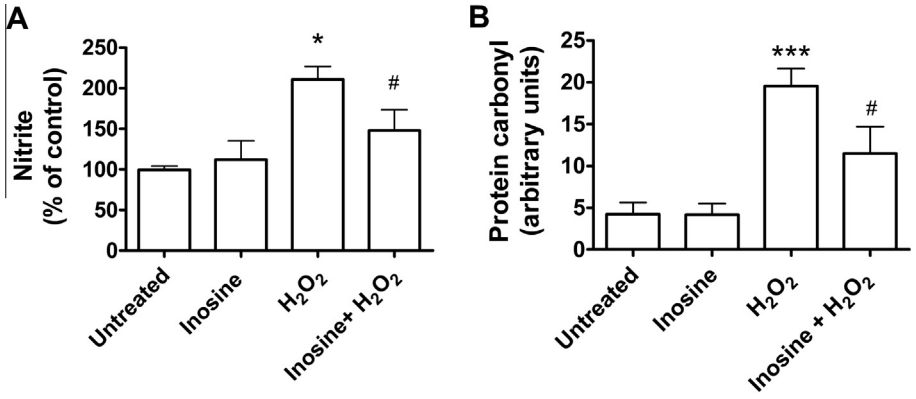

Fig. 2. Inosine reduced oxidative stress in MES 23.5 cells cultured with astrocytes. (A) Effect of inosine treatment (0–100 μM) on 200 μM H_2O_2 induced NO_2^- release in the medium of MES 23.5–astrocytes co-cultures at 24 h of toxic treatment. Cultures were treated with inosine 24 h before and during toxic treatment. Data represent mean ± SEM of three triplicate experiments. (B) Effect of inosine treatment (0–100 μM) on 200 μM H_2O_2 induced protein carbonylation in MES 23.5 cells cultured with astrocytes at 3 h of toxic treatment. Cultures were treated with inosine 24 h before and during toxic treatment. Data represent mean ± SEM of six replicates over three independent experiments. One-way ANOVA: $^{*}P < 0.05$ and $^{***}P < 0.001$ vs untreated and inosine (alone) values; $^{\#}P < 0.05$ vs H_2O_2 (alone) value.

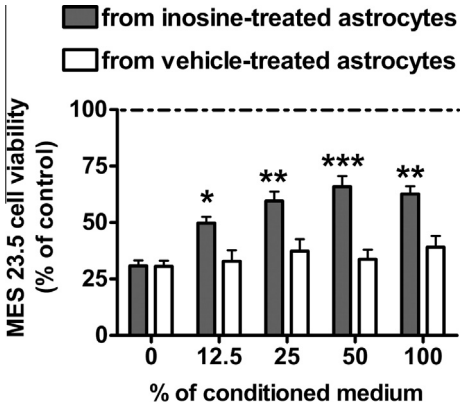

Fig. 3. Inosine-conditioned medium from astrocytes increased viability of H_2O_2-treated MES 23.5 cells. Effect of increasing concentration of cell medium collected from control (white bars) or 100 μM of inosine-treated astrocytes (gray bars) on 200 μM H_2O_2-induced cell death in MES 23.5 pure cultures. Cultures were treated with conditioned medium 24 h before and during toxic treatment. Data represent mean ± SEM of thirteen independent experiments. Two-way ANOVA analysis ($P = 0.0003$) followed by the Newman–Keuls multiple comparison test ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs respective control value).

Inosine treatment did not affect urate concentration

Extracellular inosine breakdown has been reported in astroglial cultures (Ceballos et al., 1994). To determine whether inosine degradation occurred in our cultures, purine metabolites of inosine were measured in the medium of MES 23.5–astroglial co-cultures treated with inosine. For this experiment we selected two time points: 0, when inosine was added to the cultures, and 24 h, when the cultures would be treated with toxicant. Over 24 h inosine concentration, reflecting both endogenous plus exogenous contributions, was reduced by 99% ($P < 0.0001$); over the same time period hypoxanthine and xanthine increased by 127-fold ($P < 0.0001$) and 1.5-fold ($P < 0.0001$), in comparison to time zero, respectively (Table 1). Thus, the hypoxanthine increment was 1.6-fold greater than the amount of inosine added.

Moreover, adenosine, an inosine 'precursor', increased by 4-fold ($P = 0.0001$, Table 1) over the 24 h. By contrast, urate content was not changed in the medium over the same time period ($P = 0.46$, Table 1), indicating that extracellular urate unlikely mediated inosine's effects.

