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Protection of dopaminergic cells by urate requires its accumulation in astrocytes

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Abstract

Urate is the end product of purine metabolism and a major antioxidant circulating in humans. Recent data link higher levels of urate with a reduced risk of developing Parkinson's disease (PD) and with a slower rate of its progression. In this study, we investigated the role of astrocytes in urate-induced protection of dopaminergic cells in a cellular model of PD. In mixed cultures of dopaminergic cells and astrocytes oxidative stress-induced cell death and protein damage were reduced by urate. By contrast, urate was not protective in pure dopaminergic cell cultures. Physical contact between dopaminergic cells and astrocytes was not required for astrocyte-dependent rescue as shown by conditioned medium experiments. Urate accumulation in dopaminergic cells and astrocytes was blocked by pharmacological inhibitors of urate transporters expressed differentially in these cells. The ability of a urate transport blocker to prevent urate accumulation into astroglial (but not dopaminergic) cells predicted its ability to prevent dopaminergic cell death. Transgenic expression of uricase reduced urate accumulation in astrocytes and attenuated the protective influence of urate on dopaminergic cells. These data indicate that urate might act within astrocytes to trigger release of molecule(s) that are protective for dopaminergic cells.

Keywords

MES 23.5 cells; transporter; HPLC; cell viability; uricase; transgenic

Introduction

Currently, some 90% of Parkinson's disease (PD) cases are classified as sporadic, reflecting the uncertainty of their causes. A combination of genetic and environmental factors is thought to trigger pathogenic cascades that converge to increase oxidative stress or to reduce natural antioxidant defenses, leading to cellular impairment and the neurodegeneration characteristic of PD (Ross and Smith, 2007). Dopaminergic neurons in the substantia nigra *pars compacta* are highly sensitive to oxidative stress and their selective degeneration is responsible for the progressive motor disability of PD.

Urate (2,6,8-trioxy-purine; a.k.a. uric acid) circulates in humans at concentrations that are near its limit of solubility and many fold higher than in most other mammals. In humans and apes urate is the enzymatic end product of purine metabolism due to mutations of the uricase (a.k.a. urate oxidase) gene (*UOX*) that occurred during hominoid evolution (Oda *et al.*,

2002). The resulting urate elevation has been hypothesized to have raised antioxidant levels in human ancestors and thereby lengthened their lifespans. Urate possesses antioxidant properties comparable to those of ascorbate (Ames *et al.*, 1981) and forms stable coordination complexes with iron and other metal ions (Davies *et al.*, 1986), accounting for its ability to reduce oxidative damage caused by reactive nitrogen and oxygen species (Whiteman *et al.*, 2002).

Recently, epidemiological and clinical studies have found people with higher serum levels of urate to be less likely to develop PD (Weisskopf *et al.*, 2007). Moreover, amongst PD patients those with higher urate in serum or CSF showed a slower rate of disease progression assessed clinically (Schwarzschild *et al.*, 2008; Ascherio *et al.*, 2009), or radiographically as a reduced rate of dopaminergic nerve terminal marker loss (Schwarzschild *et al.*, 2008). In PD models urate attenuated motor and dopaminergic deficits in rodents (Wang *et al.*, 2010). *In vitro*, urate reduced oxidative stress as well as cell death induced by toxicants in dopaminergic cell lines (Duan *et al.*, 2002; Haberman *et al.*, 2007), and rescued dopaminergic neurons in a model of spontaneous cell death (Guerreiro *et al.*, 2009). Similarly, we reported that urate prevented dopaminergic neuron death induced by MPP⁺ in ventral mesencephalon cultures, and conversely that enzymatically lowering urate levels exacerbated this neurotoxicity (Cipriani *et al.*, 2012). Although the mechanism of neuroprotection by urate remains largely unknown, urate rescue of spinal cord neurons from excitotoxicity has been found to depend upon an astroglial mechanism (Du *et al.*, 2007), consistent with our recent evidence that the neuroprotection conferred on cultured dopaminergic neurons by raising intracellular urate can be enhanced by co-culturing with astroglia (Cipriani *et al.*, 2012).

In the present study, we assess the role played by astrocytes in the protective effect of urate on dopaminergic cells in a cellular oxidative stress model of PD.

Materials and Methods

Mice

Transgenic (Tg) *UOx* mice (Kono *et al.*, 2010) were obtained from Kenneth L. Rock and the University of Massachusetts. Mice were backcrossed eight times on the C57BL/6 genetic background and phenotyped by measuring *UOx* activity in serum samples (Cipriani *et al.*, 2012). All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals with approval from the animal subjects review board of Massachusetts General Hospital.

MES 23.5 cell line

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 Weidong Le at Baylor College of Medicine (Houston, USA). Despite the inherent environmental (*in vitro*) and cellular (tumor cell) limitations in modelling dopaminergic neuron degeneration, the dopaminergic properties of MES 23.5 cells and their molecular responses to dopaminergic neuron toxins have been well characterized and support its relevance as a cellular model of the dopaminergic neuron degeneration in PD. MES 23.5 cells were cultured on polyornithine-coated T75 flasks (Corning Co, Corning, NY) in the Dulbecco modified Eagle medium (DMEM) (Invitrogen/Gibco), which contained Sato components (Sigma Immunochemicals), 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. The culture medium was changed every 2 days, MES 23.5 cells were subcultured

either in newT-75 flasks or plated onto polyornithine-coated plates. MES 23.5 cells were used at passage 10–20, at which we confirmed the persistence of its dopaminergic phenotype (Crawford et al., 1992) by quantifying its high content of dopamine (23 ± 3 pmol/mg protein) using HPLC with electrochemical detection (Xu *et al.*, 2010).

