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 uM) for 24 hours before addition of the oxidative stress inducer H2O2 (200uM). Twenty-four hours later, cell viability was quantified by 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 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.

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1 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* (Wu et al., 2003; Chen et al., 2002; Zurn and Do, 1988; Benowitz et al., 1998; Petrausch et al., 2000). Moreover, inosine showed antinflammatory effects in the central nervous system (CNS) and periphery (Hasko et al., 2000; Rahimian et al., 2010; Jin et al., 1997; Gomez and Sitkovsky, 2003; Shen et al., 2005). 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 inverse risk factor for PD. Clinical and population studies have found 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 (Cipriani et al., 2012b; Cipriani et al., 2012a; Zhu et al., 2011; Wang et al., 2010; Gong 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 urate level in the CNS (Rahimian et al., 2010; Spitsin et al., 2010; Ceballos et al., 1994; Scott et al., 2002). A cellular study indicated that inosine added to cortical astroglial (but not neuronal) cultures increases urate concentration in the medium (Ceballos et al., 1994).

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.

2 Material and methods

2.1 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 Massachusetts General Hospital.

2.2 MES 23.5 cell line

The rodent MES 23.5 dopaminergic cell line (Crawford et al., 1992) was obtained from Dr. Weidong Le at 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 hours and again during toxicant treatment. 200 μM H_2O_2 were added to the cultures for 24 hours and then cells were used for assays.

2.3 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 1,800 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_2-95% 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).

2.4 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 hours 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 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 hours 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.

2.5 Conditioned media experiments

Enriched-astrocytes cultures were grown on poly-L-lysine (100 µg ml^{-1})/DMEM/F12-coated 6 well-plates in DMEM plus 10% FBS. Astrocytes were let grow for three days and then 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 hours before and during H$_2$O$_2$ treatment.

2.6 Drugs

Inosine was dissolved in DMEM as 20X concentrated stocks. H$_2$O$_2$ was dissolved in PBS (0.1 M, pH 7.4) as 100X concentrated stocks. Drugs were obtained from Sigma.

2.7 Cell viability and toxicity assessments

In MES 23.5 cultures, cell viability was measured by the 3-(4,5-dmethylthiazol-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 hours. Then, the medium was changed to DMEM serum-free medium for 24 hours before H$_2$O$_2$ was added. In order to assess inosine protection, increasing concentrations of drug (0–100 µM) were loaded 24 hours before and again during toxicant treatment. After washes, 100 µl of MTT solution (0.5 mg ml^{-1} in DMEM) were added for 3 hours at 37° C. Then, MES 23.5 cells were lysed with 10ul/well of acidic isopropanol (0.01M 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 hours before and again during toxicant treatment. After washing in PBS, cultures were fixed with 4% (wt/vol) paraformaldehyde for 1 hour 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.

2.8 High-Performance Liquid Chromatography

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 filters 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 multi-channel 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.

2.9 Nitrite (NO_2^-) release

MES 23.5-astrocytes co-cultures were grown on 96-well plate as described above. After treatments, nitrite release (NO_2^-), an indicator of free radical generation, was quantified in cell medium by 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 Griess solution.

2.10 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 ug 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.

2.11 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.

2.12 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.

3 Results

3.1 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-astrocyte 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 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).

3.2 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 hours, inosine decreased the level of NO_2^- (nitrite), an indicator of free radical generation, from 2-fold to 1.4-fold of control value in cell medium ($P = 0.00139$, Fig. 2A). Moreover, at 3 hours 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).

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

We previously observed that astrocytes mediate urate's protective effect through 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 hours with 100 μM inosine improved MES 23.5 viability in a concentration-dependent manner ($P < 0.001$). This observation was confirmed by 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 ($F_{1, 151} = 3.59$, $P = 0.0079$; Figure 3).

3.4 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 hours, when the cultures would be treated with toxicant. Over 24 hours 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 hours. 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 astrocyte-enriched cultures at time 0 and 24 hours of treatment. Although adenosine increased 2-fold, intracellular concentrations of urate and other purines were not changed at 24 hours 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 hours, respectively. Hypoxanthine increased over the time up to 4-fold (*P* < 0.0001) at 24 hours in comparison to time zero and xanthine by 1.8-fold in comparison to 6 hours (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.

4 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.

