Neuroscience Letters 662 (2018) 1–5

Contents lists available at ScienceDirect

Neuroscience Letters

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

Original Research Article

Aspirin increases ferroportin 1 expression by inhibiting hepcidin via the JAK/STAT3 pathway in interleukin 6-treated PC-12 cells

Su-Na Huang[a,b], Huai-Zhen Ruan[a,**], Mesona Yung-Jin Chen[b], Gan Zhou[c,**], Zhong Ming Qian[b,*]

[a] Department of Neurobiology, College of Basic Medical Science, Third Military Medical University, Chongqing, China
[b] Laboratory of Neuropharmacology, Fudan University School of Pharmacy, Shanghai 201203, China
[c] Department of Clinical Pharmacology, Xiangya Hospital, Central South University, Changsha 410008 China

ARTICLE INFO

Keywords:
Aspirin (ASA)
Interleukin-6 (IL-6) and inflammation
Transferrin receptor 1 (TfR1)
Ferroportin 1 (Fpn1)
Ferritin light chain (Ft-L)
Hepcidin

ABSTRACT

To understand the potential mechanisms involved in the beneficial effects of aspirin (ASA) in mood disorders, Alzheimer's (AD) and Parkinson's disease (PD), we investigated the effects of ASA on the expression of iron transport proteins transferrin receptor 1 (TfR1), ferroportin 1 (Fpn1), and iron storage protein ferritin light chain (Ft-L) in interleukin-6 (IL-6)-treated PC-12 cells. We demonstrated that IL-6 alone could induce a severe decline in Fpn1 expression and cell viability, and an increase in Ft-L protein, while ASA could markedly diminish the effects of IL-6 on these parameters. We also found that IL-6 significantly increased hepcidin expression and janus kinase 2 (JAK2) and signal transducer and activator of transcription 3 (STAT3) phosphorylation, while ASA also observably suppressed these IL-6-induced effects. The data imply that ASA increases Fpn1 expression by inhibiting hepcidin expression via the IL-6/JAK/STAT3 pathway and show that the reduced content of Ft-L is due to the increased Fpn1 and subsequent iron release in the cells. The reduction of iron in neuronal cells by the increased expression of Fpn1 might be partly associated with the beneficial effects of ASA on mood disorders, AD and PD.

1. Introduction

Aspirin (ASA) is a non-steroidal anti-inflammatory drug (NSAID) which has been used for millennia to treat a wide range of maladies, including pain and inflammation [1]. Clinical studies have found evidence that high-dosing of ASA are associated with a reduced risk of Alzheimer's disease (AD) [2]. A study in mice demonstrated that ASA [3] or sodium salicylate [4] could prevent the neurotoxic effects of MPTP (1-methyl-4-1,2,3,6-tetrahydropyridine). A study in rats showed that sodium salicylate could also afford protection against rotenone-induced oxidative stress and neuronal degeneration [5]. An in vivo rat study demonstrated that ASA protects striatal dopaminergic neurons from degeneration induced by MPP+ (1-Methyl-4-phenylpyridinium ion) and 6-OHDA (6-hydroxydopamine) [6]. MPTP, rotenone, 6-OHDA and MPP+ all are neurotoxins, known to trigger oxidative stress in dopaminergic cells [4,7]. The mechanisms of how ASA protects dopaminergic neurons against oxidative damage are however, not yet fully described.

Abnormally high levels of iron have been demonstrated in a number of neurodegenerative disorders, including AD and Parkinson's disease (PD) [8–11]. Increased iron in the brain has been implicated as a major generator of reactive oxygen species [12,13]. Oxidative stress resulting from the increased iron has been widely believed to be one of initial causes responsible for neuronal death in some neurodegenerative diseases [14,15]. It has also been demonstrated that ASA affects iron metabolism by increasing ferritin synthesis in cultured bovine pulmonary artery endothelial cells [16] and it has been shown that ASA is associated with lower serum ferritin in a study with 913 elderly human participants [17].