In our previous studies we found evidence that urate's protective effect on dopaminergic cells was correlated with its increase within astrocytes (Cipriani et al., 2012b). To assess whether inosine treatment increased intracellular urate in astroglial cells its concentration was measured in inosine-treated enriched astroglial cultures at time 0 and 24 h of treatment. Although adenosine increased 2-fold, intracellular concentrations of urate and other purines were not changed at 24 h in comparison to time 0 (Table 2) and vehicle-treated cells (data not shown). Similarly, no effect was seen on extracellular urate, where inosine induced an approximately 5-fold increase in hypoxanthine concentration ($P < 0.01$, Table 3). Thus despite the expression of functional xanthine oxidase, the enzyme that converts hypoxanthine to xanthine and in turn to urate in cortical astrocytes (Ceballos et al., 1994), we did not find evidence of the conversion of inosine to urate.

Purine increase induced by inosine in mixed-cultures might play a role in inosine protective effect. To assess whether this effect was selective for mixed-cultures, inosine metabolite concentration was also measured in the medium of MES 23.5 cultures after inosine treatment. Similarly to mixed cultures, in MES 23.5 cultures inosine concentration decreased to about 30% ($P < 0.0001$) and 3% ($P < 0.0001$) of control at 6 and 24 h, respectively. Hypoxanthine increased over the time up to 4-fold ($P < 0.0001$) at 24 h in comparison to time zero and xanthine by 1.8-fold in comparison to 6 h (Table 4). Moreover, adenosine increased about 9-fold ($P < 0.0001$) in comparison to time zero. Urate concentration did not change at any tested time (Table 4). These data exclude a direct effect of inosine metabolites on MES 23.5 cells since no protective effect was found in these experimental conditions.

DISCUSSION

We report that inosine prevented oxidative stress-induced cell death in dopaminergic MES 23.5 cells cultured with astrocytes. This effect appeared to be independent of increased intracellular urate, an inosine metabolite and established antioxidant.

Table 1. Extracellular purine content at time zero (0) and 24 h in 100 μM inosine-treated MES 23.5–astrocytes co-cultures

Analites	Concentration (μM)	
	0	24 h
Adenosine	0.16 ± 0.01	0.82 ± 0.04***
Inosine	104 ± 8	0.74 ± 0.09***
Hypoxanthine	1.25 ± 0.26	160 ± 28***
Xanthine	0.34 ± 0.03	0.85 ± 0.05***
Urate	0.89 ± 0.04	0.83 ± 0.05

Purine content of co-cultures cell medium was analyzed by high-performance liquid chromatography. Data are expressed as μM. Significance was determined by Student's *t* test: $^{***}P < 0.001$ vs 0 time point value. Data are presented as mean ± SEM of eight experiments.

Table 2. Intracellular purine content at time zero (0) and 24 h in 100 μM inosine-treated enriched astroglial cultures

Analites	Concentration (nmol/g of protein)	
	0	24 h
Adenosine	130 ± 48	390 ± 137*
Inosine	607 ± 230	477 ± 204
Hypoxanthine	320 ± 77	400 ± 199
Xanthine	3 ± 1	6 ± 3
Urate	38 ± 5	26 ± 2

Purines were extracted from enriched astroglial cultures by cell trituration in extracting buffer (see methods) and measured by high-performance liquid chromatography. Data are expressed as nmol/g of protein. Significance was determined by Student's *t* test: $^{*}P = 0.012$. Data are presented as mean ± SEM of four experiments.

Table 3. Extracellular purine content at time zero (0) and 24 h in 100 μM inosine-treated enriched astroglial cultures

Analites	Concentration (μM)	
	0	24 h
Adenosine	1.7 ± 0.1	1.6 ± 0.4
Inosine	105 ± 1	9.0 ± 0.1***
Hypoxanthine	22.6 ± 0.1	127 ± 21**
Xanthine	8.5 ± 0.1	13 ± 2
Urate	20.3 ± 0.1	24 ± 4

Purine content of astrocyte medium was measured by high-performance liquid chromatography. Data are expressed as μM. Significance was determined by Student's *t* test: $^{**}P < 0.01$ and $^{***}P < 0.001$ vs 0 time point value. Data are presented as mean ± SEM of four experiments.

Table 4. Extracellular purine content in 100 μM inosine-treated MES 23.5 cells over 24 h of treatment

Analites	Concentration (μM)		
	0	6 h	24 h
Adenosine	0.28 ± 0.09	2.28 ± 0.08	2.8 ± 0.4**
Inosine	89 ± 20	30 ± 2**	2.6 ± 0.1***
Hypoxanthine	149 ± 10	680 ± 43***	780 ± 81***
Xanthine	N.D.	0.10 ± 0.04	0.28 ± 0.07*
Urate	N.D.	N.D.	N.D.

