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Peroxiredoxin 5 prevents iron overload-induced neuronal death by inhibiting mitochondrial fragmentation and endoplasmic reticulum stress in mouse hippocampal HT-22 cells

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ABSTRACT

Iron is an essential element for neuronal as well as cellular functions. However, Iron overload has been known to cause neuronal toxicity through mitochondrial fission, dysregulation of Ca^{2+}, endoplasmic reticulum (ER) stress, and reactive oxygen species (ROS) production. Nevertheless, the precise mechanisms of iron-induced oxidative stress and mitochondria- and ER-related iron toxicity in neuronal cells are not fully understood. In this study, we demonstrated that iron overload induces ROS production earlier in the ER than in the mitochondria, and peroxiredoxin 5 (Prx5), which is a kind of antioxidant induced by iron overload, prevents iron overload-induced mitochondrial fragmentation mediated by contact with ER and translocation of Drp1, by inhibiting ROS production and calcium/calcineurin pathway in HT-22 mouse hippocampal neuronal cells. Moreover, Prx5 also prevented iron overload-induced ER-stress and cleavage of caspase-3, which consequently attenuated neuronal cell death. Therefore, we suggested that iron overload induces oxidative stress in the ER earlier than in the mitochondria, thereby increasing ER stress and calcium levels, and consequently causing mitochondrial fragmentation and neuronal cell death. So we thought that this study is essential for understanding iron toxicity in neurons, and Prx5 may serve as a new therapeutic target to prevent iron overload-induced diseases and neurodegenerative disorders.

1. Introduction

Iron is a trace element important for the regulation of brain as well as cellular functions, and is involved in ATP production, DNA synthesis, production of neurotransmitters, and myelin formation. In the brain, the iron requirement is higher than that in other organs because of the high-energy needs (Connor and Menzies, 1996; Connor et al., 2001; Kell, 2009; Rouault, 2001). However, there is increasing evidence that an imbalance in iron homeostasis can result in production of reactive oxygen species (ROS), which can lead to serious damage of DNA, proteins, and lipids (Chen et al., 2016; Connor et al., 2001; Salvador, 2010). Many cases of brain iron accumulation have been reported to cause oxidative damage, mitochondrial dysfunction, and inflammation. Iron accumulation has also been known to be involved in various neurodegenerative disorders, such as Parkinson's disease, Huntington's disease, and Alzheimer's disease (Sipe et al., 2002; Urrutia et al., 2014; Ward et al., 2014). However, the precise mechanisms underlying iron accumulation-induced neuronal toxicity are not fully understood.

In biological systems, iron is a key element in the production and metabolism of ROS. If free iron is increased by iron accumulation, it can catalyze the decomposition of hydrogen peroxide (H_2O_2) to form hydroxyl radicals ($HO^{\cdot}$), via Fenton's reaction. ROS generated by iron can cause damage to biomolecules such as lipids, proteins, and DNA, and can also stimulate ryanodine receptor-mediated calcium release, thereby contributing to neurodegeneration via excessive calcium release (Hidalgo et al., 2007; Jomova and Valko, 2011; Valko et al., 2016). Peroxiredoxins (Prxs), which are a family of antioxidant enzymes, can eliminate H_2O_2 and have been reported to participate in various intracellular oxidative signaling pathways (Kang et al., 2005; Knoops et al., 2011; Rhee et al., 2005). While Prx overexpression provides protection to various cells, including neuronal cells, knockdown of Prxs generally makes the cells more sensitive to cell death induced by oxidative stress (Hampton and O'Connor, 2016; Park et al., 2015a). These studies have indicated that Prxs play important roles in redox sensitive signaling. However, the relationship between Prxs and iron-induced oxidative stress has been rarely studied to date.

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The mitochondria are important organelles involved in various cellular functions, and are highly dynamic organelles that continuously fuse and divide, referred to as fusion and fission, respectively. Imbalance of mitochondrial dynamics in neurodegenerative diseases is associated with cell death and neuronal synaptic loss (Cho et al., 2010; Knott et al., 2008). Inappropriate supply of iron caused by dysregulated iron homeostasis is associated with loss of respiratory capacity and mitochondrial DNA damage. In addition, various neurodegenerative diseases are closely related to mitochondrial dysfunction and iron dysregulation (Gao et al., 2009; Horowitz and Greenamyre, 2010). Our previous studies have indicated that iron overload causes mitochondrial fragmentation through calcium/calcineurin-mediated dephosphorylation of Drp1 at Ser637 in hippocampal neurons (Lee et al., 2016). However, more research is needed to elucidate the precise mechanisms underlying the iron overload-induced mitochondrial fragmentation and oxidative stress.

Recent studies have reported that iron overload increases the levels of intracellular Ca^{2+} and affects the calcineurin-dependent regulation of nuclear factor in activated T-cells during cardiomyopathy (Khamseekaew et al., 2016; Lin et al., 2013). The increase in intracellular Ca^{2+} induced by iron stimulates ryanodine receptor (RyR), which is one of the endoplasmic reticulum (ER) calcium channels (Hidalgo et al., 2007; Sanmartin et al., 2014). Several studies have indicated that iron-overload is involved in ER stress in heart- and liver-injury rat models and in high-fat diet and alcohol mouse models (Lou et al., 2009; Tan et al., 2013). In addition, the ER-associated protein, inverted formin 2, has been shown to participate in mitochondrial fission (Korobova et al., 2013). Therefore, we hypothesized that iron overload increases ER stress and ER-mitochondria interaction, and consequently causes mitochondrial fragmentation in neuronal cells.

