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Apolipoprotein E 4 triggers multiple pathway-mediated Ca^{2+} overload, causes CaMK II phosphorylation abnormity and aggravates oxidative stress caused cerebral cortical neuron damage

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Abstract. – OBJECTIVE: Apolipoprotein E (APOE) gene polymorphism is correlated closely with resistance to brain damage. This study aims to investigate the effects of APOE4 on oxidative stress damaged cerebral cortical neuron.

MATERIALS AND METHODS: Primary cerebral cortical neurons were isolated from APOE gene knock-out mice ($APOE^{-/-}$ mice). Oxidative stress damaged $APOE^{-/-}$ mouse cerebral cortical neuron model was established. Three experimental designs (experiment 1, 2, 3) were conducted by employing several methods. Lactate dehydrogenase (LDH) and superoxide dismutase (SOD) analysis were employed for neurotoxicity assessment. Flow cytometry and transferase-mediated deoxyuridine-triphosphate-biotin nick end labeling (TUNEL) were used to examine neuron apoptosis. Immunohistochemistry and Nissl staining were used to identify neuron morphology. Western blot was used to detect phosphorylated CaMK II (p-CaMK II) and cleaved caspase 3 expression. Ca^{2+} levels in neurons were also examined by detecting fluorescence intensity.

RESULTS: APOE4 treatment (Vehicle + APOE4) significantly aggravates oxidative stress damaged cerebral cortical neuron by increasing LDH levels and decreasing SOD activities, induces neuron apoptosis compared to Vehicle group ($p < 0.05$). APOE4 treatment significantly enhanced Ca^{2+} levels compared to Sham group ($p < 0.05$), MK801 treatment (Vehicle + APOE4 + MK801) significantly decreased Ca^{2+} levels compared to the Vehicle+APOE4 group at 12 h and 24 h ($p < 0.05$). APOE4 triggers CaMK II phosphorylation, caspase 3 activation and neurons apoptosis. Both of MK801 and KN93 inhibit CaMK II phosphorylation, decreases caspase 3 activation, and suppresses neurons apoptosis

CONCLUSIONS: APOE triggers Ca^{2+} overload through NMDAR and CaMK II signaling pathway, both of which cause Ca^{2+} concentration increasing, CaMK II phosphorylation abnormity, and finally aggravate oxidative stress damaged neurons apoptosis.

Key Words:
Apolipoprotein E4; APOE4, Oxidative stress, Cortical neuron damage, CaMK II, NMDAR, Apoptosis.

Introduction

For a long time, the occurrence and development of brain damage are extensively considered to be unrelated to the genetic factors[1,2]. However, the functions of the genes and associated products caused by the brain damage response, such as apolipoprotein E (APOE), have been widely studied in the recent years[3-5]. Radojicic et al[6] reported that the APOE gene polymorphism is correlated closely with the resistance to brain damage, and characterizes with subtype specificity. Mori et al[7] found that comparing with the wild-type mice, the vulnerability of brain tissues is significantly increased in APOE gene-knockout mice, which is consistent with the clinical case-analysis conclusion that APOE4 gene correlates with poor prognosis of brain damage[8,9]. Chen et al[10] found that APOE isoforms affect the neuron apoptosis through delayed rectifying K^+ channel post the brain injury of an animal model.

Hoe et al[11] also reported that APOE4 mediates the calcium influx through activating the N-meth-

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yl-D-aspartate receptor (NMDAR). The later study[12] also indicated that APOE interacts with NMDAR by forming the multiple-protein complex and undergoing certain conditions. Some researchers APOE may characterize with subtype specificity and affect the NMDAR, and produce the difference of calcium channel permeability. Finally, the APOE-NMDAR complex causes different degrees of Ca^{2+} accumulation in neurons surrounding the damage regions of cerebral, induces different degree of apoptosis, and further affects the disease progression and prognosis[13].

Totally, APOE gene-subtypes correlate with the resistance to brain damage and cause different endings of damaged nervous system. However, the role and mechanism of the APOE gene-subtypes in physiology and brain damage have not been fully clarified, which needs to be studied intensively. Therefore, this work aims to investigate the effects of APOE4 on the oxidative stress damage cerebral cortical neuron model.

Materials and Methods

APOE Gene Knock-out Mice

The APOE gene knock-out mice ($APOE^{-/-}$ mice) were purchased from Laboratory Animal Center of Peking University Health Science Center, Beijing, China. The $APOE^{-/-}$ mice were fed at Animal Center of Chongqing Medical University, Chongqing, China. The rats were housed in the environment of the light/dark cycle of 12 h/12 h at the room temperature. All the experiments were approved by the Ethics Committee of Chongqing Medical University, Chongqing, China. The present study was conducted according to the guidance of Care and Use of Laboratory Animals of HNI.

Primary Culture for Cerebral Cortical Neuron of $APOE^{-/-}$ Mice

The primary cerebral cortical neurons cultures were established from pups within 24 h from the $APOE^{-/-}$ mice birth. The $APOE^{-/-}$ mice were sacrificed by decapitation, and the brain of mice was immediately removed from the skulls and incubated in the ice-cold D-Hank's balanced salt solution. The blood vessel was removed and cut into pieces adequately. The pieces were treated with the 0.125% trypsin in the D-Hank's balanced salt solution for 20 min at 37°C to obtain the cell suspension. Then, the digestion of the trypsin was terminated by using the fetal bovine serum (FBS, Gibco BRL. Co. Ltd., Grand Island, NY, USA), and the cell suspension was centrifuged at the speed of 1000 r/min for 10 min. The cell suspension was then re-suspended in the Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F12) supplementing with the heat-inactivated 10% FBS at 37°C and 5% CO_2. Twenty-four hours post the culture, the DMEM/F12 medium was replaced with the serum-free DMEM/F12 medium. Forty-eight hours post the culture, the 10 μmol/L cytarabine was added into the medium, and 1/2 medium was transferred with the interval of 2-3 days.

