Azadirachtin Induces Primary Hippocampal Neurons Apoptosis Through Calpain Pathway

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Abstract: The function of azadirachtin A inducing neuron apoptosis was revealed and the major apoptotic pathway has been investigated. After cultured hippocampal neurons had been exposed to azadirachtin as intended concentration for 24 h., Hoechst staining showed that azadirachtin A induced primary hippocampal neuron apoptosis with a dose dependent manner. Neurons exposed to azadirachtin were damaged seriously in cytoskeleton abnormality detected by MAP2 immunoassaying. When neurons exposed to the mixed solution of azadirachtin A (40 µM) and calpain inhibitor II (ALLM) 25 µM, it was detected by both Hoechst staining and laser confocal micrographs that the neuron damage was alleviated and the apoptotic rate was reduced if compared with only azadirachtin A solution treatment. Western blotting results revealed that the bands of α- spectrin cleaved products (145kD, 150 kD) became stronger while the bands of P35 became weaker after azadirachtin A exposure. It means that the calpain activity in the neurons increases under the azadirachtin A exposure. These studies indicated that neuron apoptosis induced by azadirachtin was involved in calpain pathway.
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1.Introduction

Azadirachtin, a tetranortritaritarpinoid, is the major active ingredient isolated from neem seed kernel. This plant product has a diverse range of bioactivities, such as insecticidal, anthelmintic, antifeedancy and insect growth regulation effects [1,2,3] .Preparation of azadirachtin was widely used to control insect by reducing their feeding, survival, and reproduction [4] .In addition, azadirachtin was reported to have the functions of antifertility , antidenguevirus, antimalarial and treatment of

Diabetes [5]. Due to the residual contamination of chemical pesticides to agricultural crops and food commodities, the use of azadirachtin as an alternative to synthetic pesticides has increased significantly [6,]. Although azadirachtin is low acute toxicity to mammalian species, with an LD_{50} more than 5000 mg/kg in the rat [7], the possibility of future hazards should not be ignored considering of the wide scale use of azadirachtin in agriculture and the possible use in medicine [8]. As to the cytotoxic mechanisms, there are some clues about azadirachtin on mammalian physiology and toxicology [9,10]. Available evidence indicates that azadirachtin acts primarily on the central nervous system where azadirachtin alters the programming or interferes with the releasing mechanism of neurohormones. Neurophysiological modulation of phagostimulant and, in some cases, deterrent chemoreceptor functions appear to be the main mechanism involved in the potent antifeedant actions of azadirachtin [11]. Azadirachtin also.

Inhibits vitellogenesis, a process of insect egg development. This dose dependent effect involves direct cytotoxic effects and general functional disruption of endocrine in neuron docrine systems. John Akudugu[12] has cultivated six human glioblastoma cells and presented at 28 mM azadirachtin, 25 to 50% lethal glioblastoma cells have been detected. It has been found that the nuclear DNA is a critical target for azadirachtin as evidenced by the formation of micronuclei. A period of 40 hours is required to detect the influence of azadirachtin on DNA. The results of John Akududu made us presume that azadirachtin may induc e neurons apoptosis. The present study has been conducted to assess the inference of azadirachtin on hippocampus in apoptosis. Not only can these experimental results get the data about the safety of azadirachtin on human, but it can be also helpful to understand the action mechanism of azadirachtin on insects .To our knowledge, no previous studies have been carried out to determine the apoptotic actions of azadirachtin on neurons.

2. Materials and methods

Neuronal cultures

Cultures of primary hippocampal and cortical neurons from E17-19 rats were made from fetal Wistar rats and cultured. Briefly, brains were dissected on ice in HBSS (Sigma), and dissociated with 0.25% trypsin.

Neurons were seeded to a density of 2.7 x 10^3 /mm^2 in 12mm type coverslips previously coated with poly-D-lysine (100 µg / ml). After 24 h. *in vitro*, the neurobasal growth medium containing 10% fetal bovine serum was replaced with neurobasal medium without serum, but with B27 (Sigma) supplement. The cultures were maintained in a humidified CO_2 incubator (5% CO_2, 37 ℃). Immunocytochemical staining of the cultures for microtubule associated protein 2 (MAP2) and glial fibrillary acidic protein (GFAP) indicated > 95% of the cells were neurons with minimal contamination by glia.

