Journal of Trace Elements in Medicine and Biology 34 (2016) 62–69

Contents lists available at ScienceDirect

Journal of Trace Elements in Medicine and Biology

journal homepage: www.elsevier.com/locate/jtemb

Iron-induced oxidative stress activates AKT and ERK1/2 and decreases Dyrk1B and PRMT1 in neuroblastoma SH-SY5Y cells

Elizabeth Bautista, Paula Vergara, José Segovia*

Departamento de Fisiología, Biofísica y Neurociencias, Centro de Investigación y de Estudios Avanzados del IPN, Mexico

ARTICLE INFO

Article history:
Received 6 August 2015
Received in revised form 6 October 2015
Accepted 23 November 2015

Keywords:
Oxidative stress
Iron
ERK1/2
AKT
PRMT1
DyrK1B
c-jun
Smad

ABSTRACT

Iron is essential for proper neuronal functioning; however, excessive accumulation of brain iron is reported in Parkinson's, Alzheimer's, Huntington's diseases and amyotrophic lateral sclerosis. This indicates that dysregulated iron homeostasis is involved in the pathogenesis of these diseases. To determinate the effect of iron on oxidative stress and on cell survival pathways, such as AKT, ERK1/2 and DyrK1B, neuroblastoma SH-SY5Y cells were exposed to different concentration of $FeCl_2$ (iron). We found that iron induced cell death in SH-SY5Y cells in a concentration-dependent manner. Detection of iNOS and 3-nitrotyrosine confirms the presence of increased nitrogen species. Furthermore, we found a decrease of catalase and protein arginine methyl-transferase 1 (PRMT1). Interestingly, iron increased the activity of ERK and AKT and reduced DyrK1B. Moreover, after $FeCl_2$ treatment, the transcription factors c-Jun and pSmad1/5 were activated. These results indicate that the presence of high levels of iron increase the vulnerability of neurons to oxidative stress.

1. Introduction

Iron catalyzes the formation of reactive oxygen species (ROS) through the Haber–Weiss and Fenton reactions [1,2]. Therefore its deregulation increases the generation of ROS and the concomitant loss of neuronal functioning. Iron accumulates as a function of aging and alterations of its homeostasis occurs in several neurodegenerative diseases [3,4]. High concentration of iron is found in the *substantia nigra* (SN) of Parkinsonís patients [5] and ferritin in the cerebrospinal fluid of patients with multiple sclerosis [6,7].

On the other hand, in SH-SY5Y cells, derived from a human neuroblastoma, the overexpression of the divalent metal transporter 1 (DMT1) promotes cell death by increasing the uptake of Fe^{2+} [8]. In SH-SY5Y cells, iron-overload produces the generation of ROS and reactive nitrogen species (RNS) such as hydroxyl radical and nitric oxide (NO) [9]. Therefore, the presence of antioxidant enzymes such as superoxide dismutase (SOD) and catalase are essential for the regulation NO levels [10]. In physiological conditions there are inhibitors of the production of NO such as NG-dimethyl-L-arginine (ADMA) which is generated from methylation of the arginine residue by protein arginine methyl-transferase 1 (PRMT1) [11–13]. Interestingly, besides synthesizing the endogenous inhibitors of NOS, PRMT1 is also required for the efficient activation of Smad1/5. Moreover, iron induces the expression of hepcidin through the phosphorylation of Smad1/5/8 [14]. Hepcidin is a protein that participates in iron homeostasis and its levels are decreased in Alzheimer's patients [15,16].

As previously mentioned, iron causes cell death. Extracellular signal-regulated kinases 1 and 2 (ERK 1/2), protein kinase B (PKB, also known as AKT) and dual-specificity tyrosine-phosphorylation-regulated kinase 1B (Dyrk1B) are crucial for cell proliferation, differentiation, survival and apoptosis. Mitogen-activated protein kinases (MAPKs) including ERK1/2, p38 MAPK, and c-Jun NH2-terminal kinase (JNK) are activated by oxidative stress. Specifically, it has been previously reported that iron induces the activation of AKT and ERK1/2 [17,18]. Finally, recent studies have linked the activation of ERK and AKT with the expression of Dyrk1B, a protein that promotes cell survival [19–21]. However, the effects of oxidative stress on DyrK1B have not been determined.

