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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.
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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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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

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