Oxidative Stress Damage $APOE^{-/-}$ Mouse Cerebral Cortical Neuron Model Establishment

The cerebral neurons were cultured for 7 days and incubated with 150 μM H_2O_2 for 40 min. Then, the H_2O_2 was removed and continue to culture for 24 h and observe the status of the cerebral cortical neurons.

Experiment 1: The cerebral neurons were cultured for 7 days and identified by using the neuron-specific enolase (NSE) staining and Nissl staining according to the previous study[14]. Then, the neurons were applied to establish oxidative stress damage neuron model as listed in Figure 1A. The cerebral cortical neurons were divided into 4 groups, including Sham group, Vehicle group (oxidative stress damage group), Vehicle + APOE3 group (oxidative stress damage neurons treated with 5 μg/ml APOE3), Vehicle + APOE4 group (oxidative stress damage neurons treated with 5 μg/ml APOE4). The lactate dehydrogenase (LDH) and superoxide dismutase (SOD) were examined. And the flow cytometry examination was also conducted.

Experiment 2: The neurons were applied to establish oxidative stress damage neuron model as listed in Figure 1B. The cerebral cortical neurons were divided into 5 groups, including Sham group, Vehicle group, Vehicle + APOE3 group, Vehicle + APOE4 group and Vehicle + APOE4 + MK801 group. The neurons in every group were treated with flou-3 at 6 h, 12 h, 24 h, and 48 h, respectively, and the laser confocal microscope was used to detect the Ca^{2+} fluorescence intensity.

Experiment 3: The neurons were applied to establish oxidative stress damage neuron model as listed in Figure 1C. The cerebral cortical

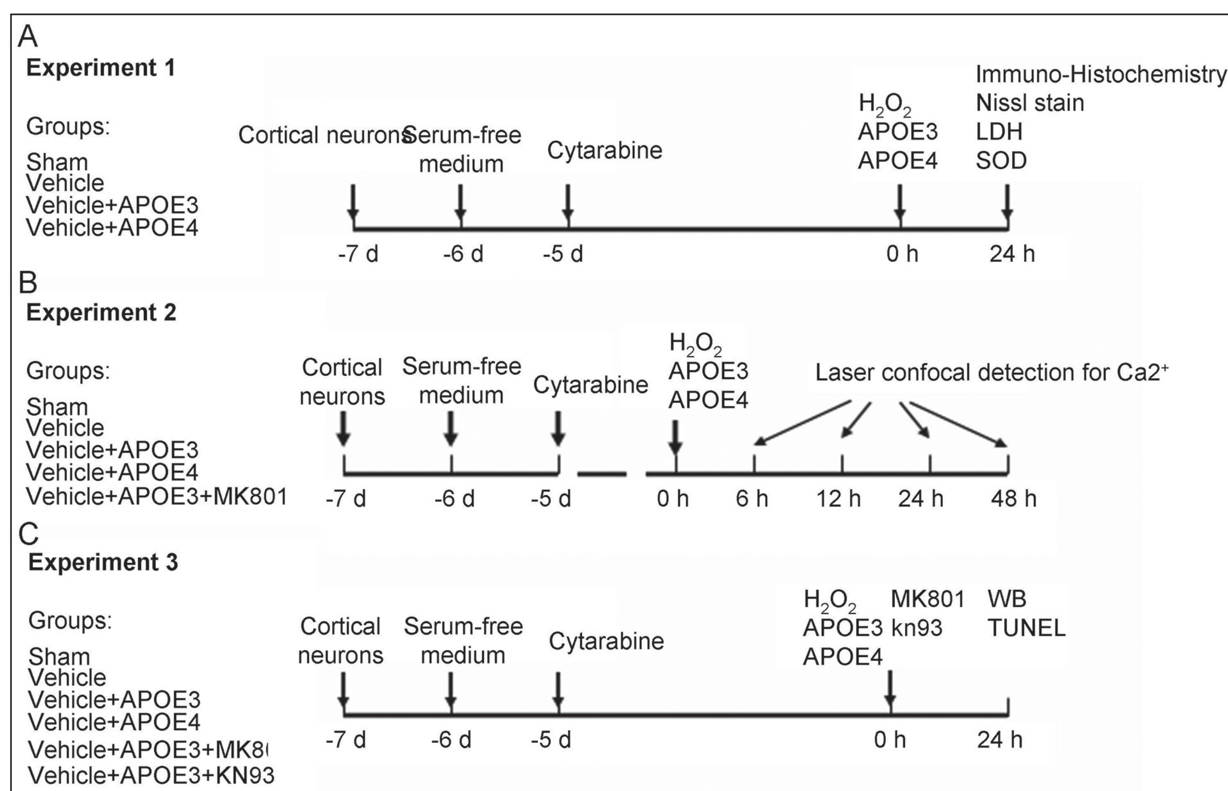

Figure 1. Experimental designs and animal groups classification. WB: Western blot.

neurons were divided into 5 groups, including Sham group, Vehicle group, Vehicle + APOE3 group, Vehicle + APOE4 group, Vehicle + APOE4 + MK801 group and Vehicle + APOE4 + KN93 group. The CaMK II phosphorylation and cleaved caspase 3 expression were analyzed by using the Western blot assay. Meanwhile, the neuron loss and apoptosis were also examined by using transferase-mediated deoxyuridine-triphosphate-biotin nick end labeling (TUNEL) staining and computer image analysis.

Neurotoxicity Assessment

The neurotoxicity was evaluated by measuring the LDH and SOD in the culture medium. As a measurement of neurotoxicity, the levels of LDH and SOD in the culture medium of cell were determined with an LDH assay kit (Jian Cheng Biotechnology, Nanjing, China) and SOD assay kit (Jian Cheng Biotechnology, Nanjing, China) according to the manufacturer's instruction.

