Indirubin-3′-monoxime suppresses amyloid-beta-induced apoptosis by inhibiting tau hyperphosphorylation

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Graphical Abstract

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Abstract

Indirubin-3′-monoxime is an effective inhibitor of cyclin-dependent protein kinases, and may play an obligate role in neuronal apoptosis in Alzheimer’s disease. Here, we found that indirubin-3′-monoxime improved the morphology and increased the survival rate of SH-SY5Y cells exposed to amyloid-beta 25–35 (Aβ25–35), and also suppressed apoptosis by reducing tau phosphorylation at Ser199 and Thr205. Furthermore, indirubin-3′-monoxime inhibited phosphorylation of glycogen synthase kinase-3β (GSK-3β). Our results suggest that indirubin-3′-monoxime reduced Aβ25–35-induced apoptosis by suppressing tau hyperphosphorylation via a GSK-3β-mediated mechanism. Indirubin-3′-monoxime is a promising drug candidate for Alzheimer’s disease.

Key Words: nerve regeneration; indirubin-3′-monoxime; amyloid-beta; Alzheimer’s disease; neuronal apoptosis; tau hyperphosphorylation; phosphorylated glycogen synthase kinase-3β; phosphorylated c-Jun N-terminal kinase; neural regeneration

Introduction

The recognition of pathologic features in patients with Alzheimer’s disease (AD) has provided clues to the mechanisms of neuronal apoptosis, and drawn attention to new prospects for AD therapy. A number of experimental models have shown that neuronal death occurs alongside the elicitation of proteins involved in the cell cycle (Lim and Qi, 2003; Colacurcio et al., 2015). Damaged neurons, instead of continuing with the cell cycle after mitosis, initiate abortive processes that result in apoptotic cell death (Xu et al., 2008; Absalon et al., 2013). Cyclin-dependent kinase (CDK) is involved in such processes, and CDK inhibitors might reduce neuronal loss in AD (Zhang et al., 2004; Johnson et al., 2005). There is an urgent need to develop safe, effective, and selective CDK inhibitors that can pass the blood-brain barrier.

Indirubin is a selective CDK inhibitor, which suppresses the activities of CDK1, CDK2, and CDK5. However, it has poor water solubility and liposolubility (Absalon et al., 2013). Indirubin-3′-monoxime (IMX), also a CDK inhibitor, has a low molecular weight and better solubility than indirubin (Zahler et al., 2010; Liao and Leung, 2013). It is nontoxic and acts by competition with adenosine triphosphate at the catalytic site of CDKs (Shelton et al., 2004).
The compound was found to suppress tau phosphorylation in SHβ cells expressing human tau 23 (Leclerc et al., 2001) and, in cerebellar granular neurons, it reduced apoptosis initiated by withdrawal of potassium (Xie et al., 2004). Systemic administration of IMX (20 mg/kg; 3 times per week for 2 months) in APP transgenic mice attenuated spatial memory deficits and decreased presenilin 1 (PS1) mutations in several AD-like phenotypes (Ding et al., 2010).

The aim of the present study was to investigate the neuroprotective effect of IMX against amyloid-beta (Aβ)-induced apoptosis in cultured SH-SY5Y neuroblastoma cells. In addition, we explored the effect of IMX on tau hyperphosphorylation and putative related mechanisms.

Materials and Methods

SH-SY5Y cell culture

SH-SY5Y cells (Beijing Union Medical College Cell Center, Beijing, China) were grown on RPMI 1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 15% (v/v) fetal bovine serum (Gibco BRL), 2 mM L-glutamine, 100 µg/mL streptomycin (Sigma, St. Louis, MO, USA), and 100 U/mL penicillin (Sigma) in a humidified atmosphere at 5% CO2 and 37°C. The medium was replaced every 2 days; cells were passaged every 3–4 days.

Cell viability assay

Aβ peptide fragment 25–35 (Aβ25-35; Sigma) was dissolved in sterile deionized water and stored at −20°C. IMX (Sigma) was dissolved in dimethyl sulfoxide to 10 mM, stored in aliquots at −20°C, and diluted in medium as necessary. The cells were divided into five groups in a 96-well plate: control (untreated); Aβ (20 µM Aβ25-35); IMX0.2 (0.2 µM IMX + Aβ25-35); IMX0.5 (0.5 µM IMX + Aβ25-35); and IMX1 (1.0 µM IMX + Aβ25-35). Cells in the Aβ groups were incubated with Aβ25-35 at 37°C for 7 days. Cells in the IMX groups were incubated at 37°C for 2 days. We used 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium (WST-8, Cell Counting Kit-8; Dojindo, Kumamoto, Japan; Tsukatani et al., 2011) to examine the effect of IMX on Aβ25-35-induced changes in SH-SY5Y cells. After treatment, the medium was replaced by Dulbecco’s modified Eagle’s medium and the cells were washed twice with cold PBS and resuspended in 200 µL cold 1× binding buffer. Annexin V–FITC (10 µL) and propidium iodide (PI; 5 µL) were admixed and incubated in the dark at room temperature for 15 minutes, then 300 µL binding buffer was added. Cells were quantified instantly in a flow cytometer (FACS Calibur, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA), using emission filters at 525 and 575 nm. Cells negative for both annexin V and PI (V−/PI−) were considered normal, whereas a V+/PI− result was considered a criterion of early apoptosis, and V+/PI+ was assumed to indicate late apoptotic-like cell death and necrosis. A minimum of 10,000 cells per experiment were assayed in the three separate trials.