Astroglia-enriched cultures

Astroglial cultures were prepared from the brain of 1- or 2-day-old neonatal mice with modifications to previously reported procedures (Saura *et al.*, 2005). Cerebral cortices were carefully stripped of their meninges and digested with 0.25% trypsin for 15 min at 37°C. Trypsinization was stopped by adding an equal volume of 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. The suspension was pelleted, re-suspended in culture medium and triturated to a single cell suspension by repeated pipetting followed by passage through a 70 µm-pore mesh. Cells were seeded at a density of 1,800 cells per mm² on poly-L-lysine (100 µg ml⁻¹)/DMEM/F12-coated flasks and cultured at 37°C in humidified 5% CO₂-95% air. Medium was fully changed on the fourth day and then every other day. Cultures reached confluency after 7–10 days *in vitro*.

In order to remove oligodendrocytes and microglial cells, flasks were agitated at 200 X g for 20 min in an orbital shaker. Following shaking medium was changed and flasks were again agitated at 100 X g for 18–20 hours. Floating cells were washed away and cultures were treated with 10 µM cytosine arabinoside (Ara-C) for 3 days. Our astroglial cultures comprised > 95% astrocytes, < 2% microglial cells and < 1% oligodendrocytes. No neuronal cells were detected (Suppl. Fig. 1A–C).

In order to prepare astroglia-enriched cultures from UOx wild-type (WT) and Tg pups individual cultures were prepared from cortices of each pup. The rest of the brain was used for phenotyping by western blotting. Brain tissue extracts were considered WT when they were negative to UOx staining and Tg when they showed a band at 32 kDa corresponding to UOx. Cultured cell phenotypes were confirmed by measuring UOx activity in the cell medium.

Co-cultures

Astroglia-enriched cultures were prepared as described above. After Ara-C treatment astrocytes were detached from flasks by mild trypsinization (0.1% for 1 min) and re-plated on pre-coated plates in DMEM plus 10% FBS. Astrocytes were allowed to grow for two days before MES 23.5 cells were seeded on top of them at a concentration of 600 cells per mm². MES 23.5 cells were detached from astrocytes by pipetting before processing for dopamine and protein carbonyl assays.

Co-cultures were imaged by an Olympus BX50 microscope with a 20X/0.50 objective and Olympus DP70 camera. Images were processed with DP controller software (Olympus) and merged with ImageJ (NIH).

Conditioned medium experiments

Enriched astroglial cultures were treated with 100 µM urate, or vehicle. Twenty-four hours later conditioned media were collected and filtered through a 0.2 µm membrane to remove cellular debris and immediately used for following experiments. MES 23.5 cells were treated with increasing proportions of conditioned medium 24 hours before and during H₂O₂ treatment. In UOx experiments the enzyme was added to astrocytes for 15 hours before conditioned medium collection.

Drug treatment

Urate was dissolved in DMEM as 20X concentrated stocks. H₂O₂ was dissolved in PBS (0.1 M, pH 7.4) as 100X concentrated stocks. Probenecid and hydrochlorothiazide (HCTZ) were dissolved in ethanol and pyrazinoate (PZO) in DMEM 50X concentrated stocks. Drugs were obtained from Sigma.

Cell viability

In MES 23.5 cultures, cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma) (Hansen *et al.*, 1989). MES 23.5 cells were seeded onto polyornithine-coated 96-well plates (600 cells per mm²) and grown for at least 24 hours until the cells became 70–80% confluent. The medium was changed to DMEM serum-free medium for 24 hours before increasing concentrations (50–800 μM) of H₂O₂ were added to the culture medium. In order to assess protection by urate, increasing concentrations (0–100 μM) were loaded 24 hours before and again during toxicant treatment. After three washes with DMEM, 100 μl of MTT solution (0.5 mg ml⁻¹ in DMEM) were added for 3 h at 37°C. Then MES 23.5 cells were lysed with acidic isopropanol (0.01M HCl in absolute isopropanol) to extract formazan, which was measured spectrophotometrically at 490 nm with a Labsystems iEMS Analyzer microplate reader. The *n* for each treatment refers to the number of triplicate data points, which were usually obtained from separate 96-well plates.

In co-cultures, living MES 23.5 cells were quantified by immunocytochemistry. After treatments astrocytes-MES 23.5 co-cultures were fixed with 4% paraformaldehyde for 1 hour at room temperature. Then cells were loaded with a blocking solution (0.5% albumin, 0.3% Triton-X in phosphate buffered saline) for 30 min at room temperature and then incubated with an Alexa 488-cojugated antibody specific for neuronal cells (1:200, overnight at 4°C; FluoroPan Neuronal Marker). The following day fluorescence was read at 535 nm by means of a microplate reader.

High-Performance Liquid Chromatography

Cells were scraped in a solution of 150 mM phosphoric acid, 0.2 mM EDTA, and 1 μM 3,4-dihydroxybenzylamine (DHBA; as internal standard) and chromatographed by a multi-channel electrochemical/UV HPLC system as previously described (Burdett *et al.*, 2012).

Western blot assay

Cells were scraped in ice-cold RIPA buffer (Sigma), boiled for 5 min in an appropriate volume of 6 X loading buffer, loaded (50 μg of proteins per well) into a 12% SDS-PAGE gel and run at 120 mV. Proteins were then transferred electrophoretically onto 0.2 μm nitrocellulose membranes (Biorad Laboratories) and saturated for 1 hour at room temperature with blocking buffer (5% non-fat dry milk in Tris buffered saline, 0.1% TWEEN-20). To detect urate transporter expression membranes were probed overnight with the following primary antibodies: rabbit glucose transporter 9 (GLUT9)-specific polyclonal antibody (1:2000; Abcam Inc), mouse organic anion transporter 1 (OAT1)-specific monoclonal antibody (1:200; Abbiotec), goat urate transporter 1 (URAT1)-specific polyclonal antibody (1:200; Santa Cruz, Inc). Kidney or brain extract was used as positive control. Proteins were visualized using chemiluminescence (Immobilon; Millipore). To detect UOx expression samples were prepared as described above and loaded into a 10% SDS-PAGE gel. Membranes were probed overnight with a rabbit UOx-specific polyclonal antibody (1:200; Santa Cruz, Inc). Liver extract was used as positive control.

