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Deciphering the pathways that protect from IL-13-mediated potentiation of oxidative stress-induced dopaminergic nerve cell death

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ABSTRACT

The majority of Parkinson's disease (PD) cases are sporadic with only about 10% of PD patients having a family history of the disease suggesting that this neurodegenerative disorder is the result of both environmental and genetic factors. Both oxidative stress and neuroinflammation are thought to contribute to PD. Previously, we showed that the activation of interleukin 13 receptor alpha 1 (IL-13Rα1) increases the sensitivity of dopaminergic neurons to oxidative damage both in cultured cells and in animals. In this study, we investigated the pathways involved in the IL-13-mediated potentiation of oxidative stress-induced dopaminergic cell death using a combination of cell survival assays and Western blotting with appropriate antibodies. In addition, siRNA was used to examine the role of 4E-BP1 in this cell toxicity paradigm. We show that activation of both the Jak-Stat and PI3 kinase-mTOR pathways play key roles in the promotion of cell death by IL-13 in the presence of mild oxidative stress. The Jak 1/2 inhibitor ruxolitinib, the mTOR inhibitor rapamycin and the PI3 kinase inhibitor LY294002 all prevented the potentiation of cell death by IL-13. Moreover, 4E-BP1, a target of mTOR, appeared to mediate the protective effects of rapamycin. Together, these results indicate that multiple signaling pathways downstream of IL-13Rα1 activation play a role in the toxic effects of IL-13 in dopaminergic neurons in the presence of mild oxidative stress and suggest that any of these pathways might provide potential targets for the treatment of PD.

1. Background

Parkinson's disease (PD) is a chronic, progressive neurodegenerative disease and the second most common after Alzheimer's disease [1]. In the US, PD affects 1% of the population over age 60 and it is estimated that 60,000 new cases will be diagnosed this year. The clinical symptoms are mainly due to the progressive decrease in dopamine signaling in the basal ganglia that is caused by the loss of the dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc). Although a number of genes have been shown to cause familial PD, these contribute to only ~10% of PD cases with the remainder being sporadic and of unknown origin. While the precise causes of PD still remain to be determined, chronic neuroinflammation is one of the hallmarks of the disease.

Our recent studies on neuroinflammation in the context of PD suggested that the IL-13/IL-13Rα1 system plays a role in disease development and/or the progression [2,3]. Interestingly, the interleukin 13 receptor alpha 1 (IL-13Rα1) gene lies within the PARK12 locus of PD susceptibility. Moreover, the PARK12 locus is located on the X chromosome which may be related to the observation that PD has a higher incidence in men as compared to women. Even more interesting is the observation that the expression of IL-13Rα1 in the brain is specific to the DA neurons of the ventral tegmental area (VTA) and of the SNc, the area of the brain most affected by PD. Indeed, double immunostaining studies showed that ~80% of the SNc neurons that express the DA marker tyrosine hydroxylase also express IL-13Rα1 [2].

Further evidence for a role for IL-13Rα1 in PD was obtained using the bacterial lipopolysaccharide (LPS) mouse model of PD [2]. We demonstrated that mice that were deficient in IL-13Rα1 were protected from DA neuronal loss when compared to their wild type littermates suggesting that the IL-13/IL-13Rα1 system could exert a neurotoxic effect under certain conditions [2]. Experiments done using the mouse DA nerve cell line MN9D showed that IL-13 alone did not have toxic effects on cells. However, when IL-13 was administered to the cells in

Abbreviations: BSA, bovine serum albumin; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorofluorescein diacetate; mTOR, mammalian target of rapamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI3-kinase, phosphoinositide 3 kinase; ROS, reactive oxygen species; Rux, ruxolitinib; tBOOH, t-butyl hydroxide; TBS, tris-buffered saline

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the presence of marginally toxic doses of oxidants, it increased cell death in a dose dependent manner. These results suggested that activation of IL-13Rα1 could contribute to the vulnerability of DA neurons under inflammatory conditions when both cytokines and reactive oxygen species (ROS) are produced.

The binding of IL-13 to IL-13Rα1 induces the tyrosine phosphorylation of the receptor [for review see [3]] thereby activating its kinase activity which results in the phosphorylation of the downstream substrates of its signaling cascades. IL-13 primarily activates two different intracellular signaling cascades: the Jak-Stat and the phosphatidylinositol 3′-kinase (PI3 kinase) pathways [for review see [4]]. Upon IL-13 stimulation of cells, Stat6 is phosphorylated on tyrosine and then forms a homodimer that translocates to the nucleus where it promotes gene transcription [4]. Tyrosine phosphorylation of IRS-2 leads to the activation of PI3 kinase and Akt [4]. However, whether either of these pathways contributes to the potentiation of oxidative stress-induced DA nerve cell death is not known. In this study, we describe experiments designed to determine the signaling pathways activated by IL-13 that contribute to the potentiation of oxidative stress-induced cell death in DA neurons.