Western blot assay

To evaluate the effects of Aβ25-35 and IMX on the phosphorylation of tau, glycogen synthase kinase 3β (GSK-3β) and c-Jun N-terminal kinase (JNK) in SH-SY5Y cells, the cells were incubated with a mixture of 20 µM Aβ25-35 and 0.5 µM IMX for 6 hours. They were then washed twice with cold PBS, and lysed in cell lysis buffer for 30 minutes on ice. The soluble portion was produced by centrifugation (15,000 × g for 15 minutes at 4°C). Protein concentrations were established using the bicinchoninic acid method (Yalamati et al., 2015) (Pierce Biotechnology, Rockford, IL, USA). Aliquots of total protein were boiled for 10 minutes in loading buffer and subsequently separated in 10% sodium dodecyl sulfate-polyacrylamide gel. Next, the proteins were transferred onto nitrocellulose membranes by electroporation (Immobilon TFP, Millipore Corp., Bedford, MA, USA) using a Trans-Blot system (Bio-Rad, New York, NY, USA). The membranes were then blocked with 5% nonfat milk in Tris-buffered saline with Tween-20 (TTBS; 10 mM Tris-HCl, 150 mM NaCl, 0.2% Tween-20) for 1 hour at room temperature. Samples were incubated with monoclonal primary antibodies (β-actin, 1:10,000, Sigma; polyclonal anti-tau pS199, 1:1,000, BioSource Int., Camarillo, CA, USA; anti-tau pT205, 1:1,000, Bioworld Technology (St. Louis Park, MN, USA); Ser-9-phosphorylated GSK-3β (p-GSK-3β Ser9), phosphorylated JNK (p-JNK), and JNK, all 1:1,000; Cell Signaling Technology, Beverly, MA, USA) at 4°C overnight. The membranes were washed twice with TTBS, and incubated with the secondary antibody (anti-rabbit-horse-radish peroxidase, 1:5,000; Cell Signaling Technology) at room temperature for 1 hour. Proteins were then visualized using the ECL Advanced Western Blotting Detection kit (Amersham Biosciences Ltd., Amersham, UK), and the mean optical density of each band was calculated using a Fluor-S MultiImager (Bio-Rad Laboratories (Shanghai) Co., Ltd., Shanghai, China) with Quantity One software (Bio-Rad Laboratories (Shanghai) Co., Ltd.).

Statistical analysis

The data, presented as the mean ± SEM, were analyzed using SPSS 11.0 software (SPSS Inc., Chicago, IL, USA). Means were compared by one-way analysis of variance and the least significant difference post hoc test. P < 0.05 was considered statistically significant.
Results

IMX enhanced the viability of SH-SYSY cells exposed to Aβ<sub>25-35</sub>

Under an inverted phase contrast microscope (Olympus Optical Co., Ltd., Tokyo, Japan), SH-SYSY cells in the control group appeared well-formed than those in the Aβ group. Pretreatment with different concentrations of IMX markedly improved morphology after Aβ<sub>25-35</sub> exposure (Figure 1A).

Cell viability after Aβ<sub>25-35</sub> exposure was significantly lower than in control cells (P < 0.001). However, pretreatment with IMX (0.5 µM and 1.0 µM) increased cell viability in a concentration-dependent manner (P < 0.01; Figure 1B).

IMX reduced neuronal apoptosis triggered by Aβ<sub>25-35</sub> in SH-SYSY cells

Flow cytometry showed that there were more apoptotic cells after exposure to 20 µM Aβ<sub>25-35</sub> than in the control group (P < 0.01), but significantly fewer in the IMX group (12.4 ± 2.02%) than in the Aβ group (20.33 ± 2.02%; P < 0.01). This indicates that IMX protected cells against Aβ-induced apoptosis (Figure 2).