Flow Cytometry Assay

The neurons were harvested and stained by using the flow cytometry assay and Annexin V-FITC/PI double staining detection kit (Sigma-Aldrich, St. Louis, MO, USA) according to the instruction of manufacturer. All of the flow cytometry assays were performed at least repeat for three times. The cell early apoptosis and late apoptosis were calculated by using the CELL QUEST software (BD Biosciences, Franklin Lakes, NJ, USA).

TUNEL Analysis

Apoptosis in the cerebral cortical neurons was determined at the time points by TUNEL analysis. Specifically, an *in situ* cell death detection kit (Roche, Basel, Switzerland) was used according to the manufacturer's instructions. Four fields were randomly selected, and 100 cells were counted. The number of apoptotic cells was noted.

Immunohistochemistry Assay

Immunohistochemistry assay for the neurons was performed as described previously[15]. The cells were fixed by using 4% paraformaldehyde and then placed in 3% H_2O_2 for 5 min to inhibit endogenous peroxidase activity. Antigen retrieval was performed by boiling for 20 min in 0.01 M

sodium citrate buffer (pH 6.0), and nonspecific antibody binding was blocked by incubating the sections in phosphate-buffered saline containing 5% goat serum. The sections were incubated overnight at 4°C with rabbit anti-rat NF-200 antibody (1:200; WuHan Boster Biological Technology, Wuhan, China), washed, incubated for 30 min at 37°C with biotin-labeled goat-anti-rabbit IgG (WuHan Boster), and then incubated for 10 min at 37°C with streptavidin-biotin complex. The color was developed by the addition of 3,3′-diaminobenzidine (DAB).

Nissl Staining

We prepared 0.1% cresyl violet solution by mixing 0.1 g cresyl fast violet mixed in 100 ml deionized H_2O. We added 10 drops of glacial acetic acid just before use and filter. We defatted the tissues by soaking in a 1:1 alcohol/chloroform mixture overnight. We rehydrated the slices in 100% alcohol followed by a 95% alcohol/5% deionized H_2O mixture. We stained in cresyl violet for 3-5 min. We rinsed in distilled water. We soaked in 95% ethyl alcohol for 5-30 min. We check microscopically for staining. We dehydrated in 100% alcohol for five minutes. We replaced alcohol and repeated it. We cleared in xylene for five minutes. We replaced xylene and repeated it. We mounted with resin medium.

Western Blot Analysis

Western blot assay was performed as described previously[16]. The neurons were centrifuged for 3 min at 300 × g to remove the supernatant and collect cells. The cells were stored appropriately at -80°C immediately until analysis. Proteins extraction from whole-cell lysates were obtained by gently homogenizing them in radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, China) with further centrifugation at 14000 × g at 4°C for 30 min. The supernatant was used as whole-cell protein extract, and the protein concentration was determined using a detergent compatible assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein (50 mg) were loaded on a sodium lauryl sulfonate polyacrylamide gel electrophoresis (SDS-PAGE) gel. After proteins were electrophorezed and transferred to a nitrocellulose membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK), the membrane was blocked and incubated with the goat anti-mouse NSE polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:500), goat anti-mouse caspase-3 polyclonal antibody (Santa Cruz Biotechnology, 1:500) at 4°C overnight. Then, the nitrocellulose membranes were incubated with secondary antibodies (Santa Cruz Biotechnology) for 1 h at room temperature. The immunoblots were then probed with an ECL Plus chemiluminescence reagent kit (Beyotime Biotechnology Shanghai, China), and visualized with the imaging system (Model: 4000, Bio-Rad, Hercules, CA, USA). The data was analyzed by the software ImageJ (Frederick, MD, USA).

Ca^{2+} Fluorescence Intensity Evaluation

Before evaluation, the Fluo-3 AM (at final concentration of 3 μM) was added into the neurons for 30 min at 37°C and washed with PBS for 20 min. After the treatment of Fluo-3 AM, the Ca^{2+} fluorescence intensity was evaluated under the excitation wavelength of 488 nm and emission wavelength by using the laser confocal microscope (BD Biosciences, Franklin Lakes, NJ, USA). The images were analyzed and captured by using the software Image J (Frederick, MD, USA).

Statistical Analysis

SPSS software 19.0 (IBM SPSS Statistics for Windows, Armonk, NY USA) was used to analyze the data in this study. The data were illustrated as mean ± standard deviation (SD) and were obtained from at least six independent experiments. The Student's *t*-test was used to analyze the differences between the groups. The $p < 0.05$ was considered as the statistical significance.

Results

Identification for Cerebral Cortical Neurons of $APOE^{-/-}$ mice

The cerebral cortical neurons illustrated the morphology of triangle or elliptocytosis, and characterized with outstretched neuron synapses (Figure 2A, B), 3-4 days post the neuron culture. Five to six days post the neuron culture, the neuron volume was further enlarged; the neuron synapses were further stretched. Meanwhile, the neuron synapses and branches inter-connected to form a sparse network in the culture medium (Figure 2A, B).

Moreover, the NSE staining results showed that the cytoplasm and synapses were stained as tan-colored, and nucleus was not stained with

