International Journal of Sciences

Research Article Volume 7 – September 2018 (09)

TG could Modulate FPN1 in MES 23.5 Cells by Hepcidin

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Abstract: ***Objective*** To observe the effects of thapsigargin (TG) on ferroportin1 (FPN1) in MES23.5 dopaminergic cell. ***Methods*** MES23.5 cells were treated with TG (0.1 μM or 1 μM) for 24h. the mRNA levels of hepcidin were measured using real-time PCR; The protein levels of CHOP, XBP-1s and FPN1 were measured using western blots. ***Results*** CHOP and XBP-1s expression was significantly higher in the TG-treated group than that in the control group; hepcidin mRNA levels in TG-treated group were up-regulated, this increase was to a larger extent in the 1 μM TG group when compare with the 0.1 μM TG group; however, FPN1 expression was significantly lower in the TG-treated group, this decrease was to a larger extent in the 1 μM TG group when compare with the 0.1 μM TG group. ***Conclusion*** TG could modulate FPN1 in MES23.5 cells by hepcidin.

Keywords: Parkinson's Disease; MES23.5 Cells; ER Stress; Hepcidin; FPN1

1. Introduction

ER stress is a pathological sate that caused by accumulation of misfolded or unfolded proteins, abnormal glycosylation caused by glucose deficiency, abnormal cholesterol metabolism, disorder of calcium metabolism and hypoxia. In the initial stage of stress, cells can make an adaptive response, unfolded protein response (UPR), which can improve protein folding ability properly or abnormal protein degradation ability by regulating transcriptional programs, and there is a result of reducing abnormal aggregation of proteins and maintaining endoplasmic reticulum homeostasis[1-3]. However, under chronic ER stress, the ER stress exceeds the cell's own regulatory range, accumulation of misfolded proteins and persistent UPR activates the pro-apoptotic signaling pathways and cause cell death ultimately[4, 5].

Parkinson's disease (PD) is the second most common central nervous system neurodegenerative disease in the world, which seriously affect the middle-aged health, and there is a high incidence rate every year[6, 7]. The pathological hallmarkers of PD are the selective degeneration of dopaminergic (DA) neurons in the substantia nigra compacta (SNc) and the appearence of intracellular protein inclusions, called Lewy Bodies (LBs)[8]. Although the etiology and pathogenesis of PD have not yet been elucidated, many evidences indicated oxidative stress, mitochondrial dysfunction, inflammation, endoplasmic reticulum (ER) stress, and abnormal deposition of iron may resulting in damage of DA neurons[9-12]. ER stress is one of the main toxic mechanisms of PD[13-15].

Hepcidin is a key regulator of systemic iron homeostasis[16]. It was reported that hepcidin was regulated by ER stress in hepatocytes[17]. Hepcidin maintains systemic iron homeostasis by regulating ferroportin1(FPN1). Hepcidin inhibits the release of iron (mainly from red blood cells, macrophages, hepatocytes and placental cells) into plasma by binding to FPN1 to induce intracellular iron or degradation of plasma[18, 19]. Hepcidin is not only expressed in the liver, but also in the brain. In the present study, we investigated whether hepcidin was regulated by ER stress in nerve cells. Thus, targeting ER stress may be a promising neuroprotective strategy for management of PD.

2. Materials and methods

2.1 MES23.5 cell culture and treatment

MES23.5 Cell Culture Medium

91 mL of DMEM/F-12, 5 mL of fetal bovine serum, 1 mL of glutamine, 2 mL of 50×Sato's solution, and 1 mL of cyan/streptomycin solution were mixed.

MES23.5 cell culture and treatment

The cell culture flask was treated with 100 μg/mL polylysine. The MES23.5 cells were quickly transferred from liquid nitrogen to a 37°C water bath.

Published at: http://www.ijsciences.com/pub/issue/2018-09/
DOI: 10.18483/ijSci.1805; Online ISSN: 2305-3925; Print ISSN: 2410-4477

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After being completely dissolved, MES23.5 cells were mixed and suspended in a complete culture solution, and then inoculated into a culture flask pre-plated with polylysine, and placed at 37℃, 5% CO^2 incubator. At the time of the experiment, the cells were seeded at $1 \times 10^5/cm^2$ at 6 × plate. and the cells were treated in groups when the cell density reached 80%, as follows:

Control group: treated with serum-containing whole medium for 24 h;

0.1 μM TG group: incubated with TG (0.1 μM) for 24 h;

1 M TG group: incubated with TG (1 μM) for 24 h.

2.2 Western blotting

Wash precooling PBS for 3 times, add lysate for 30 minutes on ice, then centrifuged at 12000 r/m for 20 minutes, transfer the supernatant to in the new tube, the protein content was determined by the BCA method. The protein by SDS-PAGE electrophoresis and electrotransfer to PVDF membrane. With a volume fraction of 0.10 milk powder for 2 h at room temperature, then add CHOP (1 : 300), XBP-1s(1 : 500), β-actin (1 : 10000), breeding at 4 ℃ for a night, using 1 : 10000 diluted sheep anti-rabbit HRP-IgG secondary antibody was incubated at room temperature for 1 h, after which ECL luminescence showed Shadow, after scanning, use LSUVP Vision worksTM LS software to analysis of the results.

