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Research article

Possible involvement of iron-induced oxidative insults in neurodegeneration

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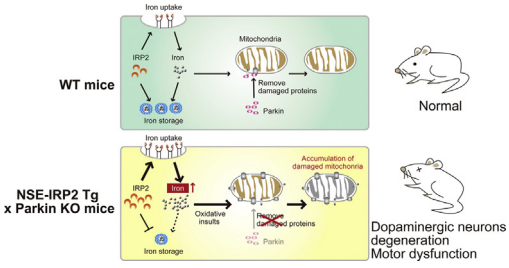
HIGHLIGHTS

- Increase of IRP2 accumulates iron that can provoke mitochondrial oxidative insults.
- Mitochondrial oxidative insults are induced in neurons in IRP2 transgenic (Tg) mice.
- Parkin appears involved in removal of iron-induced mitochondrial oxidative insults.
- IRP2 increase degenerates dopaminergic neurons synergistically with loss of Parkin.
- The IRP2 Tg mice may be useful to probe the roles of iron in neurodegeneration.

GRAPHICAL ABSTRACT



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ABSTRACT

Involvement of iron in the development of neurodegenerative disorders has long been suggested, and iron that cannot be stored properly is suggested to induce iron toxicity. To enhance iron uptake and suppress iron storage in neurons, we generated transgenic (Tg) mice expressing iron regulatory protein 2 (IRP2), a major regulator of iron metabolism, in a neuron-specific manner. Although very subtle, IRP2 was expressed in all regions of brain examined. In the Tg mice, mitochondrial oxidative insults were observed including generation of 4-hydroxynonenal modified proteins, which appeared to be removed by a mitochondrial quality control protein Parkin. Inter-crossing of the Tg mice to Parkin knockout mice

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perturbed the integrity of neurons in the substantia nigra and provoked motor symptoms. These results suggest that a subtle, but chronic increase in IRP2 induces mitochondrial oxidative insults and accelerates neurodegeneration in a mouse model of Parkinson's disease. Thus, the IRP2 Tg may be a useful tool to probe the roles of iron-induced mitochondrial damages in neurodegeraration research.

1. Introduction

Iron is an essential nutrient but can also be toxic because iron can readily cycle between ferrous (Fe^{2+}) and ferric (Fe^{3+}) in physiological settings and oxidizes proteins and nucleic acids via generation of free radicals. Dysregulation of iron metabolism causes some neurodegenerative diseases [21] and iron progressively accumulates in the lesions of sporadic neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease [17,18]. Therefore, tight regulation of iron metabolism appears to be critical for maintenance of neuronal cells [2,21].

Iron homeostasis is mainly regulated by coordinated expression of molecules involved in iron uptake and storage. Iron availability is regulated at the post-transcriptional level through the interactions between the iron-responsive elements (IREs) on mRNAs encoding proteins involved in iron metabolism and mRNA-binding proteins called iron regulatory proteins (IRPs) [14]. Binding of IRPs to IREs on the mRNA of the iron uptake protein, transferrin receptor1 (TfR1) enhances translation of TfR1, whereas binding of IRPs to the IRE on the mRNA of the iron storage protein, ferritin suppresses its production. Iron stored in ferritin is not toxic, because Fe^{3+} stored in ferritin cannot be converted to Fe^{2+}. Therefore, augmented expression of IRPs leads to an increase in iron uptake and a decrease in iron storage, which result in an increase of iron that cannot be stored safely and able to oxidize and damage cellar components [10]. There are two IRPs (IRP1 and IRP2) and IRP2 is abundant in brain as compared to other organs [9].

To examine the effects of iron in the integrity of neurons in mice, we generated transgenic (Tg) mice that express IRP2 in neurons [4]. We show increase in IRP2 induces mitochondrial oxidative insults and accelerates neurodegeneration.

2. Material and methods

2.1. Antibodies

The anti-myc (4A6 and 9E10) were purchased from Millipore and Roche, respectively. The following antibodies were obtained as indicated: 4-hydroxynonenal (4-HNE) (JaICA and Alpha diagnostic); β-actin, Tom20 (Santa Cruz Biotechnology); PINK1 (Novus); HA (Covance); COX III core1 (Invitrogen); and tubulin, Tyrosine hydroxylase (TH) (Cedarlane). Anti-IRP2 has been described [1].

2.2. Plasmids and cell culture

HA- and GFP-human Parkin was subcloned into pDNA3.1 (invitrogen) and pTRE2 (Clontech), respectively. p220-IRP2-myc has been described previously [6]. pNSE-IRP2-myc was generated by subcloning the human IRP2-myc cDNA into pNSE [4]. pcDNA3.1-HA- or pTRE2-GFP-Parkin were stably introduced in HEK293 or HeLa cells, respectively using Lipofectamine 2000 (Invitrogen). Parkin expression was induced by addition of 1 μg/ml doxycycline (DOX) for 48 h in HeLa cells that expressed GFP-Parkin in a DOX-dependent manner. IRP2-myc under the control of dexamethasone (DEX) (p220-IRP2-myc) was induced by treatment with 80 nM DEX for 48 h.

2.3. Immunoblotting, immunoprecipitation and fluorescence microscopy

These analyses were performed as described previously [19]. Quantifications were performed by Fluoview (Olympus) and BZ-II Analyzer (Keyence).

2.4. Assessment of mitochondrial membrane potential

Cells were treated with 25 nM MitoTracker Orange for 10 min at 37 °C.

2.5. Generation of NSE-IRP2 Tg mice

NSE-IRP2 transgenic mice were generated by microinjection of pNSE-IRP2-myc into E0.5 mouse embryos from a C57BL/6J × DBA2/J F1 background. Parkin KO mice have been described [15]. These mice were backcrossed to C57BL/6J mice (Charles River Japan) more than ten times. All the experiments using mice were carried out according to the Guidelines for Animal Experimentation, Juntendo, Osaka, and Kyoto University.

2.6. Southern blotting

Southern blotting was performed as previously described using human IRP2 cDNA as a probe [19].

2.7. RNA electrophoretic mobility shift assay (EMSA)

EMSA was performed as described previously [6].

2.8. Histochemical and morphological analyses

Brain sections were stained with the appropriate primary antibodies, followed by development using HISTOFINE (Nichirei) and a metal-enhanced diaminobenzidine (DAB) substrate kit (Pierce). Toluidine blue staining and electron microscopy were performed as described previously [7].

2.9. Fe^{2+} staining

Brains were perfused consecutively with 50 mM hydrogen sulfide and 4% paraformaldehyde, embedded in paraffin. Sections were immersed in a solution of 5% $K_3[Fe(CN)_6]$ and 5% HCl followed by immersion in 0.05% DAB and in 1% H_2O_2 plus 0.05% DAB.

2.10. Measurement of striatal dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA)

Dissected striata were analyzed using a reverse-phase C18 column (150 × 4.6 mm; Tosoh) on an HPLC system (ESA Biosciences) with a coulometric 8-electrode electrochemical detection system.