In the present study, we investigated the effect of $FeCl_2$ (iron) on oxidative stress in SH-SY5Y cells, this was based on previous studies that demonstrated the ability of $FeCl_2$ as an inducer of ROS

Abbreviations: ADMA, NG-dimethyl-L-arginine; Dyrk1B, dual-specificity tyrosine-phosphorylation-regulated kinase 1B; ERK1/2, extracellular signal-regulated kinases 1 and 2; GPx, glutathione peroxidase; IREs, iron regulatory element; IREs, iron regulatory elements; JNK, (c-Jun *N*-terminal kinase); MAPKs, mitogen-activated protein kinase; NO, nitric oxide; ONOO-, peroxynitrite; SN, substantia nigra; pGST, glutathione S-transferase Pi; PRMT1, protein arginine methyl-transferase 1; ROS, reactive oxygen species; SOD, superoxide dismutase.

* Corresponding author at: Departamento de Fisiología, Biofísica y Neurociencias, Centro de Investigación y de Estudios Avanzados del IPN, Av. IPN #, 2508, México 07360, D.F., México. Fax: +52 55 5747 3754.
E-mail address: jsegovia@fisio.cinvestav.mx (J. Segovia).

http://dx.doi.org/10.1016/j.jtemb.2015.11.005
0946-672X/

[9,22,23]. Thus, we found that iron induces cell death by increasing oxidative stress and decreasing catalase and PMRT1. We decided to evaluate the effects of iron on different signaling pathways related to cell survival such as AKT, ERK1/2, DyrK1B and c-Jun. We demonstrated that oxidative stress induced the activation of AKT and ERK 1/2 and reduced the levels of Dyrk1B. Here, we also described that iron increases the levels of c-Jun an important mediator of apoptosis. Finally, our data support the notion that iron influences its own regulation since it activates Smad1/5 which in turn could induce the expression of hepcidin.

2. Materials and methods

2.1. Cell culture and treatments

SH-SY5Y cells were cultured in complete DMEM medium (Dulbeccoís minimum essential medium; Life Technologies) supplemented with 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 50 U/mL penicillin and 50 mg/mL streptomycin (Life Technologies) and maintained in a humidified 5% CO_2 atmosphere at 37 °C. Medium was replaced every three days. After two days in culture, cells were incubated with 0, 25 μM, 50 μM, 100 μM, 500 μM 1 mM, 5 mM, 10 mM and 15 mM of $FeCl_2$ for 24 h for cell viability assays (MTT and trypan blue). In agreement with other studies, we established low or high concentrations based on MTT and trypan blue assays. We consider 25, 50 and 100 μM as low iron concentrations; since they only have effect on mitochondrial function (effect no greater than 30%) and not in cell viability. Previous reports showed that iron concentrations of 100–200 μM induced cell death by increasing intracellular iron thus concentrations starting at 500 μm were considered high because they significantly decrease cell viability [8,17,18,23,24].

2.2. MTT assay

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. SH-SY5Y cells were cultured in 24-well plates (2×10^5 cells/per well), and in the presence of different concentrations of $FeCl_2$ described above. After 24 h, cells were incubated with MTT (0.5 mg/mL) for 4 h at 37 °C, medium was removed and the blue formazan crystals were dissolved with DMSO (250 μL). Absorbance was measured at 570 nm with a reference wavelength of 620 nm (Bio-Rad: iMark™, Hercules, CA). All experiments were performed in triplicate. The absorbance values are an indirect measure of mitochondrial activity and are expressed as percentage relative cell viability with respect to the control group.

2.3. Trypan blue exclusion assay for cell death

The trypan blue exclusion assay was used to evaluate the cell death induced by $FeCl_2$ treatment. SH-SY5Y cells in a 24-well plate (2×10^5 cells/per well) were incubated with the different concentrations of $FeCl_2$ described above. After 24 h, cells were stained with 0.25% trypan blue solution.