Hoechst staining and cell apoptotic rate measurements

For Hoechst staining, after treated in the absence or presence a certain dose of azadirachtin A (A ldrich) for 24h., immunostaining neurons were labeled with DNA dye Hoechst 33258 (1µg/ml, 1min) (Sigma). Cell morphology was observed under the microscope and scored for healthy and apoptotic nuclear morphology. Cells were scored positive if they had DNA fragmentation or condensed nuclei (25% loss of nuclear surface versus control). Representative graphs are shown for experiments where at least 150 to 300 cells in 4 randomly fields.

MAP2 and TUNEL double labeling hippocampal neurons

Primary hippocampal neurons grow on poly-D-lysine coated 12mm type coverslips . After neurons were cultured in the absence or presence of intended concentration azadirachtin, calpain inhibitor II (ALLM，purchased from Calbiochem) and caspase inhibitor III(Ac-DEVD-CMK ，calbiochem) for 24 h, cells were washed three times with PBS and fixed with 4% paraformaldehyde in PBS (pH 7.4) for 15 min, rinsed three times with PBS, and permeabilized with 0.5% Triton-X-100 /PBS for 20 min, then blocked in PBS supplemented with 10% BAS blocking reagent(Sigma) at 37 ℃ for 1 h. Primary anti-MAP2 antibody (1:20,000 dilution, Bo¨ ehringer Mannheim) was applied in 1% BAS / PBS overnight. Cells were washed three times and secondary TRITC-conjugated anti-mouse antibody (Sigma) was added at 1: 200 dilution in PBS / 1% BSA blocking reagent for 1h. Coverslips were washed three times .DNA fragmentation was detected by using terminal deoxynucleotidyl transferase (TdT) mediated dUTP biotin nick end labelling (TUNEL) assay. TUNEL staining was performed using an In Situ Cell Death Detection kit, POD (Boehringer Mannheim), then incubated with 0.02% (w/ v) 3,3’ diaminobenzidine and 0.1% (w/ v) (NH_4)$_2$ Ni(SO_4)$_2$ dissolved in 0.05 M *Tris*–HCl buffer(pH 7.6)containing 0.01% (v/ v) H_2O_2 for 2 h .These procedures ended with mount medium on coverslips. MAP2 (red) and TUNEL (green) that double labeled hippocampal neurons were observed by laser confocal micrographs (LMS 510 –V2.3 model, Carl Zeiss Co.,Ltd.) . The fragment of neurons DNA was detected by TUNEL positive while neuron cell apoptosis took place. The Statistic method of apoptotic rate labeled by TUNEL was the same as by Hoechst.

Western blotting analysis

Cell were washed with PBS for two times, then scraped from the culture dish and sonicated on ice in 1% SDS buffer. Equal amounts of total cell protein were boiled in sample buffer for 3 min, loaded on a SDS/12% polyacrylamide gel and transferred to nitrocellulose. Blots containing 5% dry milk were blocked in TBST (10mM *Tris*-HCl pH 8, 150mM NaCl, 0.5% Tween) and probed with the anti-P35 (Santa Cruz) as well as a spectrin primary antibodies, respectively. Incubation with the appropriate secondary antibody conjugated to horseradish peroxidase (Sigma), detection was performed with an enhanced chemiluminescence (ECL) detection system (Amersham). To ensure even loading of protein samples, each blot was stripped with strip buffer (1.5M *Tris*-HCl pH 6.8, 2% SDS, 0.8% 2-mercaptoethanol) at 60 ℃ for 60 minutes followed by a 30 minutes wash in TBST at 25 ℃ and probed by GAPD

3.Results

For Hoechst staining, after immunostaining neurons being labeled with DNA dye Hoechst 33258 (1µg/ml, 1min) and being treated with 4 µM azadirachtin neurons, most of the hippocampal neurons labeled by Hoechst demonstrated a normal size of the nucleus (fig. 1A–B), After 24 h of treatment with 40 µM azadirachtin, the dying neurons exhibited typical apoptotic morphology such as condensed chromatin, shrunken nuclei, apoptotic bodies. More apoptotic neurons were observed at the group when treated with 400 µM azadirachtin A, and they appeared in Hoechst staining imagine as a small bright point indicated by arrowheads (fig. 1 C and D). The Hoechst staining statistic results revealed that high concentration of azadirachtin A induced primary hippocampal neuron apoptosis as a dose dependent manner (fig.2).