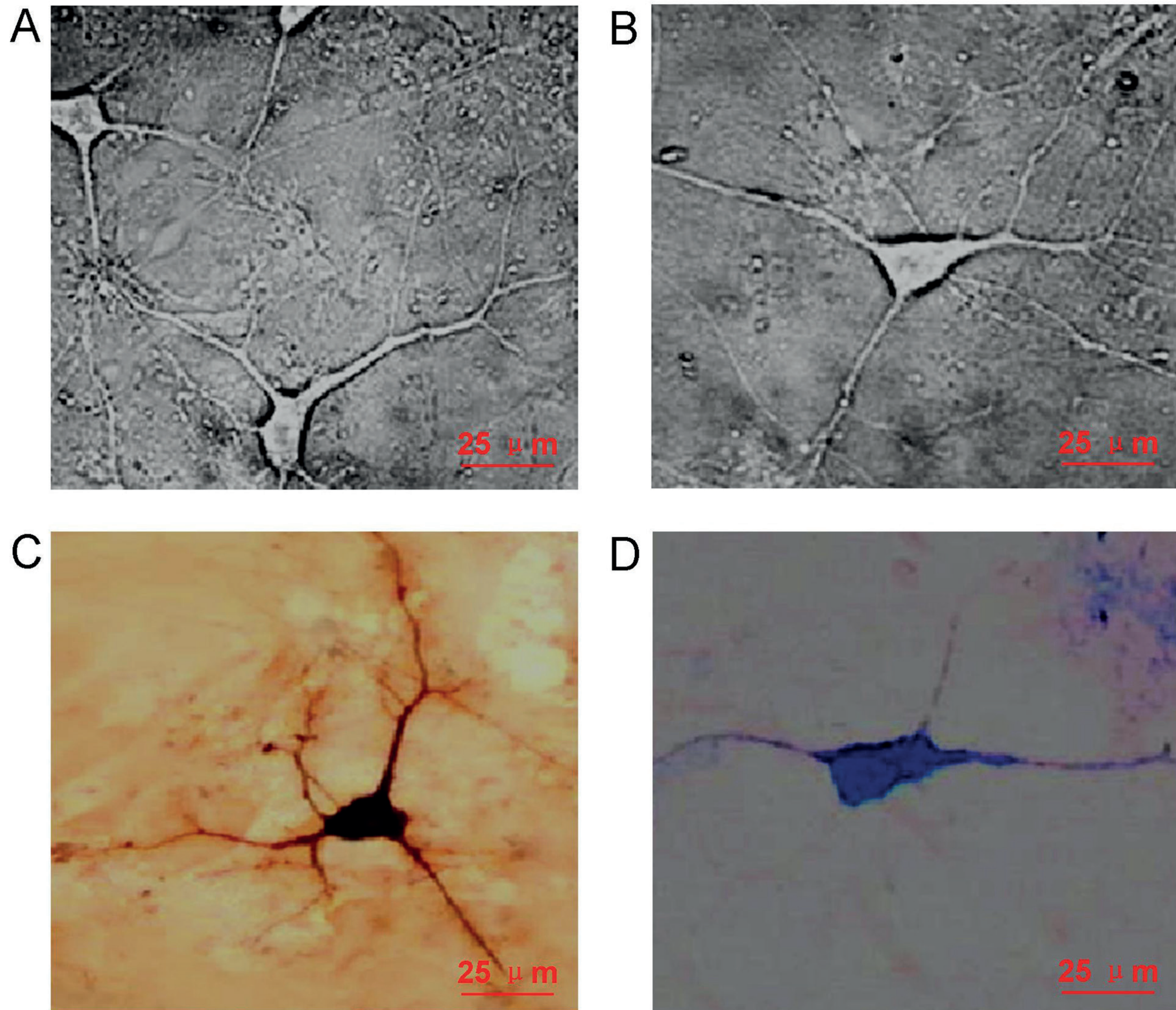

Figure 2. Identification for the cerebral cortical neurons. ***A*** and ***B,*** Images for the neuron morphology observed by using inverted phase contrast microscope. ***C,*** Immunohistochemistry assay for neuron morphology. ***D,*** Nissl staining for the Nissl bodies in neurons.

any colors (Figure 2C). The Nissl staining images indicated that the Nissl bodies mainly distributed in the cytoplasm of neurons, and were stained as blue. Due to the limited counts of Nissl bodies in the nucleus, the staining of nucleus was relatively lighter (Figure 2D).

APOE4 Aggravates Cerebral Cortical Neuron Damage of Oxidative Stress Mouse Model

In this research, the LDH levels were also examined to investigate the effects of oxidative stress on LDH levels. The results showed that the LDH levels in Vehicle group were higher significantly compared to the Sham group (Figure 3A, $p < 0.05$). The LDH levels in Vehicle + APOE3 group was lower significantly compared to Vehicle group, LDH levels in Vehicle + APOE4 group was higher significantly compared Vehicle group and Vehicle + APOE3 group (Figure 3A, $p < 0.05$).

Meanwhile, the SOD activity was also examined in the cerebral cortical neurons. The results indicated that the SOD activities of Vehicle group, Vehicle + APOE3 and Vehicle + APOE4 group were significantly decreased compared to the Sham group (Figure 3B, $p < 0.05$). The APOE3 treatment (Vehicle + APOE3 group) significantly increased SOD activity compared to Vehicle group (Figure 3B,