Purine content of astrocyte medium was measured by high-performance liquid chromatography.
Data are expressed as μM. Student's *t* test, $n = 8$, $^{*}P < 0.05$ vs 6 h value. One-way ANOVA followed by Newman–Keuls test: $^{**}P < 0.01$ and $^{***}P < 0.001$ vs 0 time point value. Data are presented as mean ± SEM of eight experiments.

As a close structural homolog of adenosine, inosine may confer protection by direct mechanisms, activating multiple subtypes of adenosine receptors that are known to modulate cell death. Several studies have implicated A_1, A_{2A} or A_3 receptors as mediators of inosine effects in the setting of inflammatory or ischemic injury (Jin et al., 1997; Gomez and Sitkovsky, 2003; Shen et al., 2005; Rahimian et al., 2010). For example, inosine was found to reduce ischemic brain injury in rats likely via an adenosine A_3 receptor-dependent pathway (Shen et al., 2005).

In vitro studies showed inosine to be protective in models of hypoxia (Litsky et al., 1999) and glucose–oxygen deprivation (Haun et al., 1996) where it mediated adenosine protective effects. Inosine has been shown to protect neurons with a neurotrophic effect, promoting axonal regeneration *in vivo* and *in vitro* (Zurn and Do, 1988; Chen et al., 2002; Wu et al., 2003) and inducing the expression of axonal growth-associated genes (Benowitz et al., 1998; Petrausch et al., 2000). This neuroprotective effect can be exerted with a receptor-independent mechanism, for example, activating the cytoplasmic protein kinase Mst3b as shown in the setting of stroke or traumatic brain injury in rodents (Zai et al., 2011). *In vitro* and *in vivo* studies showed inosine to have anti-inflammatory effects in inflammatory or ischemic injury (Jin et al., 1997; Hasko et al., 2000; Gomez and Sitkovsky, 2003; Shen et al., 2005; Rahimian et al., 2010). Moreover, a clinical study raised the possibility that inosine may have antioxidant properties improving structural and neurological impairment in multiple sclerosis patients (Markowitz et al., 2009).

A previous study reported that inosine protection against chemical hypoxia was dependent on the presence of astrocytes in cultures (Litsky et al., 1999). Similarly, we show that inosine's protective effect on dopaminergic cells was mediated by astrocytes, suggesting a mechanism more complex than a direct protective effect exerted by inosine. Moreover, the rapid inosine degradation occurring in cultures would suggest more of an indirect effect of inosine, which would be consistent with stimulated production and release of an astrocytic protective factor(s) (Imamura et al., 2008).

The rapid elimination of exogenous inosine and increase in its precursor and metabolites are also consistent with the possibility that a purine related to inosine mediates its protective effect. Treatment with inosine at a high concentration relative to endogenous levels increased the concentration of its precursor adenosine in co-cultures, suggesting either conversion of inosine into adenosine (Murray, 1971) or feedback inhibition of adenosine deaminase (Meyskens and Williams, 1971) leading to reduced degradation of endogenous adenosine. Extracellular adenosine in turn may act on its own receptors to enhance survival of dopaminergic neurons in cultures (Michel et al., 1999) or it can be taken up by neurons (Hertz and Matz, 1989).

Alternatively, increased metabolism of inosine may have mediated its protective effect. Inosine breakdown protected cells subjected to glucose deprivation or hypoxia-reoxygenation preserving cellular ATP content (Jurkowitz et al., 1998; Shin et al., 2002; Módis et al., 2009; Szoleczky et al., 2012). Intracellular inosine (and adenosine by way of inosine) was shown to be transformed to hypoxanthine and ribose 1-phosphate by purine nucleotides phosphorylase (Jurkowitz et al., 1998). In turn, ribose 1-phosphate was converted to an intermediate that can enter the anaerobic glycolytic pathway providing the ATP necessary to maintain cell integrity (Jurkowitz et al., 1998). Inhibition of the enzyme purine nucleoside phosphorylase notably prevented the neuroprotective effect of inosine in glial cells and mixed astrocyte-neuronal cultures (Jurkowitz et al., 1998; Litsky et al., 1999). Moreover, this pathway can represent the primary energy source for erythrocytes lacking functional glucose transporters (Young et al., 1985). In our study we found that the hypoxanthine increment was about 24-times higher in MES 23.5-astrocytes co-cultures than in MES 23.5 cells alone after inosine treatment. Purine nucleoside phosphorylase is highly expressed in astrocytes (Ceballos et al., 1994); thus the presence of astrocytes in cultures might provide conditions sufficient for enhanced ATP production during the toxic insult. This raises the possibility that the anaerobic glycolytic pathway might contribute to the protective effect of inosine on dopaminergic cells during oxidative stress. A role for this pathway and the associated production of hypoxanthine by increased purine nucleoside phosphorylase activity in astrocytes may also account for the observed hypoxanthine increase in molar excess of

exogenous inosine introduced. Regardless of whether altered cellular energy metabolism induced by inosine breakdown or a specific metabolite of inosine is protective, these scenarios support the hypothesis that inosine treatment might induce release of factor(s) from astrocytes to protect dopaminergic cells.