In order to normalize the values of stained bands β-actin was detected on the same blot run. Membranes were stripped by strong agitation with 0.2 N NaOH (10 min at room

temperature), blocked in blocking buffer for 1 hour and probed for 2 hours at room temperature with anti- β -actin antibody (1:2000; Sigma). Membranes were incubated with horseradish peroxidase-conjugated rabbit-specific (1:2000; Pierce Biotechnology), mouse-specific (1:2000; Pierce Biotechnology; Rockford, IL) or goat-specific antibody (1:2000; Biorad Laboratories) and developed as above. Bands were acquired as JPG files; densitometric analysis was performed by ImageJ software (NIH).

Nitrite (NO_2^-) release

NO_2^- is an indicator of free radical generation and it is a major unstable product of nitric oxide and molecular oxygen reactions. After treatment 100 μl of supernatant were added to 100 μl of Griess reagent (Sigma) and spectrophotometrically read at 540 nm with a microplate reader. Blanks were prepared by adding medium containing toxicants and/or protectants to Griess solution.

Protein carbonyl protein assay

Oxidized proteins were detected using the Oxyblot assay kit (Chemicon) according to the manufacturer's instructions. Briefly, protein carbonyl groups were derivatized with 2,4-dinitrophenylhydrazine (DNPH), subjected to 10% SDS-PAGE and transferred electrophoretically onto 0.2 μm nitrocellulose membranes. Membranes were loaded with an antibody specific to the DNP moiety of the proteins and developed using chemiluminescence.

Protein detection

Protein concentration was measured in 4 μl of each sample using Bio-Rad Protein Assay reagent (Biorad Laboratories) and measured spectrophotometrically at 600 nm with a microplate reader.

Statistical analysis

Statistical analyses were performed by GraphPad Prism version 4.00 (GraphPad Software). Unpaired Student's *t*-test was used when only two group samples were compared. ANOVA analysis, followed by Newman Keuls or Bonferroni post-hoc test, was used when more than two group samples were compared. Values were expressed as mean $\pm$ SEM. Differences at the $p < 0.05$ were considered significant and indicated in figures by symbols explained in legends.

Results

Astrocyte-dependent protection of dopaminergic cells by urate

To reproduce an oxidative stress model of PD (Sherer *et al.*, 2002; Anantharam *et al.*, 2007) we incubated MES 23.5 cells with increasing concentrations of H_2O_2 . Treatment for 24 hours with H_2O_2 decreased cell viability in a concentration-dependent manner (Fig. 1A) with about 60% of cell death at 200 μM , which was the H_2O_2 concentration chosen for following experiments.

To evaluate the effect of urate on H_2O_2 -induced cell death urate was added to cultures 24 hours before and during H_2O_2 application. Urate treatment tended to decrease H_2O_2 -induced cell death over a concentration range of 0–100 μM (Fig. 1B) without a statistically significant effect.

Du and coworkers (Du *et al.*, 2007) reported that urate's protective effect on primary spinal cord neurons was dependent by the presence of astrocytes in cultures. To assess whether

urate protects dopaminergic cells cultured with astrocytes against oxidative stress, its effect was tested on MES 23.5-astrocytes co-cultures treated with H₂O₂. In order to minimize confounding effects by astroglial established inherent protection on dopaminergic cells (Yu and Zuo, 1997), H₂O₂ toxicity was assessed in co-cultures established at astrocytes/MES 23.5 cells ratios of 0:1, 1:1 and 1:5. Astrocytes cultured with MES 23.5 cells at a ratio of 1:5 did not prevented H₂O₂-induced death in MES 23.5 cells (Fig. 1C); the same ratio was employed in following experiments. H₂O₂ did not affect astrocyte viability up to the highest tested concentration of 200 μ M (data not shown). Urate added to co-cultures 24 hours before and during H₂O₂ application conferred significant, dose-dependent protection on H₂O₂-treated dopaminergic cells (Fig. 1D, E–G).

Urate decreased reactive oxygen species (ROS) production and protein oxidation

To determine if protection is associated with reduced oxidative stress and protein damage, we measured ROS in cell media from H₂O₂-treated co-cultures of MES 23.5 cells and astrocytes. H₂O₂ raised the concentration of NO₂[−] (nitrite) in the medium over time (Fig. 2A). Urate significantly decreased medium NO₂[−] concentration in H₂O₂- treated cultures at 24 hours of treatment (Fig. 2B). As an index of oxidative damage, protein carbonyl levels were measured in MES 23.5 cells (after removal from astrocytes) and found to be increased by H₂O₂ over time (Fig. 2C). Urate attenuated the increase in protein oxidation at 3 hours of treatment with H₂O₂ (Fig. 2D).

Astrocytes mediate protection by urate without physically contacting dopaminergic cells

MES 23.5 cells were treated with increasing percentages of medium collected from vehicle-treated (control) or urate-treated astrocytes. Medium from control astrocytes did not increase viability of H₂O₂-treated MES 23.5 cells at any concentration, whereas conditioned medium from astrocytes treated for 24 hours with 100 μ M urate significantly increased MES 23.5 viability in a concentration-dependent manner.

To address a possible direct effect of carry-over urate on MES 23.5 cells, we added UOx or vehicle to astroglial cultures after 24 hours of treatment with 100 μ M urate. UOx-catalyzed (> 99.9%) elimination of urate from the conditioned medium was confirmed by HPLC measurements of 0.020 ± 0.003 μ M versus 98 ± 12 μ M urate 15 hours after addition of UOx versus vehicle, respectively ($p < 0.0001$). The protective effect of conditioned medium was only slightly attenuated by UOx, indicating that carry-over urate could not account for most of the protection conferred by urate-treated astrocyte-conditioned medium (Fig. 3B). The finding is consistent with our earlier observations that urate alone had no appreciable effect on dopaminergic cell viability.