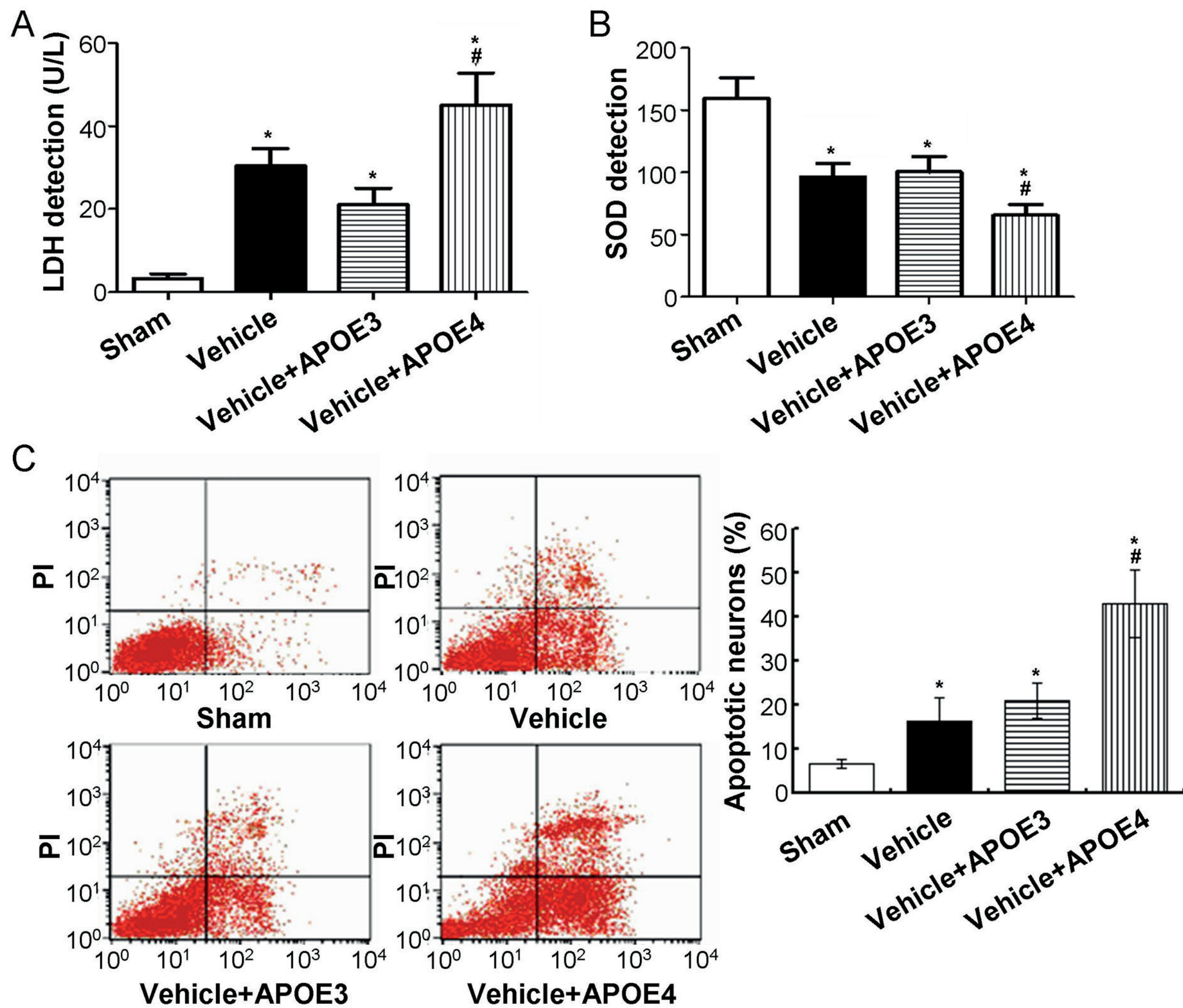

Figure 3. Neurotoxicity and apoptosis assessment for the neurons undergoing APOE4 treatment. ***A,*** LDH levels examination. ***B,*** SOD activity evaluation. ***C,*** Apoptosis rate analysis by using flow cytometry assay. $*p < 0.05$ *vs.* Sham group, $^{\#}p < 0.05$ *vs.* Vehicle group.

$p < 0.05$). However, the APOE4 treatment (Vehicle + APOE4 group) significantly decreased the SOD activity compared to Vehicle group (Figure 3B, $p < 0.05$).

APOE4 Induces Apoptosis of Cerebral Cortical Neuron of Oxidative Stress Mouse Model

The cytometry analysis results showed that the apoptotic neuron rates in Vehicle, Vehicle + APOE3 and Vehicle + APOE4 group were significantly increased compared to Sham group (Figure 3C, $p < 0.05$). However, the Vehicle treatment (Vehicle + APOE4 group) significantly increased the apoptotic neuron rate compared to the Vehicle group (Figure 3C, $p < 0.05$).

MK801 Alleviates APOE4 Induced Ca2+ Levels Increasing in Damaged Cerebral Cortical Neuron of Oxidative Stress Mouse Model

The Ca^{2+} levels in both Vehicle group and Vehicle+APOE3 group were higher compared to Sham group, but there were no significant differences (Table I, Figure 4A, $p > 0.05$). The Vehicle + APOE4 group significantly enhanced the Ca^{2+} levels compared to Sham group (Figure 4A, $p < 0.05$) at different time points. Meanwhile, the MK801 treatment (Vehicle + APOE4 + MK801 group) significantly decreased the Ca^{2+} levels compared to the Vehicle + APOE4 group at early time points of 12 h and 24 h (Figure 4A, B, $p < 0.05$). However, the effects of MK801 were not played at the late time points of 24 h and 48 h.

Table I. The Fluorescent intensity of intracellular calcium in neurons.

	Sham	Vehicle	Vehicle + APOE3	Vehicle + APOE4	Vehicle + APOE4 + MK
6 h	5.46 ± 1.65♦	19.36 ± 4.45▲	19.22 ± 4.08★	137.52 ± 12.52▼	45.08 ± 7.63
12 h	6.06 ± 0.87♦	20.06 ± 5.31▲	17.78 ± 3.14★	137.34 ± 14.67▼	49.72 ± 9.01
24 h	5.7 ± 1.15♦	19.82 ± 4.28▲	20.52 ± 5.55★	144.26 ± 8.43▼	88.08 ± 8.32
48 h	6.34 ± 0.85♦	24.06 ± 4.29▲	23.96 ± 3.9★	158.62 ± 14.49▼	87.34 ± 10.65

♦$p < 0.05$, Sham *vs*. Vehicle, Vehicle + APOE3, Vehicle + APOE4, Vehicle + APOE4 + MK. ▲$p < 0.05$, Vehicle *vs*. Vehicle + APOE4, Vehicle + APOE4 + MK. ★$p < 0.05$, Vehicle + APOE3 *vs*. Vehicle + APOE4, Vehicle + APOE4 + MK. ▼$p < 0.05$, Vehicle + APOE4 *vs*. Vehicle + APOE4 + MK.

APOE4 Triggers CaMK II Phosphorylation, Caspase 3 Activation and Neurons Apoptosis

The Western blot assay showed that the APOE4 treatment (Vehicle + APOE4 group) significantly increased the phosphorylated levels of CaMK II (Figure 5A, B) and increased the caspase 3 activity (Figure 5A, C) compared to the Sham group (all $p < 0.05$). Meanwhile, there were no effects of APOE3 treatment on the phosphorylation of CaMK II and caspase 3 activity of Vehicle neurons (Figure 5A, B, C, $p > 0.05$).