2.3 Real-time fluorescence quantitative polymerase chain reaction (PCR)

Total RNA was isolated from the MES23.5 cells using the Trizol reagent. Follow Thermo's Reverse Transcription Kit, the illustrated procedure is reverse transcribed. Then the cDNA is amplified. Proceed as follows primer sequences:

Hepcidin forward: 5'-GCCTGAGCAGCACCACCTAT-3';

Hepcidin reverse: 5'-AGCATTTACAGCAGAAGATGCAGA-3'.

GAPDH forward: 5'-AAATGGTGAAGGTCGGTGTGAAC-3';

GAPDH reverse: 5'-CAACAATCTCCACTTTGCCACTG-3'.

2.4 Statistical analysis

All data were processed using GraphPad Prism 6.02, and analyzed statistically using one-way ANOVA between groups. $P<0.05$ was considered to be statistically significant.

3. Results

3.1 Upregulation of CHOP and XBP-1s induced by TG in MES23.5 cells

MES23.5 cells were treated with TG (0, 0.1 μM or 1 μM) for 24 h, the protein levels of CHOP and XBP-1s were measured using western blots. CHOP and XBP-1s are specific markers of ER stress. Results showed the protein expression of CHOP and XBP-1s increased significantly in MES23.5 cells, compared with the control group (Fig. 1).

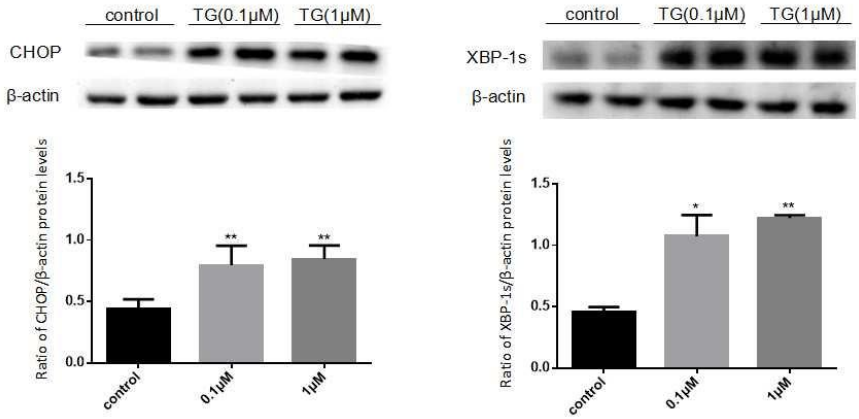

Fig.1 Protein levels of CHOP and XBP-1s in TG-treated MES23.5 cells.

Western blots were applied to detect the protein expression of CHOP and XBP-1s in MES23.5 cells. Increased expressions of CHOP and XBP-1s were observed in 0.1 μM and 1 μM TG-treated cells. β-actin was used as a loading control. Statistical analysis. Data were presented as the ratio of CHOP and XBP-1s to β-actin. ($^{*}P<0.05$, $^{**}P<0.01$, compared with control.)

3.2 TG induces hepcidin mRNA expression by ER stress

To confirm the relationship between ER stress and hepcidin, we performed further experiments in MES23.5 cells treated with TG, the mRNA levels of hepcidin were measured by using real-time PCR. Data showed that the mRNA expression of hepcidin increased significantly at 24 hrs after TG treatment in MES23.5 cells, compared with the control group (Fig. 2).

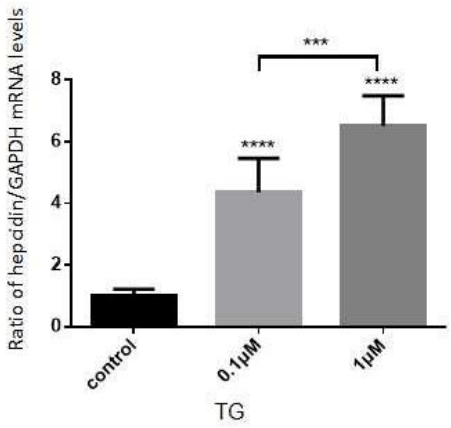

Fig.2 mRNA levels of hepcidin in TG-treated MES23.5 cells.