These findings led us to speculate that ASA might affect the expression of other iron metabolism proteins, in addition to ferritin, thereby then regulating cell iron levels. This proposed involvement of ASA in iron metabolism might be associated with its neuro-protective effect and its beneficial effects in AD and PD. To find out the potential mechanisms by which ASA protects dopaminergic neurons against oxidative damage, we investigated the effects of ASA on the expression of iron transport proteins transferrin receptor 1 (TfR1), ferroportin 1 (Fpn1), storage protein Ft-L (ferritin light chain), hepcidin and also JAK2 (janus kinase 2) and STAT3 (signal transducer and activator of transcription 3) in Interleukin 6 (IL-6)-treated PC-12 cells. PC-12 cells were used in the present study, because the cells are used extensively as a model for dopaminergic neurons [18] that can synthesize, store and

* Corresponding author at: Laboratory of Neuropharmacology, Fudan University School of Pharmacy, 826 Zhang Heng Road, Shanghai 201203, China.
** Corresponding authors.
E-mail addresses: hzruan163@163.com (H.-Z. Ruan), zhougan@csu.edu.cn (G. Zhou), qianzhongming@fudan.edu.cn (Z.M. Qian).

http://dx.doi.org/10.1016/j.neulet.2017.10.001
Received 16 August 2017; Received in revised form 16 September 2017; Accepted 2 October 2017
Available online 04 October 2017
0304-3940/ <outputStr5>

secrete dopamine as well as other transmitters [19], while IL-6 was selected because it is one of the major mediators in lipopolysaccharide (LPS)-induced inflammation [20,21].

2. Materials and methods

2.1. Chemicals

Unless otherwise stated, all chemicals were obtained from the Sigma Chemical Co., St. Louis, MO, USA. Mouse anti-human TfR1 was purchased from Invitrogen, Carlsbad, CA, USA, rabbit polyclonal anti-mouse Fpn1 from Chemicon International, Temecula, CA, USA, rabbit polyclonal anti-Ft-L from Protein-tech, Chicago, IL, USA, rabbit monoclonal anti-phospho-JAK2, rabbit monoclonal anti-JAK2, rabbit polyclonal anti-phospho-STAT3, and mouse monoclonal anti-STAT3 antibodies from Cell Signaling Technology, Inc., Danvers, MA, USA, and goat anti-rabbit or anti-mouse IRDye 800 CW secondary antibody from LI-COR Bio Sciences, Lincoln, Nebraska, USA. BCA Protein Assay Kit and Revert Aid First Strand cDNA synthesis Kit were purchased from Thermo Scientific, Waltham, MA, USA and TRIZOL Reagent from Life Technologies, Carlsbad, CA, USA. All experimental protocols were performed according to the Animal Management Rules of the Ministry of Health of China, and approved by the Animal Ethics Committees of Fudan University.

2.2. PC12 cells

PC12 cells (rat adrenergic neural tumour phaeochromocytoma cell line) were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) in a 5% CO2 incubator (TC2323) at 37 °C [22].

2.3. Assessment of cell viability

Cell viabilities were measured using an MTT assay as described previously [23,24]. Optical density (OD) was measured at the 570 nm wavelength by the use of an ELX-800 microplate assay reader (Bio-tek, Winooski, VT, USA).

2.4. Quantitative real-time PCR

The extraction of total RNA and preparation of cDNA were performed using TRIZOL reagent and reverse transcription kit respectively according to the manufacturers' instruction. The specific primers used for PCR were: hepcidin forward, 5′-gaaggcaagatggcactaagca-3′; hepcidin reverse, 5′-tctcgtctgttgccggagatag-3′; and β-actin forward, 5′-aaatcgtgcgtgacatcaaaga-3′ and β-actin reverse, 5′-gccatctcctgctcgaagtc-3′ [25]. Quantitative real-time PCR was conducted with a CFX96 PCR Instrument (Bio-Rad, USA) using specific primers and a SYBR Premix II kit (Takara, Dalian, LN, China). The CT values of each target gene were normalized to that of the β-actin mRNA. Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method [26].