Exogenous urate treatment increased intracellular urate content

Although intracellular antioxidant actions of urate might explain its observed attenuation of H₂O₂-induced oxidative damage, Guerreiro *et al.* (Guerreiro *et al.*, 2009) concluded that urate may act as an extracellular antioxidant to protect dopaminergic neurons. Similarly, the astrocyte-dependence of protection found in the present study leaves uncertain the site targeted by urate.

To determine whether urate entered MES 23.5 cells and astrocytes, intracellular urate was measured in dopaminergic and astroglial cells treated with vehicle or urate for 24 hours. *Exogenous* urate raised intracellular urate content from 0.81 ± 0.30 to 5.09 ± 0.44 nmol/mg of protein ($p < 0.01$) and from 0.14 ± 0.06 to 0.38 ± 0.04 nmol/mg of protein ($p < 0.01$) in MES 23.5 cells and astrocytes, respectively (see also Suppl. Table 1 and 2), at the time when toxicant treatment would have been initiated. No statistically significant effect on its

precursors was found either intracellularly (Suppl. Table 1 and 2) or extracellularly (data not shown).

To assess if urate was metabolized by UOx in MES 23.5 cells we treated the cell line with increasing concentrations of oxonate, a UOx selective inhibitor. Oxonate did not affect urate content in MES 23.5 cells at any given concentration (Supplementary Fig. 2A). This result was supported by western blotting analysis, which detected no staining for UOx in both MES 23.5 cells and astrocytes (Supplementary Fig. 2B).

Intracellular urate increase is required for dopaminergic protection

To determine whether urate accumulation into MES 23.5 cells and astrocytes is transporter-mediated, protein expression of urate transporters known to be key regulators of urate levels in rodents (Hosoyamada *et al.*, 2004; Preitner *et al.*, 2009) was investigated. Immunostaining for URAT1 and GLUT9 was positive in MES 23.5 cells and astrocytes, while immunostaining for OAT1 was negative in both cell types (Fig. 4A). To investigate whether any of these transporters played a role in increasing intracellular urate levels, cells were loaded with urate immediately after one of the following drugs: PZO, the active metabolite of pyrazinamide (a URAT1 inhibitor), probenecid (a URAT1 and GLUT9 inhibitor) and HCTZ (a URAT1 and OAT1 inhibitor).

HPLC determinations showed that HCTZ significantly reduced urate accumulation in a concentration-dependent manner in MES 23.5 cells, whereas probenecid and PZO had no effect (Fig. 4B). By contrast, PZO, probenecid and HCTZ markedly reduced urate accumulation in astrocytes in a concentration-dependent manner (Fig. 4C).

To determine if intracellular urate accumulation was required for urate's protective effect we conducted viability experiments pretreating mixed cultures with HCTZ, PZO or probenecid together with urate 24 hours before toxicant treatment. PZO and HCTZ prevented dopaminergic protection induced by urate in a concentration-dependent manner; a similar effect was seen with 0.5 mM probenecid (Fig. 4D). PZO, HCTZ, and probenecid did not affect susceptibility of MES 23.5 cells to H₂O₂ in urate-untreated cultures (data not shown).

Transgenic urate degradation in astrocytes reduces protection by conditioned medium

To exclude possible secondary pharmacological effects of urate-lowering transport inhibitors, we also took a genetic approach to lowering urate through enzymatic degradation within astrocytes. To that end, astrocytes were prepared from mice overexpressing *UOx* (*UOx* Tg) (Kono *et al.*, 2010). Cultured Tg astrocytes expressed UOx protein, which was undetectable in WT astrocytes (Fig. 5A and B). UOx expression reduced urate basal levels in Tg compared to WT astrocytes (Fig. 5C). Because the UOx transgene we employed (Kono *et al.*, 2010) can lead to secretion of the enzyme (Fig. 5D), we assessed the extent to which extracellular urate was catabolized in Tg and WT astrocyte cultures after addition of medium containing 100 μ M urate. We found that urate was not altered for at least 8 hours in the medium, though by the end of the 24 hour treatment period a small but significant (21%) reduction was appreciated (Fig. 6A). The intracellular urate content in Tg astrocytes was reduced compared to WT astrocytes after 8 hours of exposure to urate (Fig. 6B). Thus transgenic *UOx* expression in astrocytes produced marked and rapid reduction primarily of intracellular urate. To assess whether reduced urate accumulation affected the protective effect of conditioned medium on MES 23.5 cells, medium was collected from urate-treated WT and Tg astrocytes and immediately used to pretreat MES 23.5 cells. Cell viability of MES 23.5 cells pretreated with medium collected from urate-treated Tg astrocytes was reduced in comparison to cell viability of MES 23.5 cells pretreated with medium collected

from urate-treated WT astrocytes, suggesting a critical role for intracellular urate in the release of a soluble astrocyte-derived protective factor (Fig. 6C).

Discussion

In cultures, urate markedly enhanced survival of dopaminergic neurons in a model of spontaneous cell death (Guerreiro *et al.*, 2009) and reduced oxidative stress as well as cell death induced by toxicants (Duan *et al.*, 2002; Haberman *et al.*, 2007; Zhu *et al.*, 2011; Cipriani *et al.*, 2012). Urate's protective effects have been also found *in vivo*, where urate prevented dopaminergic cell death in a rodent model of PD (Wang *et al.*, 2010).