Furthermore, the TUNEL assay results indicated that the apoptosis neurons in Vehicle group were

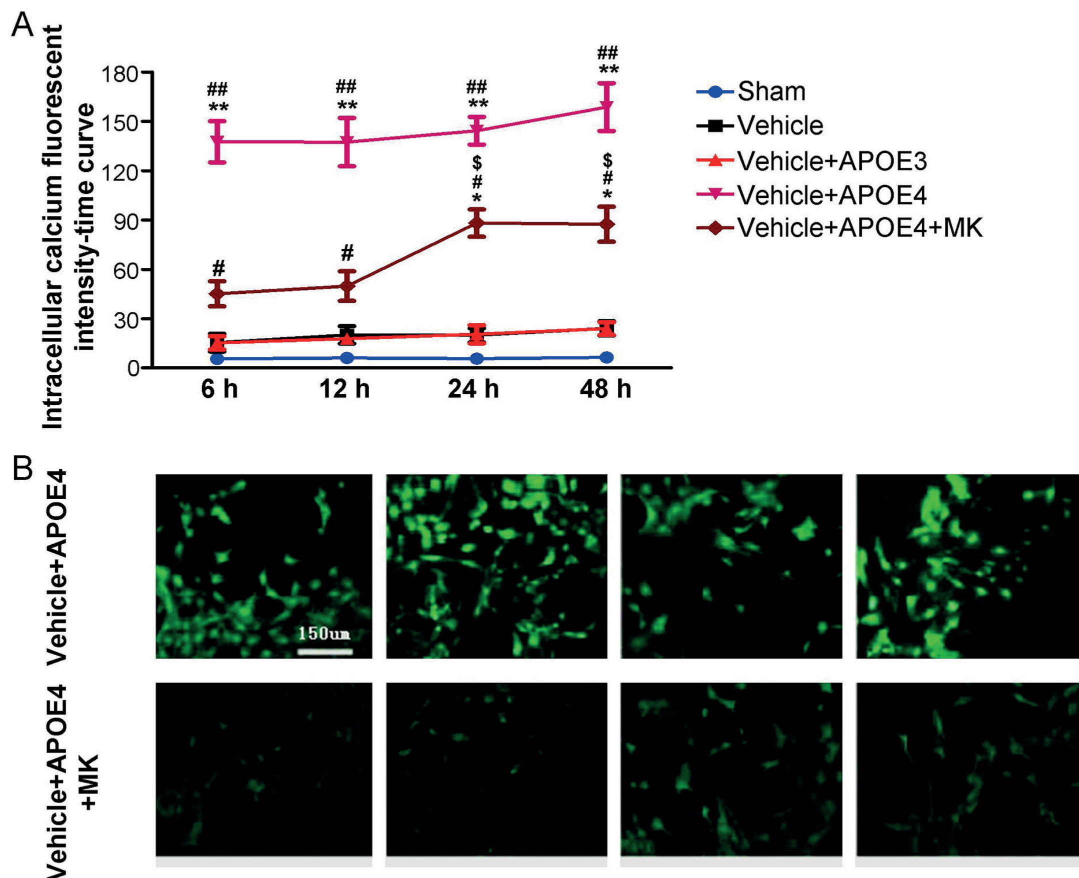

Figure 4. Intracellular calcium fluorescent intensity-time change curve. ***A,*** Statistical analysis for the intracellular calcium fluorescent intensity in different groups. ***B,*** The GFP fluorescence stained images of neurons in Vehicle + APOE4 group and Vehicle + APOE4 + MK group at different time points. *$p < 0.05$ and **$p < 0.01$ *vs*. Sham group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *vs*. Vehicle group, $^{\$}p < 0.05$ *vs*. Vehicle + APOE4 group.