We previously found that iron overload causes mitochondrial fragmentation via Ca^{2+}/calcineurin-dependent dephosphorylation of Drp1 (Ser637) in mouse HT-22 hippocampal neuronal cells (Lee et al., 2016). In this study, we further investigated the precise mechanism underlying iron overload-induced mitochondrial fragmentation with regard to oxidative stress and ER stress in HT-22 cells that were exposed to ferric ammonium citrate (FAC). We found that FAC-induced iron overload preferentially increased oxidative stress in the ER earlier than in the mitochondria, and caused mitochondrial fragmentation that was accompanied by increased ER-mitochondria contact and Drp1 translocation. In addition, Prx5 plays a key role in iron toxicity, which causes neuronal cell death accompanied by ER stress and mitochondrial fragmentation, by eliminating ROS in hippocampal neuronal cells.

2. Materials and methods

2.1. Chemicals and reagents

FAC was obtained from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), Hanks' balanced salt solution (HBSS) and penicillin/streptomycin were purchased from Welgene (Daegu, Korea).

2.2. Cell culture and treatment

HT-22 cells, derived from HT-4 cells that were immortalized from primary mouse hippocampal neuronal cultures (Davis and Maher, 1994), were maintained in DMEM that was supplemented with 10% fetal bovine serum (Thermo Fisher Scientific Inc., MA, USA) and 1% penicillin/streptomycin at 37 °C in a humidified 5% CO_2 incubator (Panasonic Corporation, Osaka, Japan). The cells were grown to a density of 1×10^4 in a 6-well plate (SPL Life Sciences Co., Pocheon-si, Korea) for 24 h at 37 °C prior to initiating the experiments.

2.3. Measurement of intracellular calcium

HT-22 cells (1×10^5) were cultured in 12-well plates for 24 h, and FAC was then added. Measurement of intracellular Ca^{2+} was assessed with Fluo-4 AM (Thermo Fisher Scientific). The culture medium was carefully removed from each well and washed with HBSS (Welgene). Cells were then incubated with Fluo-4 AM solution (5 μM), which was dissolved in phenol red-free DMEM, for 15 min at 37 °C. After removing the medium in time, each well was washed with phosphate-buffered saline (PBS). Ca^{2+} images were obtained using a DE/DMI 3000B fluorescence microscope (Leica, Wetzlar, Germany). Quantification of Ca^{2+} was performed with ImageJ software (NIH, Bethesda, MD, USA).

2.4. Plasmid construction

DsRed2-Mito gene was obtained from pDsRed2-Mito (Clontech, CA, USA). HyPer-cyto gene was obtained from pHyPer-cyto (Evrogen, Moscow, Russia), and the mitochondria-specifc HyPer (Mito-HyPer) and ER-specific HyPer (ER-HyPer) genes were generated by fusing the mitochondrial and ER targeting sequence with HyPer-cyto, respectively. DsRed2-Mito, mouse Prx5, Mito-HyPer, and ER-HyPer were amplified by polymerase chain reaction (PCR) using LA Taq polymerase (TaKaRa, Shiga, Japan). These genes were cloned into pCR8/GW/TOPO (Thermo Fisher Scientific), and inserted into pLenti6.3/V5-DEST (Thermo Fisher Scientific) using LR clonase (Thermo Fisher Scientific). Constructed vectors were confirmed by restriction mapping and DNA sequencing.

2.5. Transfection and selection of stably transduced HT-22 cells

HT-22 cells (1×10^5) were seeded in 6-well plates and grown for 24 h prior to transfection. Plasmids (1 μg), such as pLenti6.3-DsRed2-Mito and pLenti6.3-Prx5 were transfected into HT-22 cells using effectene (Qiagen, CA, USA) according to the manufacturer's instructions. After transfection for a day, DsRed2-Mito-transfected HT-22 cells were selected with 8 μg/mL blasticidin (Thermo Fisher Scientific).

2.6. RNA interference assay

HT-22 cells were grown to 50% confluency and then transfected with 10 pmol of siRNA against Prx5 (siPrx5; Bioneer, Daejeon, Korea) using Lipofectamine RNAiMAX (Thermo Fisher Scientific). The siRNA sequence is as follows: siPrx5 sense, 5′-GUCUGAGCGU UAAUGACGU-3′ and siPrx5 antisense, 5′-ACGUCAUUAACGCUCAGAC-3′.

2.7. Western blot analysis

Whole protein lysates were prepared using ice-cold PRO-PREP protein extraction solution (iNtRON Biotechnology, Seongnam, Korea). Protein quantification was performed using Bradford assay (Bio-Rad, Hercules, CA, USA), and 20–30 μg of protein lysates were separated on 10–15% sodium dodecyl sulfate-polyacrylamide gels. The proteins were then transferred onto nitrocellulose membranes (BD Biosciences, Franklin Lakes, CA, USA). The membranes were blocked with 5% skim milk (BD Biosciences) and incubated overnight at 4 °C with the following primary antibodies: anti-p-IRE1α, anti-CHOP (Abcam, Cambridge, MA, USA), anti-Prx1, anti-Prx2, anti-Prx3, anti-Prx4, anti-Prx5 (AbFrontier, Seoul, Korea), anti-β-actin, anti-Drp1, anti-CHOP, anti-ATF4, anti-GADD34 (Santa Cruz, CA, USA), anti-IRE1α, anti-Bip, anti-eIF2α, anti-p-eIF2α, anti-p-Drp1(Ser616), anti-p-Drp1(Ser637), anti-caspase3, and anti-pan-calcineurin A (Cell Signaling, Danvers, MA, USA). The membranes were washed five times with 10 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 0.1% Tween-20 (TBST) and incubated with horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG (Thermo Fisher Scientific) for 1 h at room temperature. After removal of excess secondary antibodies, the membranes were