Although considerable evidence indicates that urate is a powerful antioxidant few studies have been investigated alternative mechanisms of its protective effect. Du and coworkers (Du *et al.*, 2007) reported that the protective effect of urate on primary spinal cord neurons was dependent on the presence of astrocytes in cultures. They showed that urate induced up-regulation of the EAAT-1 glutamate transporter in astrocytes, suggesting that urate may enhance the ability of astrocytes to reduce extracellular glutamate levels around nearby neurons. Moreover, our previous studies showed that the effect of modulating intracellular urate content on the susceptibility of dopaminergic neurons to MPP⁺ treatment was amplified in cultures containing a high percentage of astrocytes in comparison to cultures where glial growth was inhibited (Cipriani *et al.*, 2012). Of note, the data reported in the present study argue against an important direct antioxidant action of urate in protecting stressed dopaminergic cells, or at least that this putative antioxidant action of urate is not sufficient to account for its benefits in this model.

In the present study, the protective role of urate on dopaminergic cells was demonstrated not only in co-cultures but also in conditioned medium experiments. The inability of UOx to prevent completely urate's effect confirmed that protection of dopaminergic cells is not induced by carryover urate on its own, but more likely by the release of soluble protective factor(s) by astrocytes in response to urate. This finding also excludes a direct interaction between H₂O₂ and urate as an explanation for how urate attenuates H₂O₂ toxicity. Similarly, the previously reported ability of urate to increase EAAT-1 expression on astrocytes and thereby reduce local glutamate buffering (Du *et al.*, 2007) could not directly explain urate's protective effect in the present study, in which the astrocyte-dependence does not require proximity between astrocytes and dopaminergic cells. Of note, the capacity of astrocytes to mediate neuroprotection by urate is not likely to be restricted to the cortical astrocytes, which we employed in this study based on their abundance relative to those in stratum or mesencephalon, consistent with enhanced neuroprotection achieved with spinal cord and ventral mesencephalon astrocytes as well (Du *et al.*, 2007; Cipriani *et al.*, 2012).

Although the identity of a putative protective factor released by urate-stimulated astrocytes remains to be determined, there is ample precedent for the inducible release of neuroprotectants from astrocytes. For example, protective effects of pramipexole on a human dopaminergic cell line were found to be mediated by astroglial release of the brain-derived neurotrophic factor (Imamura *et al.*, 2008). Similarly, grape seed extract was found to protect primary neurons against H₂O₂-induced cell death inducing IL-6 release from astrocytes (Fujishita *et al.*, 2009; Li *et al.*, 2009).

In the present study we investigated whether the protective effect of urate could be mediated by elevation of its intracellular content in dopaminergic cells and astrocytes. In agreement with our previous findings (Cipriani *et al.*, 2012), we found that *exogenous* urate elevated intracellular urate content in dopaminergic cells and astrocytes. Thus urate may have a protective effect on dopaminergic cells not only by modulating the redox status of cellular

membranes, as previously suggested by (Guerreiro *et al.*, 2009), but also by acting on intracellular targets. Increasing intracellular content by *exogenous* urate might better explain the effect induced in astroglial cultures where it was found to upregulate protein expression (Du *et al.*, 2007).

An intracellular conversion of urate to allantoin, a possible active metabolite of urate, was largely excluded by the absence of UOx expression in dopaminergic cells and astrocytes and by the lack of oxonate effect in dopaminergic cells. These data are in agreement with previous studies that reported low UOx activity in the brain (Truszkowski and Goldmanowna, 1933; Robins *et al.*, 1953). Moreover, if allantoin were the active, protective metabolite of urate then one would have expected transgenic *UOx* expression to have enhanced the protective effect of urate (by increasing its conversion to allantoin), rather than attenuating it as observed.

To investigate whether cellular urate accumulation was dependent on membrane carriers, transporter inhibitors were employed. The increase in intracellular urate content of MES 23.5 cells and astrocytes treated with urate was markedly reduced by these transport inhibitors, indicating that urate accumulation in cells was likely due to the uptake of *exogenous* urate rather than a modulation of the purine pathway. This hypothesis was also supported by the finding that *exogenous* urate did not significantly affect, intracellularly or extracellularly, the content of any other purine measured. In mixed cultures, all three of the urate transporter inhibitors tested—HCTZ, probenecid and PZO – prevented urate's protective effect. The correlation of these protective effects with the blockade of urate accumulation in astrocytes but not in MES 23.5 cells strengthens the evidence that urate increase in astrocytes is a critical first step in its protective effect on cultured dopaminergic cells. This hypothesis is supported by the finding that transgenic expression of UOx in astrocytes attenuated urate's protective effect on dopaminergic cells. Of interest, the loss of UOx enzyme during hominoid evolution (Oda *et al.*, 2002) has increased urate levels in the human body and it has been proposed to have raised antioxidant levels in human ancestors and thereby lengthened their lifespans. Verisimilarly, loss of UOx expression may have enhanced cellular antioxidant defences by not only increasing circulating levels of urate in the human body, but also presumably its intracellular content.

Urate transporters are highly expressed in the kidney where they control urate secretion and reabsorption. Urate transporters have also been found in the human and rodent brain at the level of choroid plexus and blood brain barrier (Alebouyeh *et al.*, 2003; Mori *et al.*, 2003) and localized in neuronal and endothelial cells (Bahn *et al.*, 2005; Ohtsuki *et al.*, 2004). The presence of urate transporters suggests a possible role for these carriers in regulating urate homeostasis in the brain, though their function there is unknown. Interestingly, an allelic variation in the *GLUT9* gene, associated with lower serum uric acid levels, was reported to correlate with a lower age at onset in PD (Facheris *et al.*, 2011).

Translational significance

A compelling convergence of epidemiological, clinical and initial cellular studies has suggested a potential neuroprotective effect of higher urate levels on dopaminergic neurons (Cipriani *et al.*, 2010; Shoulson, 2010) and expedited development of a phase II randomized clinical trial of inosine to elevate urate in PD (<http://clinicaltrials.gov/ct2/show/NCT00833690>). In parallel, efforts to gain mechanistic insight into protection by urate might be of considerable therapeutic as well as biological value as they could impact both the rationale and the pace of advancing to phase III clinical investigation. The present findings, in a cellular oxidative stress model of PD, provide evidence of a novel urate mechanism, possibly independent of its established antioxidant properties and support its candidacy as a neuroprotective agent for PD. They also suggest a

more intricate mechanism of action that involves an astroglial intermediate, consistent with a growing appreciation of the critical pathophysiological role for astrocytes in the cellular microenvironment of degenerating neurons in PD (Rappold and Tieu, 2010).