Effects of IMX on p-GSK-3β expression

p-GSK-3β (Ser9) expression was lower after Aβ<sub>25-35</sub> exposure than in control cells, indicating that GSK-3β activity was increased. However, p-GSK-3β (Ser9) was markedly overexpressed after pretreatment with IMX (P < 0.05; Figure 4A). These results suggest that GSK-3β is involved in the effect of IMX on Aβ-induced tau phosphorylation.

IMX treatment did not affect p-JNK expression

The expression of p-JNK in the Aβ group was markedly higher than that in the control group (P < 0.05). However, there were no significant changes after IMX treatment (Figure 4B), indicating that p-JNK is not involved in the effect of IMX on Aβ-induced tau phosphorylation.

Discussion

SH-SYSY neuroblastoma cells are a well-characterized human cell model for investigating the pharmacological effects of IMX. In the present study, we exposed the cells to 20 µM IMX...
growth, and in transfected SH-SY5Y cells, supporting the findings of a previous study (Zhang et al., 2009).

Aβ induces and maintains the pathogenic changes in AD, but tau protein also has an important role in the progression of the disease (Iqbal et al., 2014). Aβ amyloidosis triggers the starting phase of tau accumulation and phosphorylation at Ser199, Thr231, and Ser396 in APP Sw mice (Tomidokoro et al., 2004; Jung et al., 2012; Huang et al., 2014; Nicole et al., 2014; Deng et al., 2015; Zhang et al., 2015), SH-SY5Y cells, and hippocampal neurons (Lafay-Chebassier et al., 2005; Jin et al., 2011; Reifert et al., 2011; Doherty et al., 2013; Martins et al., 2013; Sui et al., 2015), Aβ markedly diminished cell viability, increased the number of apoptotic-like cells, and promoted tau phosphorylation. Inhibiting tau phosphorylation has become a viable approach to treating or even preventing AD. Here, Aβ25–35 exposure elevated the rate of tau phosphorylation at pS199 and pT205 in serum-free cultured SH-SY5Y cells, supporting the findings of a previous study (Sun et al., 2008). We also found that IMX suppressed tau phosphorylation induced by Aβ25–35. This suggests that the effect of IMX on Aβ25–35-induced neurotoxicity may be via the inhibition of tau phosphorylation.

GSK-3β, also called Tau Protein Kinase I, is a proline-directed serine/threonine kinase, which phosphorylates tau at a number of AD-relevant epitopes in vitro and in transfected cells (Hanger et al., 1992; Ishiguro et al., 1992, 1993; Mandelkow et al., 1992; Mulot et al., 1994; Sperbera et al., 1995;
Bass et al., 2015). GSK-3β may have a pivotal function in the relationship between Aβ peptides and phosphorylated tau, and triggers a pathogenic cycle in AD (Wang et al., 2006; Dobarro et al., 2013; Hoppe et al., 2013; Xian et al., 2014; Amin et al., 2015; Kim et al., 2015; Vossel et al., 2015), resulting in a more important effect than those caused by other kinases such as cdk/MAPK (Elyaman et al., 2002; Liu et al., 2002). The activation of GSK-3β is reportedly related to its low rate of phosphorylation at residue Ser9 (Stambolic and Woodgett, 1994; Wang et al., 1994; Murai et al., 1996). In the present study, we used an antibody against p-GSK-3β Ser9 to investigate the activation of GSK-3β in SH-SY5Y cells. After exposure to Aβ25–35, p-GSK-3β (Ser9) expression was decreased, indicating that GSK-3β activity was elevated. However, pretreatment with IMX led to a considerable rise in the expression of p-GSK-3β (Ser9), which suggests that GSK-3β contributes critically to the action of IMX on Aβ-initiated tau phosphorylation.

The constituents of the mitogen-activated protein kinase (MAPK) family, including extracellular signal-regulated kinase, p38 MAPK, and JNK, are enzymes of major importance in the hyperphosphorylation of tau. Because the activation of JNK is critically involved in Aβ-induced cell death (Wei et al., 2002), we investigated the effects of IMX on JNK activation after Aβ25–35 exposure. Expression of p-JNK in the Aβ25–35-exposed cells was significantly higher than in control cells. However, there were no significant changes after IMX treatment, indicating that IMX did not influence JNK in our cell system.

In summary, IMX exerted neuroprotective effects by preventing Aβ-induced damage, via a mechanism that likely involves inhibition of tau phosphorylation. The suppression of GSK-3β signaling was the most important route by which IMX suppressed phosphorylation. Taking into consideration the important role of Aβ throughout the pathogenesis of AD, our results suggest that IMX is a promising drug candidate for the treatment of AD.

Author contributions: YDZ, SGZ and XSW conceived and designed the study. XJL, SGZ, LGX and JL performed the experiments. SGZ, IPS, MQ and QD analyzed the data and wrote the paper. All authors approved the final version of the paper.

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