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N-stearoyl-L-Tyrosine inhibits the cell senescence and apoptosis induced by H_2O_2 in HEK293/Tau cells via the CB2 receptor

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ABSTRACT

Although considerable energy and money have been spent trying to inhibit Aβ production and its related metabolic enzyme activities, there are still no drug treatments available to cure even slow for Alzheimer's disease. Therefore, tau protein has been focused recently as the new target for the treatment of Alzheimer's disease. The transfected human embryonic kidney 293 (HEK 293) cells with or without Tau 411 plasmid were used to evaluate the effect of tau protein on cell viability. H_2O_2 was added to simulate microenvironment of oxidative stress (OS) during aging. N-stearoyl-L-tyrosine (Nstyr), one of the synthesized N-arachidonoylethanolamide analogues was administrated in HEK293/Tau cells during H_2O_2 insults. Cellular senescence and tau aberrant modification appeared after tau transfection and aggravated by H_2O_2 insult which detected by β-galactosidase staining analysis and western blotting analysis. The level of expression of Bcl-2 and the result of FCAS analysis indicated the appearance of cellular apoptosis. The expression of prosenescence moleculars such as p16-Rb and P53 were induced by tau transfection in HEK293 cells. Both p16-Rb and p53 senescent molecules were inhibited by Nstyr. AM251 (1 μM; an antagonist of CB1 cannabinoid receptor) or AM630 (1 μM; an antagonist of CB2 cannabinoid receptor) was used to offset the anti-senescence effects afforded by NsTyr. The anti-senescence and anti-apoptosis effect of NsTyr was completely abolished by AM630. Meanwhile, transfection of $siRNA_{CB2}$ was used to further confirm the above experimental results and it came out the similar results compared with AM630. Taken together, our results suggest that oxidative stress aggravates cellular senescence and apoptosis in HEK293/Tau, which can be reversed by Nstyr via CB2 receptor.

1. Introduction

Neurodegenerative diseases, such as Alzheimer's Disease (AD), are age-related, progressive and have a high rate of incidence in the old people [1]. One pathologic hallmark in AD is neurofibrillary tangles, which consist of aberrant forms of the microtubule-associated protein tau [2]. According to recent researches, treatment of 3 × Tg-AD mice based on Tau can clearly improve cognitive abilities. Therefore, Tau plays an essential role in the impairment of congition in neurodegeneration.

Excessive reactive oxygen species (ROS) generation is closely associated with worsen microenvironment of brain in AD patients which induces the abnormal proteins aggregation during the process of neurodegeneration [3]. Oxidative stress is also involved in post translational modifications. According to our previous researches, Neurofibrillary tangles (NFTs) induced by aberrant modification of tau protein under OS can cause cellular damage [4]. H_2O_2 is usually chosen to mimic the oxidative microenvironment during aging because of its stable, uncharged, and freely diffusible characteristic between and within cells compared with other members of ROS group [5].

Endocannabinoid system (ECS) has been shown to be implicated in different pathophysiological functions including cognition and neurodegeneration [6]. Cannabinoid receptors (CB) are widely distributed in the central nerve system. Most CB1 receptor with particular high levels distributes in the neocortex, hippocampus, cerebellum, brain stem, and basal ganglia according to molecular biological studies. While CB2 receptor has mainly involved in

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immune cells such as microglia [7]. It has been reported that the agonists of CB1 receptor and (or) CB2 receptors have potential neuroprotective effects on neurodegenerative diseases and brain stroke. Especially CB2 receptor may play an important role in Alzheimer's Disease [8].

Cellular senescence, also termed replicative senescence, was first proposed four decades ago. Cellular senescence is induced not only by telomere shortening, but also by various types of cellular stress including oxidative stress, oncogene activation, DNA damage, and chromatin abnormality [9]. So far, senescence-associated growth arrest has been shown to depend functionally on the tumor-suppressor pathways controlled by p16INK4a and pRB (retinoblastoma protein), as well as by p53. Cellular senescence, especially induced by ROS, plays an important role in age-related disease.

The endogenous arachidonate-based lipids, anandamide (N-arachidonoylethanolamide, AEA) and 2-arachidonoylglycerol (2-AG), are known as "endocannabinoids". Endocannabinoids are taken up by a transporter on the glial cell and degraded by fatty acid amide hydrolase (FAAH), which cleaves anandamide into arachidonic acid and ethanolamine or monoacylglycerol lipase (MAGL) [10]. N-fatty-acyl amino acids exist as endogenous substances and may have a role in regulating tissue functions as AEA. Endogenous N-arachidonylglycine is present in many tissues at a high concentration and has a potent inhibitory effect on fatty acid amide hydrolase (FAAH) which is primarily responsible for the degradation of AEA. Among the AEA analogues, N-fatty-acyl amino acids with a long-chain fatty acyl structure have emerged as the most attractive candidates. Among a series of N-fatty-acyl amino acids as analogues of AEA, N-stearoyl-L-tyrosine (NSTyr), elicited good neuroprotective activities, has been reported.

