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L-Carnitine attenuates H_2O_2-induced neuron apoptosis via inhibition of endoplasmic reticulum stress

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ABSTRACT

Both oxidative stress and endoplasmic reticulum stress (ER stress) have been linked to pathogenesis of neurodegenerative diseases. Our previous study has shown that L-carnitine may function as an antioxidant to inhibit H_2O_2-induced oxidative stress in neuroblastoma SH-SY5Y cells. To further explore the neuroprotection of L-carnitine, here we study the effects of L-carnitine on the ER stress response in H_2O_2-induced SH-SY5Y cell injury. Our results showed that L-carnitine pretreatment could increase cell viability; inhibit apoptosis and ROS accumulation caused by H_2O_2 or tunicamycin (TM). L-carnitine suppress the endoplasmic reticulum dilation and activation of ER stress-associated proteins including glucose-regulated protein 78 (GRP78), CCAAT/enhancer-binding protein-homologous protein (CHOP), JNK, Bax and Bim induced by H_2O_2 or TM. In addition, H_2O_2-induced cell apoptosis and activation of ER stress can also be attenuated by antioxidant N-acetylcysteine (NAC), CHOP siRNA and the inhibitor of ER stress 4-phenylbutyric acid (4-PBA). Taken together, our results demonstrated that H_2O_2 could trigger both oxidative stress and ER stress in SH-SY5Y cells, and ER stress participated in SH-SY5Y apoptosis mediated by H_2O_2-induced oxidative stress. CHOP/Bim or JNK/Bim-dependent ER stress signaling pathways maybe related to the neuroprotective effects of L-carnitine against H_2O_2-induced apoptosis and oxidative injury.

1. Introduction

Numerous studies have demonstrated the oxidative stress mediated by reactive oxygen substances (ROS) involved in pathogenesis of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and ischemic and hemorrhagic stroke and antioxidant strategy has shown promise in the treatment of both acute and chronic neurodegenerative diseases (Halliwell, 2006; Calabrese et al., 2010; Ghosh et al., 2011). In addition to oxidative stress, endoplasmic reticulum (ER) stress characterized by unfolded protein accumulation and up-expression of glucose-regulated protein 78 (GRP78), also serves as an important role in neuron apoptosis and a number of classic death signals may involve ER gateways (Logue et al., 2013; Scheper and Hoozemans, 2009). The ER-mediated pathway triggered by ER stress will leads to proapoptotic unfolded protein response including induction of CCAAT/enhancer-binding protein-homologous protein (CHOP), activation of the apoptosis signal-regulating kinase 1 (ASK1)–c-Jun-N-terminal kinase (JNK) pathway and Bim upregulation (Liu et al., 2013; Stefani et al., 2012). As an important organelle for neuronal survival and normal cellular function, ER is sensitive to alterations in cellular homeostasis such as redox imbalance in neuronal oxidative injury (Malhotra and Kaufman, 2007). When the client protein load is excessive compared with the reserve of ER chaperones, the ER stress occurred. Meanwhile, unfolded protein accumulation could produce more ROS, which may lead to oxidative stress (Gibson and Huang, 2004). Studies have demonstrated that both oxidative stress and endoplasmic reticulum stress (ER stress) have been linked to pathogenesis of many neurodegenerative diseases and lead to neuron apoptosis (Higgins et al., 2010; Kanekura

Abbreviations: H_2O_2, hydrogen peroxide; TM, tunicamycin; ROS, reactive oxygen species; DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; GRP78/Bip, glucose-regulated protein 78; MAPK, mitogen-activated protein kinase; CHOP, CCAAT/enhancer-binding protein-homologous protein; NAC, N-acetylcysteine; ER stress, endoplasmic reticular stress; ERK, extracellular-signal regulated kinase; JNK, c-Jun N-terminal protein kinase; ESR, electron spin resonance technology.

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et al., 2009). However, the cross-talk between oxidative and ER stress and whether and how ER stress participated in the neuronal oxidative injury need further study.

L-Carnitine (4-N-trimethylammonium-3-hydroxybutyric acid) is an endogenous mitochondrial membrane compound and natural dietary additives (Nalecz and Nalecz, 1996). Recently studies have reported that L-carnitine could effectively protect various cells against oxidative injury both in vitro and in vivo (Virmani and Binienda, 2004; Binienda et al., 2004; Gülçin, 2006; Binienda and Ali, 2001; Rani and Pannerelvam, 2001; Dhitavat and Ortiz, 2005; Mazzio et al., 2003), which is a potential antioxidant for oxidative stress related neurodegenerative diseases. In our previous study, we used the human neuroblastoma SH-SY5Y cell line as an in vitro model and assessed the effect of L-carnitine on hydrogen peroxide (H_2O_2)-mediated oxidative stress and neurotoxicity. Our results showed that the neuroprotective effect of L-carnitine were mediated, at least, through scavenging oxygen free radicals, prevention of oxidation of lipids, enforcement of endogenous antioxidant defense, inhibition of cell apoptosis and regulation apoptosis related gene expression of Bcl-2 and Bax (Yu et al., 2011). Given the ER location of Bcl-2 and Bax proteins and their key role in the apoptotic cascade, it is reasonable to speculate that H_2O_2 may cause ER stress and thereby contributes to apoptosis of SH-SY5Y cells and ER stress pathways may be related to the antioxidative effects of L-carnitine on H_2O_2-induced neuronal apoptosis.