In addition, our findings that urate transporters can modify purine uptake and dopaminergic cell death extend the range of translational strategies for targeting urate levels in PD. Although initial human trials aiming to raise CNS urate elevation in PD are conservatively focused on a precursor (inosine) approach, a drawback is the increased risk of gout and uric acid urolithiasis that accompanies the associated systemic rise in urate levels. Our demonstration that urate transport inhibitors commonly employed in clinical practice (e.g., probenecid and HCTZ, which lower and raise serum urate, respectively) can block urate uptake and dopaminergic cell death *in vitro* suggests that transport-targeted therapeutics may provide an alternative or adjunct to urate precursors. Thus they may avoid peripheral complications of hyperuricemia. Because the directionality of urate transport at the tissue (e.g., blood-brain barrier) as well as the cellular levels are not easily addressed in culture models, *in vivo* preclinical studies of urate transport pharmacology in the CNS and in whole animal models of PD will be an important next step.

In conclusion, we found that protection of dopaminergic cells by urate depends on its accumulation in astroglial cells that in turn release soluble protective factors. The data bolster the rationale for targeting urate elevation as a therapeutic strategy for PD and indicate that urate transporters on astrocytes might also be a pharmacological target to modulate urate levels in PD brain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

Ara-C	cytosine arabinoside
DNPH	2,4-dinitrophenylhydrazine
FBS	fetal bovine serum
GLUT9	glucose transporter9
HCTZ	hydrochlorothiazide
OAT1	organic anion transporter 1
PZO	pyrazinoate
ROS	reactive oxygen species
Tg	transgenic
UOx	uricase
URAT1	urate transporter 1

WT wild type

References

- Alebouyeh M, Takeda M, Onozato ML, Tojo A, Noshiro R, Hasannejad H, Inatomi J, Narikawa S, Huang XL, Khamdang S, Anzai N, Endou H. Expression of human organic anion transporters in the choroid plexus and their interactions with neurotransmitter metabolites. *J Pharmacol Sci.* 2003; 93:430–436. [PubMed: 14737013]
- Ames BN, Cathcart R, Schwiers E, Hochstein P. Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: a hypothesis. *Proc Natl Acad Sci U S A.* 1981; 78:6858–6862. [PubMed: 6947260]
- Anantharam V, Lehrmann E, Kanthasamy A, Yang Y, Banerjee P, Becker KG, Freed WJ, Kanthasamy AG. Microarray analysis of oxidative stress regulated genes in mesencephalic dopaminergic neuronal cells: relevance to oxidative damage in Parkinson's disease. *Neurochem Int.* 2007; 50:834–847. [PubMed: 17397968]
- Ascherio A, LeWitt PA, Xu K, et al. Urate as a predictor of the rate of clinical decline in Parkinson disease. *Arch Neurol.* 2009; 66:1460–1468. [PubMed: 19822770]
- Bahn A, Ljubojevic M, Lorenz H, Schultz C, Ghebremedhin E, Ugele B, Sabolic I, Burckhardt G, Hagos Y. Murine renal organic anion transporters mOAT1 and mOAT3 facilitate the transport of neuroactive tryptophan metabolites. *Am J Physiol Cell Physiol.* 2005; 289:C1075–84. [PubMed: 15944205]
- Burdett TC, Desjardins CA, Logan R, McFarland NR, Chen X, Schwarzschild AM. Efficient determination of purine metabolites in brain tissue and serum by high-performance liquid chromatography with electrochemical and UV detection. *J Biomed Chromatog.* 2012 In press.
- Cipriani S, Chen X, Schwarzschild MA. Urate: a novel biomarker of Parkinson's disease risk, diagnosis and prognosis. *Biomark Med.* 2010; 4:701–712. [PubMed: 20945982]
- Cipriani S, Desjardins AC, Burdett CT, Xu Y, Xu K, Schwarzschild AM. Urate and its transgenic depletion modulate neuronal vulnerability in a cellular model of Parkinson's disease. *PloS One.* 2012 In press.
- Crawford GD Jr, Le WD, Smith RG, Xie WJ, Stefani E, Appel SH. A novel N18TG2 x mesencephalon cell hybrid expresses properties that suggest a dopaminergic cell line of substantia nigra origin. *J Neurosci.* 1992; 12:3392–3398. [PubMed: 1356145]
- Davies KJ, Sevanian A, Muakkassah-Kelly SF, Hochstein P. Uric acid-iron ion complexes. A new aspect of the antioxidant functions of uric acid. *Biochem J.* 1986; 235:747–754. [PubMed: 3753442]
- Du Y, Chen CP, Tseng CY, Eisenberg Y, Firestein BL. Astroglia-mediated effects of uric acid to protect spinal cord neurons from glutamate toxicity. *Glia.* 2007; 55:463–472. [PubMed: 17203476]
- Duan W, Ladenheim B, Cutler RG, Kruman II, Cadet JL, Mattson MP. Dietary folate deficiency and elevated homocysteine levels endanger dopaminergic neurons in models of Parkinson's disease. *J Neurochem.* 2002; 80:101–110. [PubMed: 11796748]
- Facheris MF, Hicks AA, Minelli C, et al. Variation in the uric acid transporter gene SLC2A9 and its association with AAO of Parkinson's disease. *J Mol Neurosci.* 2011; 43(3):246–250. [PubMed: 20589538]
- Fujishita K, Ozawa T, Shibata K, Tanabe S, Sato Y, Hisamoto M, Okuda T, Koizumi S. Grape seed extract acting on astrocytes reveals neuronal protection against oxidative stress via interleukin-6-mediated mechanisms. *Cell Mol Neurobiol.* 2009; 29:1121–1129. [PubMed: 19381798]
- Guerreiro S, Ponceau A, Toulorge D, Martin E, Alvarez-Fischer D, Hirsch EC, Michel PP. Protection of midbrain dopaminergic neurons by the end-product of purine metabolism uric acid: potentiation by low-level depolarization. *J Neurochem.* 2009; 109:1118–1128. [PubMed: 19302482]
- Haberman F, Tang SC, Arumugam TV, Hyun DH, Yu QS, Cutler RG, Guo Z, Holloway HW, Greig NH, Mattson MP. Soluble neuroprotective antioxidant uric acid analogs ameliorate ischemic brain injury in mice. *Neuromolecular Med.* 2007; 9:315–323. [PubMed: 17999205]