2.5. Western blot analysis

Protein content was determined using the BCA protein Assay kit. Aliquots of the extract containing about 20 mg of protein were loaded and run on a single track of 10% SDS-PAGE under reducing conditions and subsequently transferred to a pure nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The blots were blocked and then incubated with primary antibodies: anti-human TfR1 (1:500), anti-mouse Fpn1 (1:1000), anti-Ft-L (1:2000), anti-phospho-JAK2 (1:1000), anti-JAK2(1:1000), anti-phospho-STAT3 (1:1000), and anti-STAT3 (1:1000) overnight at 4 °C, and then incubated with goat anti-rabbit or anti-mouse IRDye 800 CW secondary antibody (1:5000) for 2-h at room temperature. The intensities of the specific bands were detected and analyzed by the Odyssey infrared image system (Li-Cor, Lincoln, NE, USA) as described previously [27]. To ensure even loading of the samples, the same membrane was probed with mouse monoclonal anti-actin antibody at a 1:2000 dilution.

2.6. Statistical analysis

Statistical analyses were performed using Graphpad Prism. Data are presented as mean ± SEM. The differences between the means were all determined by *t*-tests or one-way analyses of variance (ANOVA). A probability value of $P < 0.05$ was taken to be statistically significant.

3. Results

3.1. ASA protected PC-12 cells from IL-6-induced damage

We first investigated the effects of ASA on the viabilities of PC-12 cells by treatment of the cells with vehicle (0.1% ethanol) or various concentrations (0.01, 0.1, 1, 2 or 4 mM) of ASA for 24 h. The MTT assay showed that there were no significant differences in cell viabilities between the cells treated with vehicle (control) or 0.01, 0.1, 1, 2 or 4 mM of ASA (Fig. 1A). To find out whether ASA could protect PC-12 cells from IL-6-induced damage, we then evaluated the effects of ASA on the viability of IL-6 treated PC-12 cells. The cells were incubated with vehicle (0.1% ethanol) for 24-h, or 0 (vehicle), 0.01, 0.1, 1, 2 or 4 mM of ASA for 18-h and then co-cultured with IL-6 (50 ng/ml) for another 6-h. It was found that treatment with IL-6 alone induced a significant reduction in cell viability (Fig. 1B). However, the viability of IL-6-treated cells increased progressively with higher concentrations of ASA being added, peaking at 1 mM, and then decreasing gradually. The viability of the cells treated with 1 mM of ASA plus 50 ng/ml of IL-6 (ASA 1 + IL-6) was significantly higher than that of the cells treated

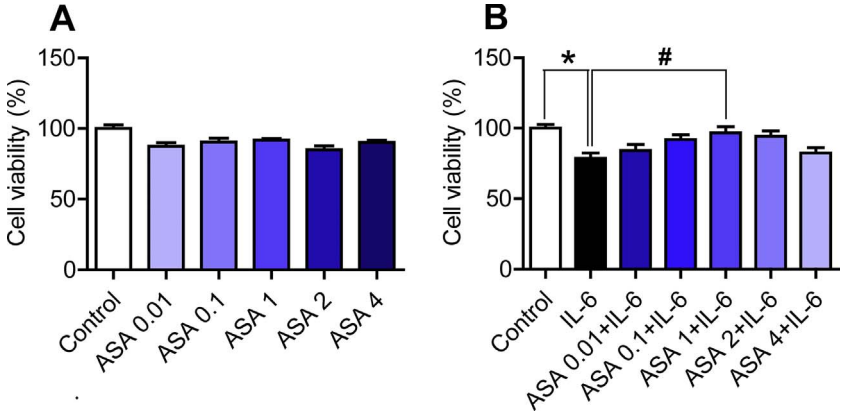

Fig. 1. ASA protected PC-12 cells from IL-6-induced damage. PC-12 cells were treated with vehicle (0.1% ethanol) or 0.01, 0.1, 1, 2 or 4 mM ASA for 24 h (A) or with vehicle (0.1% ethanol), IL-6 (50 ng/ml) for 6-h, or 0.01, 0.1, 1, 2 or 4 mM ASA for 18-h and then co-incubated with IL-6 (50 ng/ml) for another 6-h (B), and cell viability was evaluated using the MTT assay. Data are means ± S.E.M (n = 3). *p < 0.05 vs. the control; #p < 0.05 vs. the IL-6 group.