A lot of images have been gotten from laser confocal micrographs. Cultured hippocampal neurons double labeled with MAP2 (red) and TUNEL (green) after treatment for 24 h (fig.3)， a normal size of nucleus as well as fine detail of cytoskeleton were observed with MAP2 staining at the control group, but the nucleus and cytoskeleton were damaged seriously at the 40 µM azadirachtin treatment group. About half TUNEL positive hippocampal neurons were observed in these groups. When hippocampal treated with azadirachtin as well as calpain inhibitor II (ALLM) at the same time, the damage degree of nucleus and

cytoskeleton were relieved and the TUNEL positive hippocampal neurons decreased significantly comparing with the single azadirachtin treated group. The decrease of apoptotic rate implicated that calpain inhibtor II had neuron protect effect (fig. 4) and calpain pathway involved in the process of azadirachtin A inducing primary hippocampal neurons apoptosis. Another important apoptosis pathway caspase 3 has also been assayed, but caspase inhibitor III has not shown significant（P>0.05） neuron protect effect, which means caspase 3 does not close relate to azadirachtin A inducing neurons apoptosis.

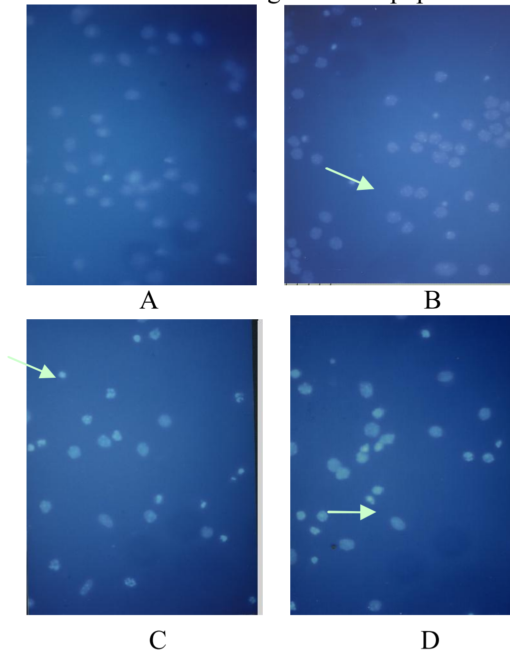

Fig. 1 Alterations in nuclear morphology induced by azadirachtin in primary hippocampal neurons. The apoptotic phenotype including changes in cell shape and hypercondensed chromatin was visualized by Hoechst 33258 staining in cultures exposed for 24 h to azadirachtin at different concentrations: 0, 4, 40 and 400 µM (A–D, respectively).

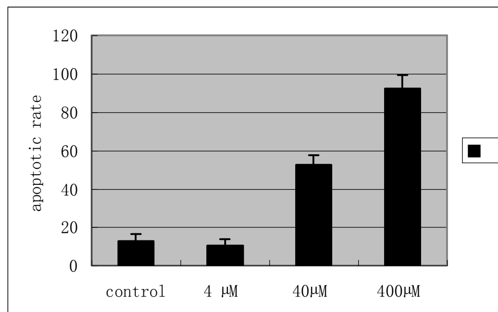

Fig. 2 Hippocampal neurons apoptotic rate induced by different concentration of azadirachtin exposure for 24h.

