Roles of Calcium, Calmodulin and Calcineurin in Hydrogen Peroxide-Induced Cytotoxicity in Huaman Neuroblastoma SH-SY5Y Cells

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Abstract

The accumulation of reactive oxygen species (ROS) leads to oxidative stress generation and induction in cell death. Recent evidence has demonstrated the role of calcium dysregulation in pathological process of neurodegenerative diseases. However, the contribution of calcium transducer, calmodulin and calcineurin in oxidative stress-induced neuron cells death has not been elucidated. This study was aimed to investigate the time-dependent effect of oxidative stress on activation in calcium-dependent cell death process. The non-radical ROS, hydrogen peroxide (H_2O_2) was used to induce oxidative stress in neuroblastoma SH-SY5Y cells. The results showed that 200 µM H_2O_2 treatment for 24 hours significantly decreased cell viability. The levels of 32 kDa-cleavage form of calcineurin was significantly increased at 2 hours of H_2O_2 treatment. Moreover, H_2O_2-treated for 8, 12, and 24 hours significantly increased the levels of calmodulin. This finding might emphasize the involvement of calcineurin and calmodulin in oxidative stress-induced cytotoxicity in neuroblastoma cells.

Keywords: Calcineurin / Calmodulin / Neuronal Cell Death

บทคัดย่อ

การสะสมของ reactive oxygen species (ROS) นำไปสู่การเกิดความเครียดแบบออกซิเดชั่น และสามารถเหนี่ยวนำให้เกิดการตายของเซลล์ ข้อมูลจากงานวิจัยในปัจจุบันบ่งชี้ว่า การทำงานที่ผิดปกติของแคลเซียมเกี่ยวข้องกับกระบวนการเสื่อมของเซลล์ประสาท แต่การกระตุ้นการทำงานของสารสื่อสัญญาณแคลเซียมเช่นแคลโมดูลิน และแคลซินูริน ในขบวนการที่ความเครียดแบบออกซิเดชั่นเหนี่ยวนำให้เกิดการตายของเซลล์ประสาทยังไม่ได้มีการศึกษาวิจัย ดังนั้นในการศึกษาวิจัยนี้จึงมุ่งที่จะศึกษาการกระตุ้นขบวนการเหนี่ยวนำการตายของเซลล์จากแคลเซียมในช่วงเวลาต่าง ๆ ภายใต้สภาวะความเครียดแบบออกซิเดชั่นในเซลล์ประสาท SH-SH5Y สภาวะความเครียดแบบออกซิเดชั่นถูกเหนี่ยวนำโดยไฮโดรเจนเปอร์ออกไซด์ ผลการทดลองพบว่าเซลล์ประสาทที่ถูกกระตุ้นด้วยไฮโดรเจนเปอร์ออกไซด์ที่ความเข้มข้น 200 ไมโครโมลาร์ นาน 24 ชั่วโมง มีการอัตราการรอดของเซลล์ลดลง และที่ระยะเวลา 2 ชั่วโมงมีการเพิ่มขึ้นอย่างมีนัยสำคัญของระดับแคลซินูรินขนาด 32 kDa นอกจากนี้ยังพบการเพิ่มขึ้นอย่างมีนัยสำคัญของของระดับแคลโมดูลินที่ระยะเวลา 8, 12 และ 24 ชั่วโมง ตามลำดับ ผลจากการศึกษาบ่งชี้ถึงบทบาทของแคลโมดูลิน และแคลซินูรินในขบวนการที่ความเครียดแบบออกซิเดชั่นเหนี่ยวนำให้เกิดการตายของเซลล์ประสาท