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 mediator of inosine effects in the setting of inflammatory or ischemic injury (Rahimian et al., 2010; Jin et al.,

1997; Gomez and Sitkovsky, 2003; Shen et al., 2005). 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* (Wu et al., 2003; Chen et al., 2002; Zurn and Do, 1988) 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 (Hasko et al., 2000; Rahimian et al., 2010; Jin et al., 1997; Gomez and Sitkovsky, 2003; Shen et al., 2005). 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, Szoleczky et al., 2012, Modis et al., 2009). 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 (Rahimian et al., 2010; Spitsin et al., 2010; Ceballos et al., 1994; Scott et al., 2002). 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 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 were elevated in the CNS of treated individuals it could produce a neuroprotective effect independent of urate.

5 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.

Acknowledgments

Abbreviations used

Ara-c	cytosine arabinoside
FBS	fetal bovine serum
ROS	reactive oxygen species
DMEM	Dulbecco's modified Eagle's medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
EDTA	ethylenediaminetetraacetic acid
DHBA	3,4-dihydroxybenzylamine

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Highlights

1. Inosine protected dopaminergic cells subjected to oxidative stress.

2. Inosine attenuated inductions of indicators of free radical generation and oxidative damage in this model.

3. Protection by inosine required astrocytes in cultures.

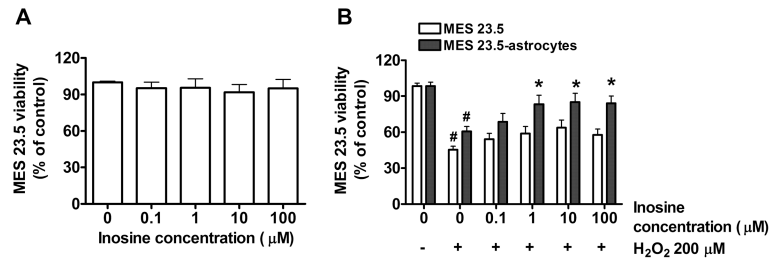4. Protection by inosine appeared to be independent of urate.