2. Materials and methods

2.1. Materials

IL13 was purchased from Peprotech. Rapamycin, LY294002 and ruxolitinib were purchased from LC Laboratories. Unless otherwise stated, other chemicals and reagents were from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture and treatments

Mouse dopaminergic MN9D cells were grown in high-glucose DMEM containing 10% fetal calf serum as previously published [2]. For toxicity assays, 10,000 cells per well were plated in 96-well plates and grown overnight. The next day, the cells were treated with 1, 5 or 10 ng/ml IL-13 alone or in the presence of 80 μM H_2O_2 or 2.5 μM t-butylperoxide (tBOOH). In some cases, inhibitors were also included. 24 h later, cell survival was determined using the MTT assay. None of the concentrations of IL-13 alone had any effect on cell survival. The concentrations of H_2O_2 and tBOOH were chosen so as to have only a modest effect on cell survival (decrease by 10–15%) as previously described [2]. Human dopaminergic SH-SY5Y cells were grown in high glucose DMEM containing 10% fetal calf serum and plated at 20,000 cells per well in 96-well dishes for the toxicity assays. The next day, the cells were treated with 1, 2.5 or 5 ng/ml IL-13 alone or in the presence of 40 μM H_2O_2 or 2.5 μM t-butylperoxide (tBOOH). In some cases, rapamycin (100 nM) was also included. 24 h later, cell survival was determined using the MTT assay. None of the concentrations of IL-13 alone had any effect on cell survival. The concentrations of H_2O_2 and tBOOH were chosen so as to have only a modest effect on cell survival (decrease by 10–15%).

2.3. Transfection

For the siRNA transfections, the MN9D cells were plated in 60 mm dishes at 500,000 cells/dish and grown overnight. The next day, the cells were transfected with 166 pmol 4EBP1 siRNA (#sc-29595) or control siRNA (#sc-37007) (Santa Cruz Biotechnology (Santa Cruz, CA)) using RNAiMAX (Invitrogen) according to the manufacturer's instructions.

2.4. Reactive oxygen species (ROS) measurement

MN9D cells were plated at 10,000 cells/well onto 96-well black walled microtiter plates. The next day, the cells were treated with IL-13 alone or in the presence of H_2O_2 or tBOOH for the indicated times. The culture medium was then exchanged for 100 μl of phenol red-free Hank's balanced salt solution containing 10 μM CM-H_2DCFDA (Invitrogen). After 30 min, the fluorescence (λ excitation = 495 nm, λ emission = 525 nM) of the cells was measured using a Molecular Devices SpectraMax M3 microplate reader. All the treatments were done in sextuplicate. The CM-H_2DCFDA fluorescence in treated cells was normalized to that in control cells not exposed to compounds or oxidant.

2.5. Protein preparation and western blotting

For Western blotting, 300,000 MN9D cells were grown overnight in 35 mm dishes prior to the indicated treatments. Total protein extracts were prepared and analyzed by SDS-PAGE and Western blotting as described previously [5]. The primary antibodies used for Western blotting were: rabbit anti-phospho-Akt (ser473) (#9271, 1/1000), rabbit anti-phospho-Stat6 (#9361, 1/1000), rabbit anti-phospho-4E-BP1 (thr37/46) (#2855, 1/10,000), rabbit anti-Akt (#9272, 1/10,000), rabbit anti-Stat6 (#9362, 1/1000), rabbit anti-4E-BP1 (#9644, 1/250,000) and HRP-conjugated rabbit anti-actin (#5125, 1/20,000) from Cell Signaling. Following washing, the Western blots were incubated for 1 h at room temperature in horseradish peroxidase-goat anti-rabbit or goat anti-mouse (Biorad, Hercules, CA) diluted 1/2500 and developed with the Super Signal reagent (Pierce, Rockford, IL). In all cases, the same membrane was re-probed for actin and/or a parallel membrane was probed with an antibody reacting with the total protein in order to provide a normalization standard. Autoradiographs were scanned and analyzed using a Biorad GS800 scanner. The experiments were repeated a minimum of three times with independent protein samples.

2.6. Statistical analysis

A minimum of three independent experiments were used for statistical analyses which were performed using InStat 3. The results were analyzed for statistically significant differences using either the analysis of variance (ANOVA) test and Tukey's post test for individual group means comparisons or the *t*-test, as appropriate.

3. Results

The simplest explanation for the potentiation of oxidative stress-induced dopaminergic cell death by IL-13 would be that IL-13 increases ROS production from a non-toxic to a toxic level. This would be consistent with reports that IL-13 can rapidly induce NOXs in intestinal epithelial cells [6] and lipoxygenases in monocytes/macrophages cells [7]. To address this question, mouse MN9D dopaminergic nerve cells were treated with increasing doses of IL-13 alone or in the presence of H_2O_2 or tBOOH. The doses of H_2O_2 and tBOOH used were based on the results of our earlier study with the MN9D cells [2]. As shown in Fig. 1A, treatment of cells with IL-13 alone for 5 or 60 min or 24 h had no significant effect on ROS levels as measured using CM-H_2DCFDA. Short-term treatment of the MN9D cells with H_2O_2 or tBOOH induced a significant increase in ROS levels (Fig. 1B). However, none of the tested doses of IL-13 further increased these ROS levels. Together, these results indicate that IL-13 does not potentiate oxidative stress-induced cell death by directly increasing intracellular ROS levels.

Since Stat6 phosphorylation is one of the hallmarks of IL-13 receptor activation, we next asked what effect H_2O_2 or tBOOH have on IL-13-induced Stat6 phosphorylation. As shown in Fig. 2, IL-13 rapidly induced the phosphorylation of Stat6 in the MN9D cells and this phosphorylation persisted for at least 4 h. Neither H_2O_2 nor tBOOH alone had any effect on Stat6 phosphorylation (Fig. 2) but both brought about a time-dependent decrease in Stat6 phosphorylation in IL-13-treated cells (Fig. 2).