There is considerable literature describing the helpful effects of AEA and its analogues on prevention of ischemia stroke, anti-inflammation and have the potential to decrease the age-related neuroinflammation [11]. However, the effects of AEA on the cell senescence and apoptosis are still somewhat unclear. In the present study, we evaluated the anti-senescence and anti-apoptosis effects of NsTyr on cell senescence caused by tau protein under OS and try to illuminate its mechanism.

2. Materials and methods

2.1. Cell culture

HEK293/tau cells were cultured in Dulbecco's modified Eagle's medium (DMEM/High Glucose) with 10% fetal bovine serum. No antibiotics were used. The cells were kept in an incubator at 37 °C in a 5% CO_2 fully humidified atmosphere. Growing HEK293 cells until 80% confluent in 12-well cell culture precoated with Poly-D-Lysine hydrobromide (PDL). Transfecting cells with or without Tau 411 plasmid according the protocol. After 24 h incubation, insulting cells with H_2O_2 at 50 μm to stimulate OS. (0.3, 1, 3) μM of Nstyr solution, 1 μM AM251, and 1 μM AM630 was respectively added to each well of, and the plate was incubated for 24 h in the incubator. Then measured with SA-β-gal Assay and Western Blot Analysis.

2.2. Transient transfections and treatment

One day prior to transfection, HEK293 cells were seeded in 12-well cell culture plate at 10^6 cells/well and incubated at 37 °C and 5% CO_2. The following day, cells were transfected with plasmids expressing Tau 441, using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were used for treatment at 24 h after transfection. For insulting of 50 μM of H_2O_2 solution to simulate OS, (0.3, 1, 3) μM of Nstyr solution,1 μM AM251, and 1 μM AM630 was respectively added to each well of, and the plate was incubated for 24 h in the incubator, then measured by SA-β-gal Assay, FACS analysis and Western Blot Analysis respectively.

2.3. Cytotoxicity assays

To do cytotoxicity experiments, cells were seeded in 96-well cell culture at 2×10^4 cells/well and transfected according to the instructions. After 24 h of transfection, (0.3, 1, 3 μM) of Nstyr solution, (1, 3, 10) μM of AM630 or AM251 and 50 μM H_2O_2 were added to cells for 24 h. Cytotoxicity was assessed by using cell counting kit-8 (Dojindo, Kumamoto, Japan). 10 μl of cck-8 solution was added to each well of, and the plate was incubated for 2 h in the incubator. Absorbance was measured at a wavelength of 450 nm with a microplate reader.

2.4. SA-β-gal assay

Cells were plated on PDL-coated coverslips. Cells were treated with different inhibitors in the presence or absence of NsTyr to determine which CB receptor antagonists may block the anti-senescence provided by 3 μM NsTyr. AM251 (a highly selective inhibitor of CB1 receptor) or AM630 (a highly selective inhibitor of CB2 receptor) was incubated respectively with cells 1 h prior to and during H_2O_2 insult. Cellular senescence was evaluated by using senescence β-Galactosidase Staining Kit (Beyotime, Haimen, China). Cells were immobilized for 15 min by β-galactosidase stain-fixative and washed with PBS three times of 5min. After eliminating PBS, the cells were incubated sequentially with working solution (10 μl solution A, 10 μl solution B, 930 μl solution C, 50 μl X-Gal solution) at 37 °C overnight. The absolute numbers of SA-β-gal$^+$ cells were quantified by counting six random fields per slides.

2.5. FACS analysis

For the detection of apoptotic cells, the FITC Annexin V Apoptosis Detection Kit II from BD Pharmingen™ (Bd Biosciences, San Diego, CA, USA) was used, according to the manufacturer's protocol. Hek293 cells were seeded at a density of 1×10^6 cells/well into 6-well plates, incubated for 24 h, and then separately infected with plasmids expressing Tau for approximately 24 h, treated with different drugs and incubated for another 24 h. Next, the cells were harvested and washed twice with cold PBS and then resuspended cells in 1 × Binding Buffer at a concentration of 1×10^6 cells/ml, transferred 100 μl of the solution (1×10^5 cells) to a 5 ml culture tube. After added 5 μl of FITC Annexin V and 5 μl PI. Then the cells gently vortexed and incubated for 15 min at RT (25 °C) in the dark. At last, added 400 μl of 1 × Binding Buffer to each tube and analyzed by flow cytometry within 1 h.

2.6. Silence of CB2 receptor via siRNA transfection

Cells were plated in six-well plates and grown to 50–70% confluence before transfection. Transfection of $siRNA_{CB2}$ (sc-39912, Santa Cruz Biotechnology, DBA, Milan, Italy) was performed at a concentration of 100 nM using RNAiFectTM Transfection Kit (Qiagen). Cells were cultured in complete media, and total proteins were isolated at 72 h for Western blot analysis of CB2 receptor