INTRODUCTION

Reactive oxygen species (ROS) is a reactive molecule and a free radical derived from molecular oxygen. Basically, the ROS is necessary to prevent the cellular macromolecule damage meanwhile the disproportion of superoxide anion production within mitochondria results in the cellular oxidative stress disturbing other biological macromolecules. ROS has been elucidated a role in cellular signaling for example gene expression, organelle damage, and cellular responses including inflammatory, cell survival, proliferation and cell death which eventually lead to aging, organ dysfunction and other pathological diseases (Kregel & Zhang, 2007). Since a free radical damage has been interpreted the association in neurodegenerative diseases. Hydrogen peroxide (H_2O_2) is used as a common ROS-induced cytotoxicity model and being the fundamental model feature developing to initiate a free radical generation (Kwon & Choi, 2006). Regarding to the neuronal cells is evidently susceptible to calcium (Ca^{2+}) activity. Hence, to maintain the proper neuronal activity requires an optimal Ca^{2+} concentration to maintain a proper physiological responses of calcium transduction proteins. (Crabtree, 2001; Wu et al., 2007). A calcium regulatory binding proteins provides feedback control calcium signaling like calmodulin (CaM) which is a ubiquitous protein mainly in Ca^{2+}-dependent manners. The complex of Ca^{2+}/CaM binding its target proteins facilitates initiation of various signaling pathways. One another calcium transducer is calcineurin (Cn) which is responsible for mediating a wide variety of cellular processes in dynamic calcium signal responses (Burkard et al., 2005). The regulation of calcium through calcineurin transcriptional and posttranslational manners suggest to be one of a critical mechanism for promoting and maintaining neuronal calcium dysregulation in age-related neurodegenerative diseases (Reese & Taglialatela, 2011; Lee et al., 2016). The potential reciprocation between calcium transducer proteins implies to play a critical role in a several pathological calcium-dependent diseases. Nevertheless, the precise mechanisms involved in the spatiotemporal control of calcineurin signaling are poorly understood. Our study aimed to investigate the dynamic expression of calcineurin/calmodulin calcium-dependent pathway under time dependent conditions with hydrogen peroxide-induced cytotoxicity in human neuroblastoma SH-SY5Y cells which possibly partially beneficial in a novel neuroprotective reagent research and development.

OBJECTIVES

To investigate the effect of hydrogen peroxide-induced cytotoxicity on the calcineurin proteolytic cleavage and calmodulin protein expression corresponding to time dependent treatment in human neuroblastoma SH-SY5Y cells.

LITERATURE REVIEW

Reactive oxygen species (ROS) is a natural byproducts of aerobic metabolism, correlated with cell proliferation through the activation of growth-associated signaling pathways (Min et al., 2008). The optimal ROS can protect the cellular macromolecule damaging through anti-oxidant mechanisms. Over expose to ROS induces oxidative stress actives which could disturb cellular organelles, cell viability, and cause variety of

pathologies, finally lead the cell to commit suicide. The bioenergetic activity of mitochondria is necessary for cellular energy production resulting in ROS formation. During electron transport chain reaction, the complex-I gives superoxide anion (O_2) products within the mitochondrial matrix whereas the complex-III derived O_2 disperses into the matrix and intermembrane space (Brand, 2010). At the same time, O_2 could spontaneously interacts with the ionic iron in the catalytic of mitochondrial enzymes and serves as a source for the highly effective radicals including H_2O_2, OH- and peroxynitrite (Pacher et al., 2007). Toxicity due to the direct and indirect effects of O_2 is mitigated by the antioxidant systems of the mitochondria. Basically, mitochondria contain superoxide dismutase, catalase and the thioredoxin system to detoxify O_2 and reverse its toxic effects against ROS generation (Duchen, 2004; Kang & Pervaiz, 2012).

The high effective radical such hydrogen peroxide (H_2O_2) has been implicated in cellular and tissue injury during pathological conditions, such as ischemia-reperfusion injury, hyperoxia, and inflammation (Cereghetti et al., 2008; Cereghetti et al., 2010). H_2O_2 stimulates a various cell with either cytokines or phorbol ester increase from the secretion of hydrogen peroxide into the extracellular space *in vitro* (Kwon & Choi, 2006). A high concentration of hydrogen peroxide can exert toxic effect on sensitive cells. H_2O_2-mediated oxidative stress generally relates to necrosis cell death and a role in apoptosis as well. The oxidative stress has been implied in many pathological relevant such as cancer, diabetes type 2, arteriosclerosis, chronic inflammation, ischemia and reperfusion injury and neurodegenerative diseases (Ott et al., 2007).