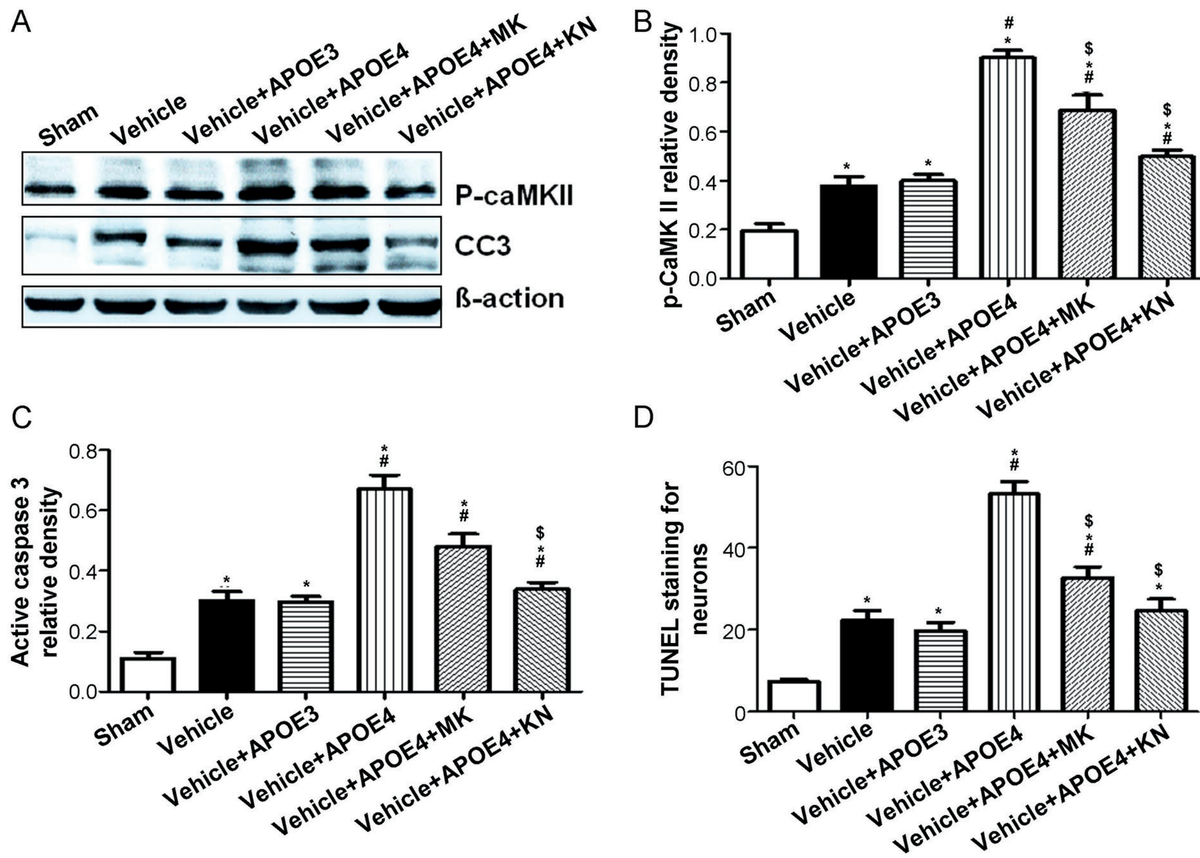

Figure 5. CaMK II phosphorylation and apoptosis examination in the cerebral cortical neurons. A, Western blot assay for the phosphorylated CaMK II (p-CaMK II), cleaved caspase 3. B, Statistical analysis for the p-CaMK II expression. ***C,*** Statistical analysis for the cleaved caspase 3 expression. D, TUNEL assay for examining the neuron apoptosis. *$p < 0.05$ *vs*. Sham group, $^{\#}p < 0.05$ *vs*. Vehicle group, $^{\$}p < 0.05$ *vs*. Vehicle + APOE4 group.

significantly increased compared to the Sham group (Figure 5A, D, $p < 0.05$). However, the APOE3 treatment inhibited the increased apoptosis neurons in Vehicle group, but the difference is not significant (Figure 5A, D, $p > 0.05$). APOE4 treatment significantly enhanced the apoptosis of cerebral cortical neurons in oxidative stress damage mouse model, compared to the Sham group and Vehicle group, respectively (Figure 5A, D, both $p < 0.05$).

MK801 inhibits CaMK II Phosphorylation, Decreases Caspase 3 Activation and Suppresses Neurons Apoptosis

In this experiment, the NDMA inhibitor, MK801, and CaMK II blocker, KN93 were used to treat the neurons. The results indicated that MK801 treatment significantly decreased the CaMK II phosphorylation (Figure 5A, B), inhibited the caspase 3 activity (Figure 5A, C) and suppressed the neurons apoptosis (Figure 5A, D) compared to the Vehicle + APOE4 group (all $p < 0.05$). Meanwhile, the KN93 could also decrease the CaMK II phosphorylation (Figure 5A, B), inhibited the caspase 3 activity (Figure 5A, C) and neurons apoptosis (Figure 5A, D) compared to the Vehicle + APOE4 group (all $p < 0.05$).

However, the inhibitive effects of KN93 treatment (Vehicle + APOE4 + KN93 group) on the CaMK II phosphorylation (Figure 5A, B), caspase 3 (Figure 5A, C) activity and neuron apoptosis (Figure 5A, D) are higher significantly compared the MK801 treatment (Vehicle + APOE4 + MK801 group) ($p < 0.05$).

Discussion

The long-time clinical practice illustrated that it's different for the resistance to the same or sim-

ilar damage in different race or nation, and even in different individuals of same race population[17]. Radojicic et al[18] reported that the different genotypes and strains of mice (AKR, BALB/c, CBA and C57BI/6) affect the resistance to the mechanical, thermal and radiation trauma. Kimura et al[19] also found that the resistance to the damage of arsenic in BALB/c and C57BL/6 is significantly different. The previous studies[7,20,21] also reported that the vulnerability of brains in APOE gene knockout mice is significantly increased compared to the wild-type mice.

APOE3 enhances the secreted amyloid precursor protein (sAPP) levels, and plays the role of neuro-protection, however, the APOE4 increases the neurotoxic beta amyloid protein levels[22,23]. In Chinese Han population, APOE4 is an important risk factor which could aggravate the trauma cased brain damage[24]. Other researches[25,26] also reported that APOE4 gene is correlated with the poor prognosis of the trauma caused brain damage, and also acts as a risk-factor for electroencephalograph (EEG) aggravation of the acute stage mild or moderate brain injury. Our work also showed that APOE3 treatment significantly decreased the LDH levels and increased SOD activity in oxidative stress damaged cerebral cortical neurons compared to Vehicle group (oxidative stress damaged neurons). The APOE4 treatment significantly increased the LDH levels and decreased the SOD activity in oxidative stress damaged cerebral cortical neurons compared to Vehicle group. Meanwhile, the APOE4 treatment significantly enhanced the apoptosis rate of neurons compared to Vehicle group. All of these findings were consistent with the previous studies[27,28], which also exhibit the association between APOE and the oxidative stress biomarkers.

Jing et al[29] transfected the APOE4 alleles into the neurons of APOE knockout mice, and found that the apoptotic neurons in APOE4 transfection group was significantly more compared to the apoptotic neurons in APOE2 and APOE3 transfection group. In our work, the APOE 4 activated the caspase 3 and aggravated the apoptosis of the cerebral cortical neurons, however, the APOE3 has not illustrated this characteristic, which results are also consistent with the previous study[30]. Therefore, we investigated the mechanisms for the APOE4 aggravated apoptosis of the oxidative stress damaged cerebral cortical neurons.