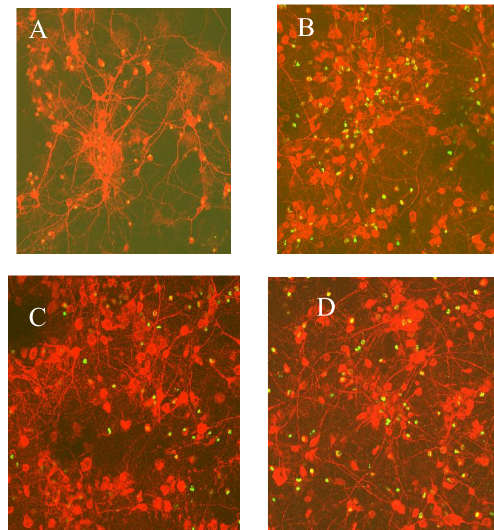

Fig. 3 Calpain inhibitor II (ALLM) 25 µM can protect neurons Cytoskeleton and inhibit neurons apoptosis induced by azadirachtin. Four images were representative laser confocal micrographs of cultured hippocampal neurons double labeled with MAP2 (red) and TUNEL (green) to detect the neuronprotect effect of calpain and caspase3 inhibiter. Apoptosis toke place in TUNEL-positive neurons. (A) Without treatment, (B) treated with azadirachtin A 40 µM for 24 h, (C) treated with azadirachtin and calpain inhibitor II (ALLM) for 24 h ,(D) treated with azadirachtin and caspase inhibitor III for 24 h. Experiments were repeated with 4 times, producing the same results.

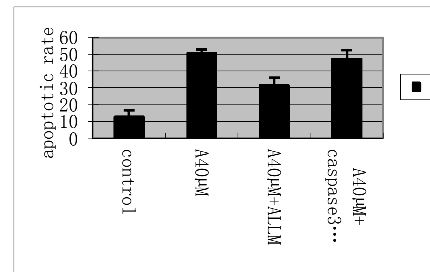

Fig. 4 Calpain inhibitor II (ALLM) inhibits neurons apoptosis induced by exposure of azadirachtin A 40 µM for 24h. These results came from the comparison between TUNEL positive neurons and normal neurons.

Western blot assayed the activity of calpain in the primary hippocampal neurons after 24 h exposure of 40 µM azadirachtin A. α- spectrin primary antibodies marked the cleaved products of α- spectrin (145kD, 150 kD) which reflected the calpain activity. The results (fig.5) revealed that cleaved α- spectrin bands of azadirachtin treated group became stronger than the bands of control group, which indicated that the calpain activity of primary hippocampal neurons increased after 40 µM azadirachtin exposure. When

treated with azadirachtin plus calpain inhibitor II (ALLM) 25 μM, the bands of cleaved α- spectrin was restored as normal as control group.

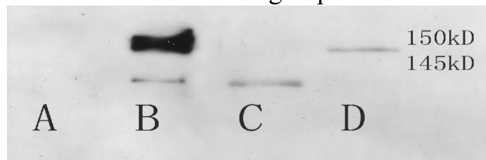

Fig. 5 Western blot assayed the activity of calpain in the primary hippocampal neurons exposed by Azadirachtin. α-spectrin primary antibodies marked the cleaved product of α-spectrin (145kD,150 kD) which is reflection of the calpain activity. (A) Control (B) azadirachtin A 40 μM. (C) azadirachtin A 40 μM +ALLM. (D) Azadirachtin A 40 μM + caspase 3 inhibitor III.

The experiment on activity of P35, one important calpain substrate, demonstrated that P35 bands of 40 μM azadirachtin exposed groups were weaker significantly than those of control group, calpain affected P35 activity through cleaving P35 to P25. But P35 bands of treated with azadirachtin groups recovered in some degree when both azadirachtin and calpain inhibitor II (ALLM) are added to the cultured hippocampal neurons (fig.6). The results above indicated that calpain pathway is involved in azadirachtin inducing primary hippocampal neurons apoptosis.

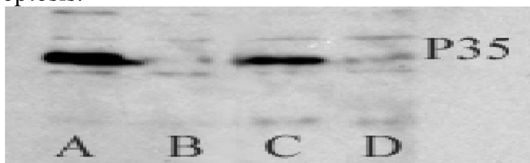

Fig. 6 The bands of P35 was detected by western blot . After azadirachtin exposure for 24 h, the p35 bands of treated with azadirachtin group (B) were weaker than those of control group (A) but recovered in some degree when calpain inhibitor II (ALLM) was added too(C). (A) control (B) azadirachtin A 40 μM. (C) azadirachtin A 40 μM +ALLM. (D) azadirachtin A 40 μM + caspase 3 inhibitor III .