- Hansen MB, Nielsen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J Immunol Methods*. 1989; 119:203–210. [PubMed: 2470825]
- Hosoyamada M, Ichida K, Enomoto A, Hosoya T, Endou H. Function and localization of urate transporter 1 in mouse kidney. *J Am Soc Nephrol*. 2004; 15:261–268. [PubMed: 14747372]
- Imamura K, Takeshima T, Nakaso K, Ito S, Nakashima K. Pramipexole has astrocyte-mediated neuroprotective effects against lactacystin toxicity. *Neurosci Lett*. 2008; 440:97–102. [PubMed: 18555604]
- Kono H, Chen CJ, Ontiveros F, Rock KL. Uric acid promotes an acute inflammatory response to sterile cell death in mice. *J Clin Invest*. 2010; 120:1939–1949. [PubMed: 20501947]
- Li XZ, Bai LM, Yang YP, Luo WF, Hu WD, Chen JP, Mao CJ, Liu CF. Effects of IL-6 secreted from astrocytes on the survival of dopaminergic neurons in lipopolysaccharide-induced inflammation. *Neurosci Res*. 2009; 65:252–258. [PubMed: 19647022]
- Mori S, Takanaga H, Ohtsuki S, Deguchi T, Kang YS, Hosoya K, Terasaki T. Rat organic anion transporter 3 (rOAT3) is responsible for brain-to-blood efflux of homovanillic acid at the abluminal membrane of brain capillary endothelial cells. *J Cereb Blood Flow Metab*. 2003; 23:432–440. [PubMed: 12679720]
- Oda M, Satta Y, Takenaka O, Takahata N. Loss of urate oxidase activity in hominoids and its evolutionary implications. *Mol Biol Evol*. 2002; 19:640–653. [PubMed: 11961098]
- Ohtsuki S, Kikkawa T, Mori S, Hori S, Takanaga H, Otagiri M, Terasaki T. Mouse reduced in osteosclerosis transporter functions as an organic anion transporter 3 and is localized at abluminal membrane of blood-brain barrier. *J Pharmacol Exp Ther*. 2004; 309:1273–1281. [PubMed: 14762099]
- Preitner F, Bonny O, Laverriere A, Rotman S, Firsov D, Da Costa A, Metref S, Thorens B. Glut9 is a major regulator of urate homeostasis and its genetic inactivation induces hyperuricosuria and urate nephropathy. *Proc Natl Acad Sci U S A*. 2009; 106:15501–15506. [PubMed: 19706426]
- Rappold PM, Tieu K. Astrocytes and therapeutics for Parkinson's disease. *Neurotherapeutics*. 2010; 7:413–423. [PubMed: 20880505]
- Robins E, Smith DE, McCaman RE. Microdetermination of purine nucleoside phosphorylase activity in brain and its distribution within the monkey cerebellum. *J Biol Chem*. 1953; 204:927–937. [PubMed: 13117870]
- Ross CA, Smith WW. Gene-environment interactions in Parkinson's disease. *Parkinsonism Relat Disord*. 2007; 13(Suppl 3):S309–15. [PubMed: 18267256]
- Saura J, Angulo E, Ejarque A, et al. Adenosine A2A receptor stimulation potentiates nitric oxide release by activated microglia. *J Neurochem*. 2005; 95:919–929. [PubMed: 16092928]
- Schwarzschild MA, Schwid SR, Marek K, et al. Serum urate as a predictor of clinical and radiographic progression in Parkinson disease. *Arch Neurol*. 2008; 65:716–723. [PubMed: 18413464]
- Sherer TB, Betarbet R, Stout AK, Lund S, Baptista M, Panov AV, Cookson MR, Greenamyre JT. An in vitro model of Parkinson's disease: linking mitochondrial impairment to altered alpha-synuclein metabolism and oxidative damage. *J Neurosci*. 2002; 22:7006–7015. [PubMed: 12177198]
- Shoulson I. Therapeutic directions for Parkinson's disease. *Mov Disord*. 2010; 25(Suppl 1):S152–4. [PubMed: 20187232]
- Truszkowski R, Goldmanowna C. Uricase and its action: Distribution in various animals. *Biochem J*. 1933; 27:612–614. [PubMed: 16745136]
- Wang LJ, Luo WF, Wang HH, Ni GH, Ye Y, Li D, Liu CF. Protective effects of uric acid on nigrostriatal system injury induced by 6-hydroxydopamine in rats. *Zhonghua Yi Xue Za Zhi*. 2010; 90:1362–1365. [PubMed: 20646589]
- Weisskopf MG, O'Reilly E, Chen H, Schwarzschild MA, Ascherio A. Plasma urate and risk of Parkinson's disease. *Am J Epidemiol*. 2007; 166:561–567. [PubMed: 17584757]
- Whiteman M, Ketsawatsakul U, Halliwell B. A reassessment of the peroxynitrite scavenging activity of uric acid. *Ann N Y Acad Sci*. 2002; 962:242–259. [PubMed: 12076979]
- Xu K, Xu YH, Chen JF, Schwarzschild MA. Neuroprotection by caffeine: time course and role of its metabolites in the MPTP model of Parkinson's disease. *Neuroscience*. 2010; 167:475–481. [PubMed: 20167258]