RESEARCH SCOPE

The human neuroblastoma SH-SY5Y cell cultures were exposed to toxic dose of hydrogen peroxide (H_2O_2) to induce oxidative stress. Cell death was investigated using cell viability assay. The levels of calcium transducer proteins were investigated using Western blot analysis.

METHODOLOGY

Cell Culture and treatment

Human neuroblastoma SH-SY5Y cells were cultured in complete media containing 45% MEM, 45% Ham's F-12, 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin at 37 °C in 95% humidified atmosphere with 5% CO_2 incubator. Before experiments, the cells were seeded at 4.5×10^5 and incubated overnight in 6-well plates until grown approximately 80% confluence. After that 200 µM H_2O_2 was freshly prepared and added to the culture medium for indicated time.

Cell viability assay

Human neuroblastoma SH-SY5Y cells were in 96-well culture plates and incubated at 37 °C in an incubator overnight. The cells were treated with 100 µM or 200 µM H_2O_2 for 2-24 hours. The control-untreated cells were incubated with culture medium for 24 hours. 10 µM of the MTT solution in PBS was added into each well. Then, the culture plates were

incubated for 4 hours. After that the solution was aspired and replaced by DMSO to dissolve formazan crystals. The optical density was measured at 570 nm of spectral wavelength by a microplate reader (Bio-Tek instrument, Winooski, VT, USA).

Western blot analysis

To observe time-dependent effect of H_2O_2-induced toxicity, the cells were treated with H_2O_2 for 2, 4, 8, 12, and 24 hours. The control cells were incubated with 10% FBS-complete media for 24 hours. After incubation, the cells were washed once with ice-cold phosphate buffer solution. The fresh ice-cold Lysis buffer (50 mM Tris pH 8, 150 mM NaCl, 0.5% sodium dodecyl sulphate 0.1% SDS; sodium dodecyl sulphate, 1% Nonidet P40, 10% glycerol and protease inhibitor cocktail set) was prepared and added into each culture well plates. Then, the cells were scraped with plastic cell scrapper on ice-cold condition. After that scraped cells were gently transferred to 1.5 ml eppendorf tube on ice and were left on ice for 15 minutes. Then, the cells were sonicated following by centrifugation for 20 minutes at 14.000 rpm at 4°C. The protein in supernatant was gently collected and aliquoted into new tubes for western blot analysis.

Statistical analysis

Statistical analysis was performed using the SPSS version 18.0 for window software (SPSS Inc., Chicago, IL, USA). The significant data was assessed using one-way analysis of variance (ANOVA) or one-way repeated measures test ANOVA followed by Tukey- Kramer test (multiple comparisons analysis). The data are expressed as mean ± SEM. The probability (*P*) value less than 0.05 was considered statistical significant different.

RESULTS

The effect of H_2O_2 on cell viability in neuroblastoma SH-SY5Y cells

The viable of SH-SY5Y cells treated with 200 µM H_2O_2 for 24 hours was significantly decreased (81±5.3% of the control value) when compared to control-untreated cells. 100 µM H_2O_2 treatment for 2, 4, 8, 12, and 24 hours did not change the cell viability. This result demonstrated that 200 µM H_2O_2 treatment for 24 hours induced cell death in neuroblastoma SH-SY5Y cells.

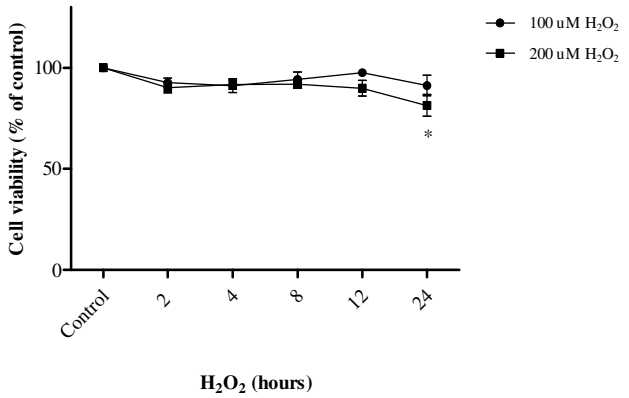

Fig. 1: The effect of H_2O_2 treatment on cell viability. The viable of cells were detected by MTT assay. SH-SY5Y cells were treated with 100 or 200 µM H_2O_2 for 2, 4, 8, 12, and 24 hours, respectively. The results are expressed as mean ± SEM of four independent experiments. *p< 0.05 compared to control-untreated cells.