To investigate the above hypothesis, here we observed the endoplasmic reticular ultra structural changes to verify the ER stress response in H_2O_2-induced or TM-induced SH-SY5Y cell apoptosis. Furthermore, the effects of L-carnitine on ER stress response, including the expression of glucose-regulated protein 78 (GRP78), CCAAT/enhancer-binding protein-homologous protein (CHOP), JNK, Bcl-2, Bax and Bim were studied. Meanwhile, the neuroprotective effects of N-acetylcysteine (NAC, ROS scavenger) and 4-PBA (a chemical chaperones and inhibitor of ER stress) on H_2O_2-induced SH-SY5Y cell injury were also observed.

2. Methods

2.1. Chemicals and reagents

H_2O_2, NAC, L-carnitine, 4-phenyl-butyrate (4-PBA), dichlorofluorescein-diacetate (DCFH-DA), Hoechst33258, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) and tunicamycin (TM) were purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). All other chemicals were of analytic grade.

2.2. Cell culture and treatment

Human neuronal-like cells, SH-SY5Y, were routinely grown at 37 °C in a humidified incubator with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin. To determine the effects of L-carnitine on H_2O_2-exposed SH-SY5Y, the subconfluent (70–80%) cells were treated with indicated doses of L-carnitine or NAC (5 mM) for 3 h or 4-PBA (5 mM) for 6 h before H_2O_2 (400 μM) or TM (10 mM) exposure. Thereafter, cells were washed with PBS to remove the extracellular L-carnitine or NAC or 4-PBA and then cells in fresh medium were exposed to the desired doses of H_2O_2 or TM. Afterwards, cells were rinsed with fresh medium (without H_2O_2) and incubated. Cells were harvested for further analysis.

2.3. Cell viability analysis

Cell viability and proliferation was determined using the MTT assay, which is a sensitive measurement of the normal metabolic status of cells. Briefly, cultured SH-SY5Y cells were initially plated in triplicate at a density of 1×10^4 cells/100 μl in 96 well plates for 24 h. The cells were pre-incubated with or without L-carnitine, NAC or 4-PBA following incubation with H_2O_2 or TM for 24 h. The cells were then incubated with 0.5 mg/ml MTT at 37 °C for 4 h. The formazan crystals generated by viable mitochondrial succinate dehydrogenase from MTT were extracted using an equal volume of the solubilizing buffer (0.01 N HCl and 10% SDS). Absorbance was measured at a wavelength of 490 nm using a Molecular Devices VersaMax microplate reader (Molecular devices, Sunnyvale, CA, USA). All experiments were performed in triplicate.

2.4. Apoptosis detection by nuclear Hoechst staining and Annexin V-FITC/PI assay

Apoptotic morphological criteria were observed by including shrinkage of the cytoplasm (round shape), nuclear condensation and membrane blebbing assayed by Hoechst 33258 staining. SH-SY5Y cells were grown in 24-well plates on poly-L-lysine coated cover slips. After different treatment for 24 h as described above, cells were fixed with 4% paraformaldehyde in PBS (120 mM NaCl, 19 mM Na_2HPO_4, 6 mM KH_2PO_4), pH 7.4, for 30 min. Cells were washed with PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 min and washed again. Cover slips were incubated with Hoechst 33258 (60 ng/ml) for 10 min. Slides were rinsed briefly with PBS, air-dried, then mounted in antifluorescein fading medium (Perma Flour, Immunon, PA, USA). Slides were analyzed under a fluorescence microscope (BX50-FLA, Olympus, Tokyo, Japan). Cells with condensed nuclei condensations were scored as apoptotic. The percentage of apoptotic cells in relation to the total number of cells was determined from 10 random fields per slide, from three independent experiments.

Apoptosis quantification was also observed by Annexin V-FITC/PI assay (McCullough et al., 2001). Briefly, after different treatment for 24 h as described above, cells were harvested by 0.25% trypsin, washed twice with cold PBS, and resuspended in 1 × binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cells/ml. Then the cells were incubated with AnnexinV- FITC and PI for 15 min at 20 °C in the dark. Samples were acquired on a FACScan flow cytometer (FACSCalibur, Becton Dickinson) and analyzed using CELLQuest software with in 1 h. Cells that were Annexin V^-/PI^+ were counted as necrotic, those that showed up as Annexin V^+/PI^+ were counted as late apoptotic or secondarily necrotic, and Annexin V^+/PI^- cells were recognized as apoptotic. Each measurement was carried out at least in triplicate.

2.5. Transmission electron microscopy

Cultured SH-SY5Y cells were initially plated in triplicate at a density of 5×10^5 cells/well in 6 well plates for 24 h and then pre-incubated with or without L-carnitine following incubation with H_2O_2 for 24 h. The cultured SH-SY5Y cells were trypsinized and collected into Eppendorff tube after washing. They were fixed by 2.5% glutaraldehyde at 4 °C and washed by PBS, fixed by osmic acid, then washed by distilled water, and dehydrated by dimethylketone. After embedment in Epon-812, the sample was cut into ultrathin sections (70 nm). The ultrathin sections were dyed with uranium acetate and plumbum citrate and examined with JEM-1200EX electron microscopy (Le Bel et al., 1992).