Figure 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 hours with increasing concentrations of inosine (0–100 μM). B) Effect of inosine treatment (0–100 μM) at 24 hours of toxic treatment with 200 μM H_2O_2 on viability of MES 23.5 cells cultured alone (white bars) or in presence of astrocytes (gray bars). Cultures were treated with inosine 24 hours before and during toxic treatment. Data represent the means ± 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).
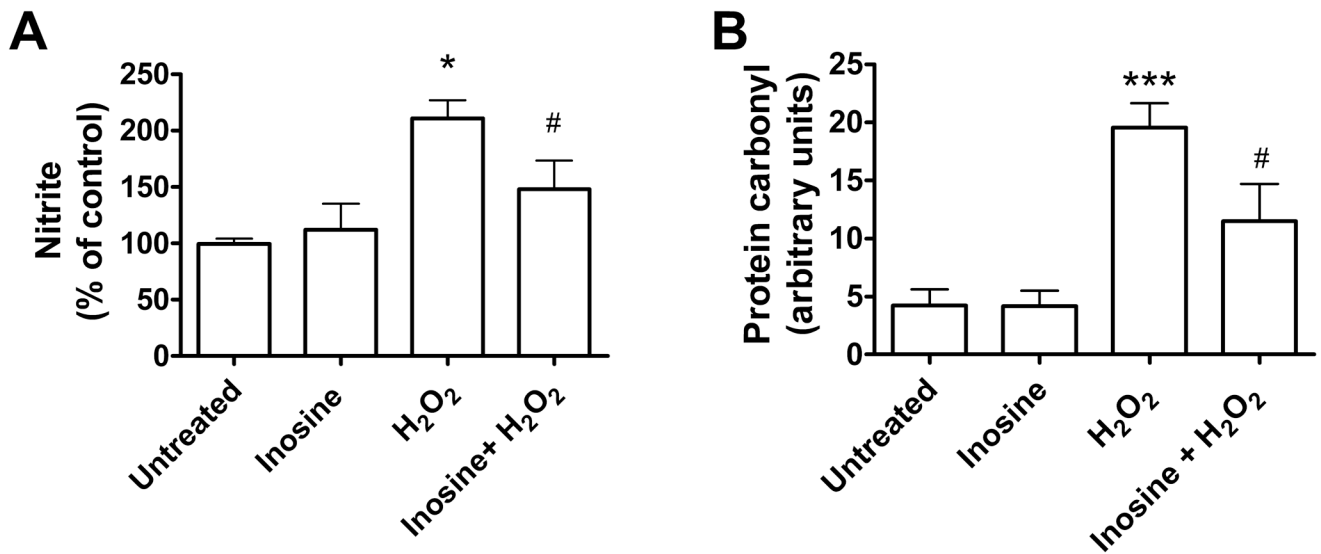
Figure 2. Inosine reduced oxidative stress in MES 23.5 cells cultured with astrocytes
(A) Effect of inosine treatment (0–100 μM) on 200 μM H_2O_2-induced NO_2^- release in the medium of MES 23.5-astrocytes co-cultures at 24 hours of toxic treatment. Cultures were treated with inosine 24 hours before and during toxic treatment. Data represent the means ± 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 hours of toxic treatment. Cultures were treated with inosine 24 hours before and during toxic treatment. Data represent the means ± 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.
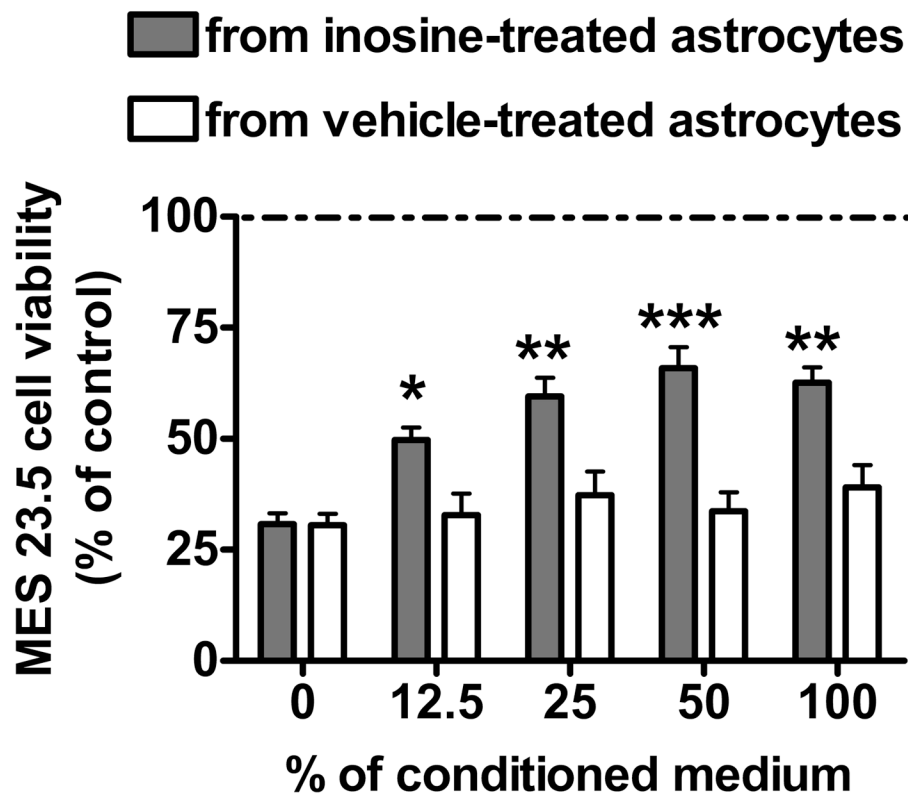
Figure 3. Inosine-conditioned medium from astrocytes increased viability of H_2O_2-treated MES 23.5 cells

Effect of increasing concentration of cell medium collected from control (white bars) or 100 μM 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 hours before and during toxic treatment. Data represent the means ± SEM of thirteen independent experiments. Two-way ANOVA analysis ($P = 0.0003$) followed by Newman-Keuls multiple comparison test (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ *vs* respective control value).

Table 1

Extracellular purine content at time zero (0) and 24 hours in 100 µM-inosine-treated MES 23.5-strocytes co-cultures.

Analites	Concentration (µM)	
	0	24 hours
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 means ± SEM of eight experiments.

Table 2

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

Analites	Concentration (nmol/g of protein)	
	0	**24 hours**
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 means ± SEM of four experiments.

Table 3

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

Analites	Concentration (μM)	
	0	**24 hours**
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 means ± SEM of four experiments.

Table 4

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

Analites	Concentration (μM)		
	0	**6 hours**	**24 hours**
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* 6h 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 means ± SEM of eight experiments.