Inosine has been shown to be converted to urate in cultures (Ceballos et al., 1994) and to elevate urate serum level in rodents and humans (Ceballos et al., 1994; Scott et al., 2002; Rahimian et al., 2010; Spitsin et al., 2010). Although we observed higher extracellular concentrations of inosine's metabolites, such as hypoxanthine, we did not find increased urate levels in media or in astrocytes. It is unlikely that an earlier increase in urate was missed due to its being metabolized to allantoin since we have already shown that cortical astrocytes and MES 23.5 cells do not express urate oxidase, the enzyme that converts urate to allantoin (Cipriani et al., 2012b). Together these observations argue against a role for urate as the mediator of inosine's protective effects in this cellular model of oxidative stress in PD. However, purine metabolism is of course different in intact humans versus murine culture models and the present findings of a urate-independent protective effect in culture do not preclude the protective effect of urate, which can be substantially elevated in people treated with inosine (The Parkinson Study Group SURE-PD Investigators et al., 2014).

In PD the degeneration of dopaminergic neurons is thought to be induced by accumulation of oxidative damage that leads to mitochondrial impairment and protein aggregation. The finding that inosine prevents oxidant-induced dopaminergic cell loss may be of substantial epidemiological and therapeutic significance for PD. A phase II clinical trial of inosine in early PD showed that inosine was safe, tolerable and effective in raising CSF and serum urate levels (The Parkinson Study Group SURE-PD Investigators et al., 2014). Our results suggest that if CNS inosine itself was elevated in the CNS of treated individuals it could produce a neuroprotective effect independent of urate.

CONCLUSIONS

Inosine had antioxidant and protective effects on dopaminergic cells with a mechanism that does not require increased urate concentration. This finding further supports inosine as a candidate for PD therapy.

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Appendix D

Association of α-synuclein gene expression with Parkinson's disease is attenuated with higher serum urate in the PPMI cohort

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Objective: To explore how urate may modulate Parkinson's disease (PD)-specific pathogenic mechanisms using clinical biomarker data. Urate is the end product of purine metabolism in humans, but also possesses potent antioxidant and neuroprotective properties. Lower serum urate is a reproducible risk factor both for developing PD and for a more rapid rate of its clinical progression.

Methods: Data were analyzed from the Michael J. Fox Foundation's Parkinson's Progression Markers Initiative (PPMI), which enrolled 218 people with early, untreated PD and 153 healthy control (HC) subjects for whom baseline blood levels of urate and α-synuclein gene (*SNCA*) transcript were available.

Results: *SNCA* transcript counts are substantially reduced (p=0.0001) in PD compared to HC among those with lower urate (below the median HC value of 5.4 mg/dL), but not appreciably different among those with higher urate (p=0.5). In further analysis fully adjusting for relevant covariates, the odds of having PD were markedly lower among individuals with more *SNCA* transcripts only if they also had lower urate (OR = 0.61 / 10^3 mRNA, p=0.002), not higher urate (OR = 0.91 / 10^3 mRNA, p=0.6), with a significant interaction between urate and *SNCA* transcripts (p=0.02).

Table: Levels of *SNCA* transcript (UTR-1) in blood are reduced in PD among those with lower but not higher serum urate in PPMI

		Lower Urate (≤ 5.3 mg/dL)***		Higher Urate (> 5.3 mg/dL)****		p for interaction
SNCA mRNA counts	HC (SD)	2188 (1182)		1941 (1227)		
	PD (SD)	1598 (874)		1810 (1086)		
	PD/HC	0.73	p = 0.0001	0.93	p = 0.5	
Odds Ratio per 10^3 mRNA	PD vs HC* (95% CI)	0.62 (0.46, 0.83)	p = 0.002	0.96 (0.72, 1.39)	p = 0.8	0.03
	PD vs HC** (95% CI)	0.61 (0.44, 0.83)	p = 0.002	0.91 (0.66, 1.27)	p = 0.6	0.02

*adjusted for age and gender

**adjusted for age, gender, BMI and transcript-related labs (WBC, RBC, Plt)

***n=187 of PPMI cohort with urate and *SNCA* mRNA at or below the serum urate median; n =182 for OR analysis.

****n=184 of PPMI cohort with urate and SNCA mRNA above the serum urate median; n=153 fro OR analysis.

Conclusions: These preliminary data suggest that the impact of α-synuclein on PD is attenuated in the presence of higher concentrations of urate.

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