2.4. Measurement of extracellular nitric oxide

Production of NO in culture supernatants was measured using the colorimetric reaction with the Griess reagent (Invitrogen G7921). Cells were treated with 0, 50, 100 and 500 μM $FeCl_2$. After 4 h, 100 μL of the supernatants were incubated with 100 μL of Greiss reagent containing 1% sulfanilamide, 2% phosphoric acid and 0.1% naphthyethylene diamide. After incubating for 20 min, absorbance was measured spectrometrically at 540 nm.

2.5. Western blot analysis

SH-SY5Y cells (1×10^6) were treated with 0, 50, 100 and 500 μM of $FeCl_2$ for 24 h, then harvested, lysed and total protein obtained in a lysis buffer containing a protease inhibitor cocktail (Complete; Roche Diagnostics, Basel, Switzerland). Protein quantification was performed using the bicinchoninic acid method (Pierce, Rockford, IL). Aliquots containing 50 μg of protein were separated on 7 or 12% SDS-PAGE gels and transferred onto PVDF membranes (Bio-Rad, Hercules, CA). Membranes were blocked for 2 h at room temperature in 5% nonfat milk/Tris-buffered saline with 0.05% Tween-20 and incubated with the primary antibodies overnight. Blots were incubated with the secondary antibody for 2 h at room temperature, followed by enhanced chemiluminescence detection (PerkinElmer Life Sciences, Waltham, MA) according to the manufacturer's instructions. Primary antibodies employed were: iNOS (SC-650), nitrotyrosine (MAB-5404), catalase (SC-34281), PRMT1 (SC-271404), SOD1 (SC-8636), pSmad2 (Cell signaling S465/S467), pSmad 1/5 (Cell signaling S463/465), c-jun (Cell signaling 60A8), DyrK1B (SC-98507), pERK (Cell Signaling; 5726), ERK (Cell Signaling, 9102; Danver, MA, USA), pAKT (SC-7985), AKT (SC-8312; Santa Cruz Biotechnology, Dallas, TX) and β-actin [25]. To quantitate the effects of iron, the ratios of the densitometric analysis were determined using the LabWorks software (UVP).

2.6. Statistical analysis

Results are expressed as means ± SEM. For statistical analysis, we used one-way ANOVA followed by Tukeyís *post hoc* multiple comparison test.

3. Results

3.1. Iron decreases the viability of SH-SY5cells

SH-SY5Y cells were treated with different concentrations of $FeCl_2$. Treatment with 25 μM of $FeCl_2$ did not decrease mitochondrial function (Fig. 1A). However, a significant reduction was observed when cells were treated with 50 μM, 100 μM, 500 μM and 1, 5 10, 15 mM (Fig. 1A). On the other hand, the effect of $FeCl_2$ on SH-SY5Y cell viability was assessed by the trypan blue exclusion assay. The results showed that 25, 50 and 100 μM of $FeCl_2$ did not reduce the cell viability, but 500 μM, 1, 5 10, 15 mM of $FeCl_2$ significantly decreased cell viability in a concentration-dependent manner (Fig. 1B). Therefore, 50, 100 and 500 μM of iron were selected to investigate its effects on oxidative stress and in different signaling pathways.

3.2. Iron increases oxidative stress

In this paper, we analyzed the expression of different markers of oxidative stress. Treatment with 50, 100 and 500 μM of $FeCl_2$ induced the expression of inducible nitric oxide synthase (iNOS) in a dose-dependent manner (Fig. 2A). Additionally, we determined the presence of 3-nitrotyrosine, a marker of reactive nitrogen species. 3-nitrotyrosine was not detected in the control or with 50 μM of $FeCl_2$ (Fig. 2A and B). However, 100 and 500 μM of iron induced an increase of 3-nitrotyrosine (Fig. 2A and B). Furthermore, treatments with 100 and 500 μM of $FeCl_2$ reduced the levels of PRMT1 a protein involved in the generation of ADMA (endogenous inhibitor of nitric oxide synthases). Additionally, we determined the levels of NO. The results show that iron increased NO levels in SH-SY5Y cells with respect to the control group (Fig. 2C).