4. Discussion

Apoptosis, which was mediated by an intrinsic death program, is the best study type of cell death [13]. Apoptosis is an essential physiological process that plays a critical role in development and tissue homeostasis. Furthermore, apoptosis is also involved in a wide range of pathological conditions and toxicological stimulation. Apoptotic cells may be characterized by specific morphological and biochemical changes, including cell shrinkage, chromatin condensation, and inters nucleosomal cleavage of genomic DNA [14, 15, 16]. This form of cell death occurs at specific stages during the development of embryonic neurons including those of the sympathetic ganglion, retina, spinal cord, hippocampus and cerebral cortex. Among these neurons hippocampus is the most sensitive to toxin [17]. The calpains form a growing family of structurally related intracellular multidomain cysteine proteinases containing a papain related catalytic domain, whose activity depends on calcium. There are two ubiquitous calpains, μ- and m-calpain, which have been extensively studied both at the DNA and protein levels, containing two subunits, a large catalytic (80 kDa) subunit and a small regulatory (28 kDa) subunit. The recently determined crystal structures of human and rat m-calpain crystallized in the absence of calcium essentially explain the inactivity of the apoptosis form by catalytic domain disruption, indicating several sites where calcium could bind causing reformation of a papain-like catalytic domain, and additionally revealing modes by which phospholipid membranes could reduce the calcium requirement. Current evidence points to a cooperative interaction of several sites, which, upon calcium binding, trigger the reformation of a papain-similar catalytic domain [18]. As an essentially ubiquitous in mammalian and insect tissue, the calpains are believed to play important roles in cytoskeletal remodeling processes, cell differentiation, signal transduction, cell cycleregulation, apoptosis and necrosis, embryonic development and long-term potentiation in the central nervous system [19,20,21]. A number of studies have shown that calpain activation precedes cell death induced by different apoptotic stimuli in various cytosolic Ca^{2+} elevated conditions [22]. When triggered by the calcium cellular signaling, calpains become activation during neuroexcitoxicity and subsequently induce degradation of cellular protein including both structure protein [23] and non-structure biochemical molecules. Degradation and/ or loss of various cytoskeletal elements such as microtubules MAP2, α-calspectin (spectrin) [24,25] together with desmin and α-actinin [26] were also noticed. On the other hand, proteolysis of regulatory proteins, such as P53, Bid, Bax, caspase, CaMKII, P35 and amyloid precursor protein (APP) [27,28,29,30]. For instance, upon neurotoxic stimulation, calpain cleaves P35, a specific activator of Cdk5, to P25, which prolongs Cdk5 activation and causes Cdk5 mislocalization. P25/Cdk5 kinase has potent ability for tau hyperphosphorylation and neuronal death [31]. Ephraim [8] detected a analogy of azadirachtin, epoxyazadiradione, cytotoxic to N1E-115 neuroblastoma (mouse), 143B.TK- osteosarcoma (human) and Sf9 (insect) cultured cell ,the results implicated high concentration calcium increased cell death. The distinct electrophysiological actions of extracellular and intracellular azadirachtin A were

investigated using cultured dorsal root ganglion neurons from neonatal rats and the whole cell variant of the patch clamp technique [32]. In those work, the sensitivity to azadirachtin of the cultured mammalian neurones and some invertebrate preparations such as insect chemosensory systems has been compared. Insect preparations have been found to respond to 1–100 nM azadirachtin. However, only at concentrations of azadirachtin between 10 and 100 μM, the significant changes in the electrophysiological properties of cultured DRG neurones have been found. It appears that some insect sensory systems are about hundreds times more sensitive to azadirachtin than rat cultured DRG neurones. From the discussion above, the presupposition about involvement of calpain pathway in azadirachtin induces primary hippocampal neuron apoptosis can be put out. When primary hippocampal neurons are exposed to high concentration of azadirachtin, in response to extracellular stimuli the level of intracellular calcium is enhanced, which leads to an activation of calpain and then trigger the proteolysis of cytoskeletal proteins, cell membrane proteins and regulatory kinases. The proteolysis of protein such as P35, tau, microtubules, MAP2, α-spectrin, lastly promote the neurons apoptosis. In conclusion, azadirachtin A can induce primary hippocampal neurons apoptosis and the neurons apoptosis induced by azadirachtin is close relative to calpain pathway.

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