The effect of H_2O_2 treatment on proteolytic cleavage of calcineurin in SH-SY5Y cells

Proteolytic cleavage of calcineurin was demonstrated by cleavage of full length calcineurin (60 kDa) into 32 kDa fragmentation using western blot analysis. 200 µM H_2O_2-treated SH-SY5Y cells for 2, 4, 8, 12, and 24 hours did not change the levels of 60 kDa-calcineurin when compared to control-untreated cell. However, H_2O_2-treated cell for 2 hours significantly increased the levels of 32 kDa-calcineurin (147±22.7% of the control value) when compared to control-untreated cells.

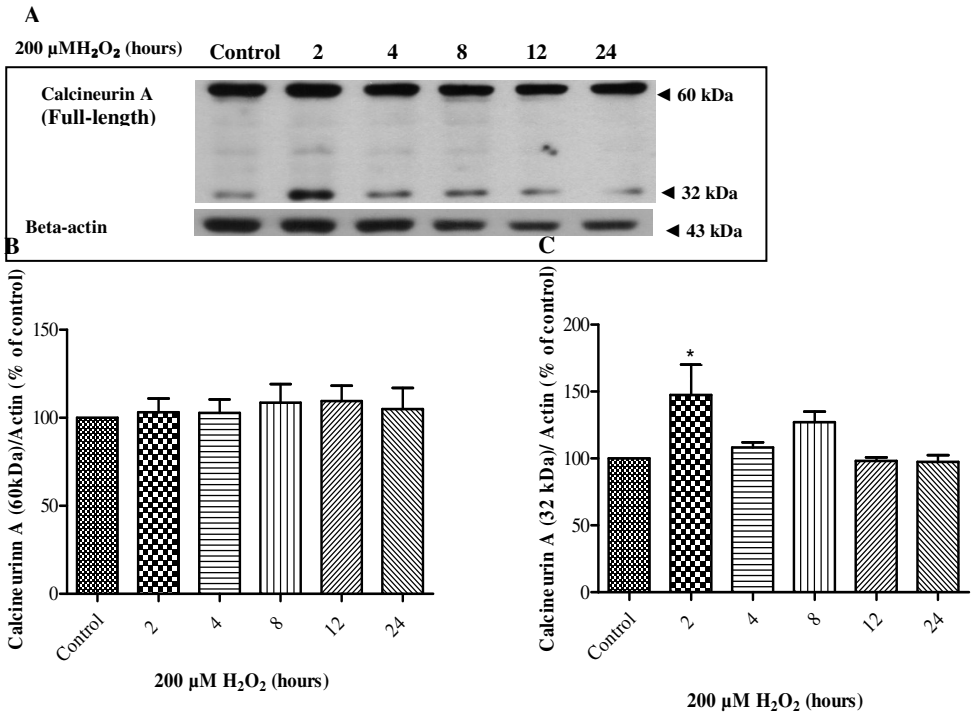

Fig. 2: The effect of H_2O_2 treatment on proteolytic cleavage of calcineurin. (A) Representative immunoblot bands of calcineurin and beta-actin from the whole cell extraction of SH-SY5Y cells. The cells were treated with 200 µM H_2O_2 for 2, 4, 8, 12, and 24 hours, respectively. The control-untreated cells were cultured for 24 hours without H_2O_2 -treatment. The quantitative analysis of protein bands of calcineurin-60 kDa and calcineurin 32 kDa were shown in B and C, respectively. *p< 0.05 compared to control-untreated cells.