- Yu PH, Zuo DM. Enhanced tolerance of neuroblastoma cells towards the neurotoxin 6-hydroxydopamine following specific cell-cell interaction with primary astrocytes. *Neuroscience*. 1997; 78:903–912. [PubMed: 9153668]
- Zhu TG, Wang XX, Luo WF, Zhang QL, Huang TT, Xu XS, Liu CF. Protective effects of urate against 6-OHDA-induced cell injury in PC12 cells through antioxidant action. *Neurosci Lett*. 2011; 506:175–179. [PubMed: 22094380]

Fig. 1.

Protection of MES 23.5 cells by urate is mediated by astrocytes. (A) Cell viability in MES 23.5 cultures after 24 hours of H₂O₂ treatment at indicated concentrations. One-way ANOVA: $n = 3$, $*p < 0.05$, $**p < 0.001$, $***p < 0.001$ vs 0 value. (B) Effect of urate ($n = 4$) treatment on 200 μM H₂O₂-induced cell death in MES 23.5 cultures. (C) Cell viability of MES 23.5 cells cultured for 24 hours with increasing H₂O₂ concentrations and astrocyte densities. Ratio between astrocytes and MES 23.5 cells is shown in symbol key. Two-way ANOVA: $n = 3$; $**p < 0.01$ vs 0 and 1:5. (D) Effect of urate treatment on 200 μM H₂O₂-induced cell death in co-cultures (1:5::astrocytes/MES 23.5). One-way ANOVA: $n = 13$; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs respective 0 value. Photomicrograph of (E) untreated, (F) H₂O₂-treated, (G) H₂O₂+100 μM urate-treated and MES 23.5 cells (green) cultured on astrocytes (red), DAPI staining was used to label nuclei (blue). Scale bar is 50 μm. The dashed line indicates the control value (100%) against which the other values were measured.

Fig. 2.

Urate reduced ROS and protein oxidation in MES 23.5 cells cultured with astrocytes. (A) NO₂⁻ release in co-culture medium after 200 μM H₂O₂ treatment for the indicated times. One-way ANOVA: $n = 15$; * $p < 0.05$ vs 0' value. (B) Effect of urate treatment on H₂O₂-induced NO₂ release at 24 hours of treatment. One-way ANOVA: $n = 16$; ** $P < 0.01$ and *** $P < 0.001$ vs control and urate values, ## $p < 0.01$ vs H₂O₂ value. (C) Protein carbonyl content in MES 23.5 cells cultured with astrocytes after 200 μM H₂O₂ treatment for the indicated times. One-way ANOVA: $n = 5$; * $p < 0.05$ vs 0 value. (D) Effect of urate treatment on H₂O₂-induced protein carbonylation at 3 hours of treatment with H₂O₂. One-way ANOVA: $n = 7$; ** $p < 0.01$ and *** $p < 0.001$ vs control and urate values; ## $p < 0.05$ vs H₂O₂ value.

Fig. 3.

(A) Effect of increasing percentages of conditioned medium from control or urate-treated astrocytes on MES 23.5 cell viability. Two-way ANOVA: $n = 15$; $**p < 0.01$ and $***p < 0.001$ vs respective control value. (B) Effect of uricase (UOx; 0.12 U/l) on conditioned medium from urate-treated astrocytes on MES 23.5 cell viability ($n = 4$). UOx or vehicle was added to astroglial cultures after 24 hours of urate treatment; conditioned media were collected after 15 more hours.

Fig. 4.

Urate's protective effect depends on its accumulation in astrocytes. (A) Western blotting shows URAT1, GLUT9 and OAT1 urate transporter expression in extracts of MES 23.5 cells and astrocytes. Kidney extract was used as positive control. (B) Urate concentration in MES 23.5 cells and (C) astrocytes treated with urate transporter inhibitors: pyrazinoate (PZO), hydrochlorothiazide (HCTZ) or probenecid (PBN), at the indicated concentrations (mM) ($n = 4-7$). (D) Effects of urate transporter inhibitors: PZO, HCTZ and PBN, at the indicated concentrations (mM), on MES 23.5 cell viability in H_2O_2 -treated co-cultures expressed as percentage of control ($n = 4-6$). One-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs 0 value.

Fig. 5.

UOx expression in transgenic (Tg) astrocytes reduced urate intracellular content. (A) Uricase (UOx) expression in Tg *UOx* astrocytes with none detected (ND) in wild-type (WT) astrocytes. (B) Western blot of UOx immunoreactivity in 50 μ g of WT and Tg astrocytes; liver extract (10 μ g) was used as positive control. Actin was used as loading control. (C) Basal intracellular urate content in WT and Tg astrocytes. Student's *t* test: $n = 5$; $*p = 0.02$. (D) Basal UOx activity in media from WT and Tg astrocytes. Student's *t* test: $n = 10$, $*p = 0.015$.

Fig. 6.

UOx expression in transgenic astrocytes reduced astrocyte-mediated protection of H₂O₂-treated MES 23.5 cells. (A) Medium urate concentration in 100 μ M urate-treated WT and Tg astroglial cultures over the time ($n = 3$; *** $P < 0.001$ vs respective WT value). Error bars were smaller than symbols for all data points. (B) Intracellular urate content in WT and Tg astrocytes after 8 hours of treatment with 100 μ M urate; Student's t test ($n = 4$, * $p = 0.02$). (C) Effect of conditioned medium collected from urate-treated WT and Tg astrocytes on viability of 200 μ M H₂O₂-treated cells ($n = 4$; * $p < 0.05$ vs respective Tg value).