Real time PCR was applied to detect hepcidin mRNA levels. Hepcidin mRNA levels were increased in TG-treated MES23.5 cells. And their difference was statistically significant between 1 μM TG group with the 0.1 μM group. ($^{***}P<0.001$, $^{****}P<0.0001$, compared with control group.)

3.3 TG down-regulates FPN1 protein expression

Western blot analysis found that the tendencies in the changes of FPN1 protein expression induced by TG were obviously different to the mRNA expression of hepcidin. FPN1 protein levels in MES23.5 cells treated with TG were significantly lower than those in the control cells (Fig. 3).

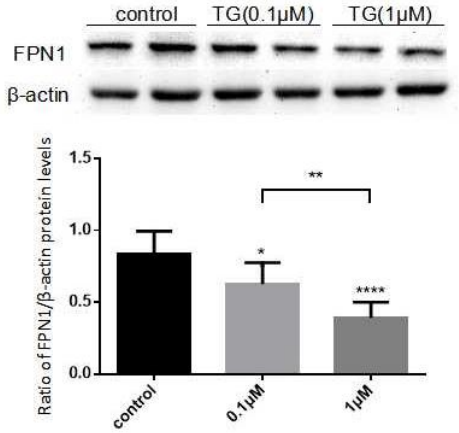

Fig.3 Protein levels of FPN1 in TG-treated MES23.5 cells.

Western blots were applied to detect the the expression of FPN1 in MES23.5 cells. Decreased expressions of FPN1 were observed in 0.1 μM and 1μM TG-treated cells. And the difference was statistically significant between 1 μM TG group with the 0.1 μM group ($^{*}P<0.05$, $^{**}P<0.01$, $^{****}P<0.0001$, compared with control).

4. Discussion

In eukaryotic cells, proteins that enter the secretory pathway must undergo processing and transport through the endoplasmic reticulum (ER). Many factors can result in ER stress, such environmental factors or increased protein synthesis, and accumulation of misfolded or unfolded proteins can cause ER stress. In this case, the cells undergo an UPR activation to retain the homeostasis of the ER, and which can further promote cell survival. However, prolonged UPR activation induces apoptosis by activating the PERK-eIF2α-ATF4-CHOP pathway[20,21]. Under normal physiological conditions, the endoplasmic reticulum transmembrane receptor PERK cannot exist in an active state alone and must be present in combination with Bip. Under ER stress, Bip is separated from PERK, which results in oligomerization and trans-autophosphorylation of PERK. As a kinase, PERK can phosphorylate eIF2α. Phosphorylated eIF-2α can inhibit the global protein translation, which lead to cell cycle to arrest in the G1 phase and also cause preferential translation of ATF4. Although it may upregulate the expression of genes responsible for restoring cellular homeostasis. Under prolonged ER stress, ATF4 may stimulate genes of CCAAT-enhancer-binding protein homologous protein (CHOP), which induces apoptosis[22].

The ER is the major reservoir for intracellular Ca^{2+} and is considered the dominant modulator of Ca^{2+} homeostasis[23]. TG, an inducer of ER stress[24], inhibit isoform of the Ca^{2+}-ATPase in ER, and elevates the free Ca^{2+} content[25]. In the present study, we showed that MES23.5 cells were treated with TG (0, 0.1 μM or 1 μM) for 24 h, CHOP and XBP-1s protein level in the TG-treated group were significantly higher than that in the control group. Our results are consistent with the experimental results of JINGYONG HUANG[26].

Hepcidin is now considered as the major player in iron homeostasis[27-30]. Hepcidin can binding to FPN1 at the cell surface through its N-terminus domain to cause FPN1 ubiquitination and internalization, ultimately result in degradation in lysosomes[31,32]. Recent studies have shown the complexity of hepcidin transcriptional regulation. As an ER stress–induced factor, CREBH activates the expression of hepcidin in hepatocytes[17]. Studies have also shown that hepcidin transcription is regulated by the SMAD1/5/8 pathway[33].

In the present study, we show that hepcidin induction by ER stressor (TG), the mRNA expression of hepcidin was up-regulated at 24 hrs after treatment with TG (0.1 μM or 1 μM) and the protein expression

of FPN1 was down-regulated in Mes23.5 cells. ER stress is closely related to Parkinson's disease, so we can maintain intracellular iron homeostasis by inhibiting ER stress and further alleviate PD. However, the underlying mechanisms of the effect of hepcidin on ER stress-induced neurotoxicity should be further studied in the future.

Acknowledgments and Fundings

This work was supported by grants from National Natural Science Foundation of China (31871049)

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