The effect of H_2O_2 treatment on calmodulin levels in SH-SY5Y cells

The expression of calmodulin was determined using western blot analysis. H_2O_2 treatment at 200 µM for 2 and 4 hours tended to increase but treatment for 8, 12 and 24 hours significantly increased the levels of calmodulin when compared to control-untreated cells, respectively**.**

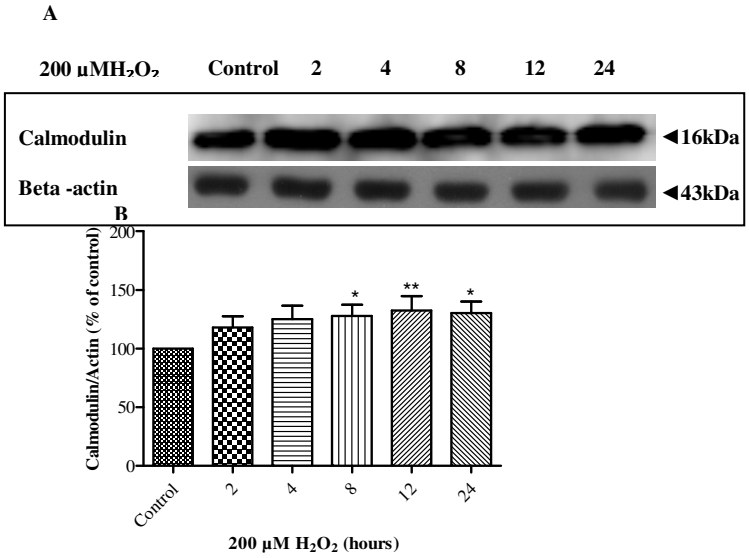

Fig. 3: The effect of H_2O_2 treatment on the levels of calmodulin in SH-SY5Y cells. (A) Representative western blot bands of calmodulin and beta-actin from whole cell extraction of SH-SY5Y cells. The density of protein band were quantified by densitometry. (B) The amounts of protein are expressed as the density of calmodulin/beta actin bands. The results are expressed as mean ± SEM of four independent experiments. *p< 0.05 compared to control-untreated (0 µM) cells and **p< 0.01 compared to control-untreated (0 µM) cells.

Conclusion and Discussion

In the present study, hydrogen peroxide (H_2O_2) can induce cytotoxicity in human neuroblastoma SH-SY5Y cells. The reduction in cell viability to 80% of control-untreated cells was observed after exposure to H_2O_2 for 24 hours. Recent evidence demonstrated that H_2O_2 treatment at 200 and 300 µM for 24 hours significantly decrease the cell viability in SH-SY5Y cells to nearby 80% and 60% when compared to control value, respectively (Tangmansakulchai et al., 2016). Nevertheless, other evidence implicated that at the low concentrations of hydrogen peroxide causes growth stimulation (Wiese et al., 1995; Davies,

1999; Gulden et al., 2010; Iloki-Assanga et al., 2015). Hence, the extracellular concentration of H_2O_2 applied in vitro and mediated cytotoxic cell death in the most cases are supposed to be at higher than in the intracellular concentration of H_2O_2. H_2O_2-induced cytotoxicity activates various cellular signaling responds under cytotoxic cell death consequently leading to ROS generation and oxidative stress. Cell death pathways activation include caspase-mediated proteolytic cell death cascade, calcium signaling on oxidative stress and proteolytic activation of calcineurin (calcium effector). Interestingly, calcineurin showed remarkably cleavage into 32 kDa forms after 2 hours of 200µM H_2O_2 treatment but this cleavage from did not change after 4 to 24 hours of treatment. The cleaved calcineurin is usually degraded by the protease. This proteosomal degradation might be the cause to bring the cleaved calcineurin levels down to the same levels of untreated cells at 4 to 24 hours. Previous study has been reported H_2O_2 exposure resulted in the cleavage of CnA into a 32kDa fragment in rat cortical neurons. In mouse primary cortical neurons shown that chloroquine (a lysosome protease inhibitor) can prevents CnA cleavage, suggesting that CnA cleavage can somehow be mediated by lysosomal proteases that have leaked into the cytoplasm after H_2O_2 exposure (Lee et al., 2007). Moreover, H_2O_2-treated for 8, 12, and 24 hours significantly increased the levels of calmodulin. It has been emphasized that calmodulin exerts its action on calcium/calmodulin-dependent calcineurin activity. This finding might emphasize the involvement of calcineurin and calmodulin in oxidative stress-induced cytotoxicity in neuroblastoma cells.

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