APOE receptor interacts with the N-methyl-D-aspartate receptor (NMDAR) by forming a multiple-protein complex[11]. Actually, the APOE gene in damaged brain affects the NMDAR with the characteristic of sub-type specificity, and causes the permeability difference of Ca^{2+}. The concentrations of Ca^{2+} in the neurons surrounding damaged regions are different, which cause the different degree of apoptosis and affect the disease progression and prognosis. Therefore, we evaluated the Ca^{2+} levels in the damaged cerebral cortical neurons by examining the Ca^{2+} fluorescence intensity. Our results indicated that APOE4 treatment triggered the increasing of the Ca^{2+} levels, which may be associated with the NMDAR and the other non-NMDA Ca^{2+} channels. Therefore, we employed the NMDAR specific blocker (or antagonist), MK801[31], to observe the effects of APOE4 on the neuron damage. The results showed that the MK801 treatment significantly decreased the Ca^{2+} levels compared to Vehicle + APOE4 group at the early time (12 h and 24 h), however, the Ca^{2+} levels were re-enhanced at late time points of 24 h and 48 h. These results suggest that there are may be some other Ca^{2+} pathways involving in the APOE4 induced cerebral cortical neuron damage.

The previous reports[32,33] investigating the down-stream signaling pathways of APOE4 aggravated neuron damage also proved that there are the other Ca^{2+} pathways. The present data indicated that APOE4 increases Ca^{2+} concentration in the damaged neurons by regulating the NMDAR signaling pathway, increases the CaMK II phosphorylation, activates the caspase 3, and finally induces the neuron apoptosis. The NMDAR blocker, MK801, could alleviate the CaMK II phosphorylation, decreases caspase 3 activation and inhibits neuron apoptosis. These findings were consistent with the previously published study[34]. We also concluded the above signal transduction pathways in Figure 6.

Moreover, we also employed the CaMK II specific inhibitor, KN93, to further investigate the role of APOE4 in damaged cerebral cortical neurons[35]. The results showed that the effects of the KN93 on the CaMK II phosphorylation, caspase 3 activation and apoptosis are significantly strengthened compared to the effects of MK801, which are consistent with the previous study[36].

Conclusions

All of the above findings hint that multiple signaling pathways involve in the effects of APOE

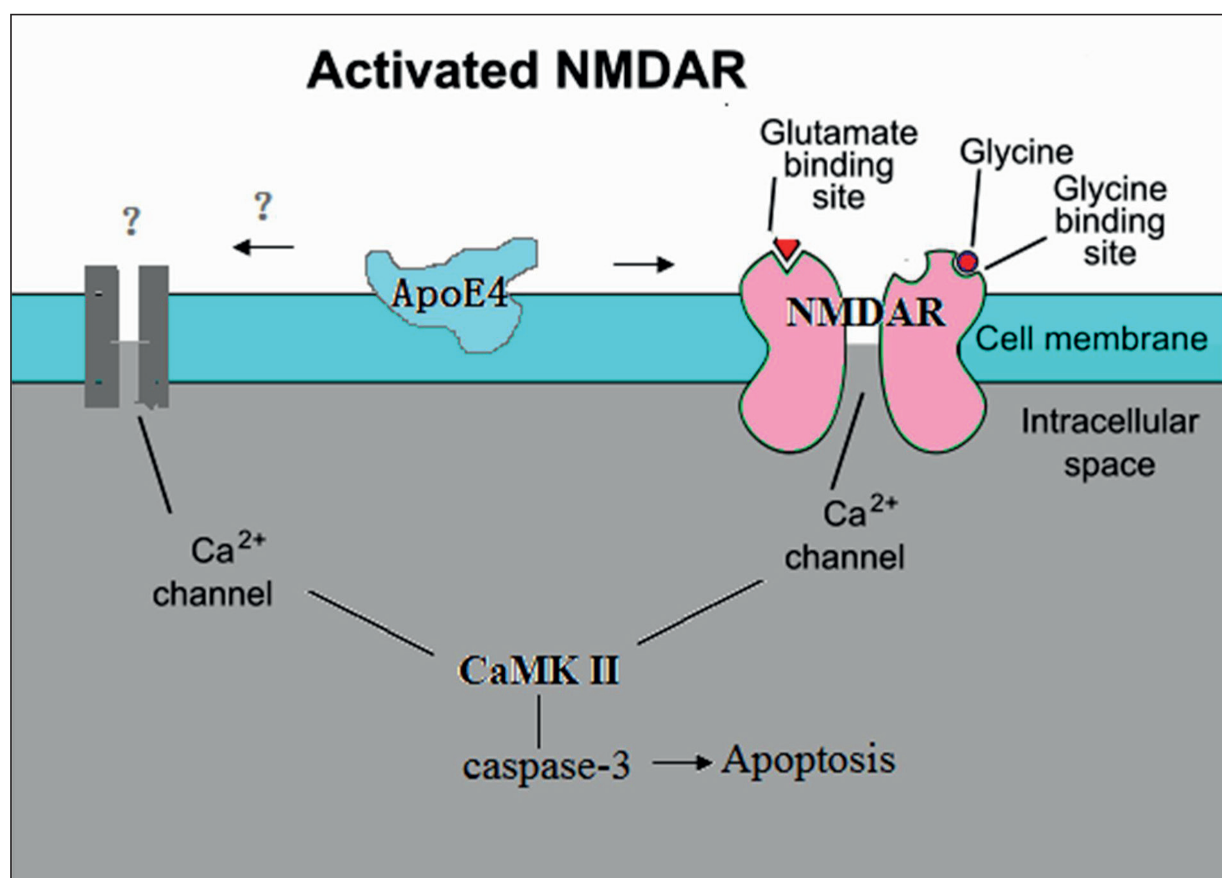

Figure 6. The sketch map for the specific APOE4 sub-type affected Ca2+ signaling pathway.

on the Ca^{2+} overload. APOE triggers the Ca^{2+} overload through NMDAR signaling and CaMK II signaling pathway, both of which cause Ca^{2+} concentration increasing, abnormity of CaMK II phosphorylation, and finally aggravate the apoptosis of the oxidative stress damaged neurons.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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