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Tilianin mediates neuroprotection against ischemic injury by attenuating CaMKII-dependent mitochondrion-mediated apoptosis and MAPK/NF-κB signaling

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ABSTRACT

Aims: Tilianin, a naturally occurring flavonoid glycoside, possesses versatile biological activities including antioxidant, anti-inflammatory, energy collecting and anti-hypoxic effects. Little is known about the mechanisms underlying the effect of tilianin against ischemic injury in neuronal cells. We aimed to determine the potential targets and mechanisms of tilianin treatment behind the crosstalk pathways induced by oxygen-glucose deprivation (OGD).

Main methods: We used an *in silico* docking model for interaction mode analysis and *in vitro* models for mechanistic exploration and target verification. Protein changes were measured using cellular immunofluorescence and ELISA techniques.

Key findings: The ability of tilianin to promote recovery of OGD-induced neurocytotoxic injury was demonstrated by maintenance of cell viability, membrane integrity and nuclear homogeneity. Tilianin treatment was also found to balance the concentrations of proapoptotic and antiapoptotic proteins that had been modified by OGD-induced mitochondrial dysfunction. Of these intersectional cascades, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) was found to bind efficiently with tilianin. This presented a certain binding score along with down-regulation of ox-CaMKII and p-CaMKII in SH-SY5Y cells affected by OGD. Importantly, after utilizing KN93, one specific CaMKII inhibitor, tilianin-mediated neuroprotection against OGD was abolished. This effect was accompanied by upregulation of mitochondrial function. Thus, the beneficial effects of tilianin toward mitochondrion-mediated apoptosis and p38/JNK/NF-κB-associated inflammatory pathways were reversed following CaMKII inhibition.

Significance: Our study indicated that attenuation of CaMKII-linked signaling mediated through mitochondria and p38/JNK/NF-κB inflammatory pathways is a key mechanism by which tilianin exerts its neuroprotective effects against cerebral ischemia.

1. Introduction

Cerebral ischemia ('ischemic stroke') is responsible for approximately 87% of all strokes, and can lead to irreversible brain damage and cardiorespiratory arrest. There is currently no licensed treatment for this disease [1]. Accumulated evidence indicates that oxidative stress initiates an integral step in the process of cerebral ischemia. This contributes to mitochondrial dysfunction, which leads to release of inflammatory cytokines and subsequent apoptosis of neurons [2,3]. Many studies show that ischemic injury destroys mitochondrial integrity and structure resulting in a series of events that include overproduction of mitochondrial reactive oxygen species (mtROS), collapse of mitochondrial membrane potential (MMP), prolonged opening of the mitochondrial permeability transition pore (mPTP) and activation of apoptotic signaling effectors such as B cell lymphoma-2 (Bcl-2), Bcl-2 associated X protein (Bax) and caspase family proteins [4,5]. Cerebral

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ischemia is also accompanied by significant inflammatory reactions including activation of mitogen-activated protein kinases (MAPKs), which induces nuclear factor kappa-B (NF-κB) [6]. Thus, regulation of these signaling pathways could help prevent and treat cerebral ischemia to help recovery of brain function.

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), a heteromeric serine/threonine-specific protein kinase, is regulated by Ca^{2+}/calmodulin complex. CaMKII is involved in many signaling cascades and is thought to be an important mediator of cerebral ischemia [7]. There are four isoforms of CaMKII (α, β, γ and δ) encoded by different genes with differential distribution and function within various tissues and cell types [8]. CaMKIIα plays a critical role in the brain especially in postsynaptic density, and studies involving CaMKIIα knockout mice demonstrated a low frequency of long-term potentiation (LTP) [9]. Many studies have illustrated that oxidative and phosphorylated forms of CaMKII are activated by both Ca^{2+} and oxidative stress at different activation sites, and to a greater extent in the initial stage of ischemia. Oxidative and phosphorylated forms of CaMKII exacerbate mitochondrial dysfunction and upregulate many key downstream proteins involved in apoptosis and inflammation signaling, which are responsible for cerebral ischemia [8]. CaMKII mediates mitochondrial damage and apoptotic cell death as well as NF-κB-mediated inflammation in rat models of cerebral ischemia [10,11]. Furthermore, CaMKII has been implicated recently as a promising drug target of neuroprotection after cerebral ischemia [12]. Therefore, our study focused on CaMKII as a crucial mediator of the molecular cascades involved in cerebral ischemia to reduce mitochondrial dysfunction and inflammatory signaling.

Tilianin (Fig. 1), a naturally occurring flavonoid, has already been proven to impact positively on cardiovascular disease, especially myocardial ischemia-reperfusion injury (MIRI) [13,14]. Published studies on tilianin suggest that it protects against MIRI through resisting oxidation damage and inflammatory reactions [15]. Flavonoids also exert well-accepted neuroprotective pharmacological effects against age-related cognitive decline and neurodegenerative diseases [16]. No study has yet been published evaluating whether tilianin has beneficial effects in cerebral ischemia or neuroprotection. Hence, we investigated the effects of tilianin on oxygen-glucose deprivation (OGD)-injured SH-SY5Y cells.

Thus, the overall aims of our work were to investigate and determine if CaMKII participates in neuroprotection, then establish the role of CaMKII in mitochondrial-mediated reactions as well as MAPK signaling modulation in neuronal cells following the use of tilianin to prevent cerebral ischemia.

2. Materials and methods

2.1. Cell culture and treatments

SH-SY5Y, a human-derived thrice cloned cell line isolated from a bone marrow biospy from a four-year-old female with neuroblastoma, was purchased from American Type Culture Collection (ATCC). Cells were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen) at 37 °C in a humidified atmosphere containing 5% CO_2.

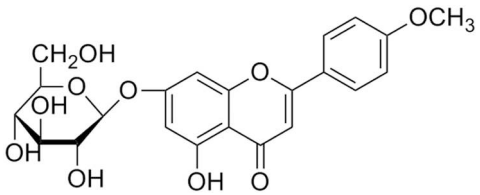

Fig. 1. Chemical structure of tilianin. The molecular formula of tilianin is $C_{22}H_{22}O_{10}$.

We used a modified OGD-injury model, which aimed to mimic the pathological changes of stroke in vitro [17]. Sodium hydrosulfite (Na_2SO_4) is an oxygen scavenger that can induce hypoxia by directly limiting the O_2 available for cellular activities [18]. We cultured SH-SY5Y cells in glucose-free DMEM with 7 mM NaS_2O_4 for 2 h to induce OGD conditions.

Tilianin was provided by the Xinjiang XibuJiasite Pharmaceutical Company (Urumqi, China), an organization affiliated to the Xinjiang Institute of Materia Medica. SH-SY5Y cells were pre-treated with different concentrations of tilianin for 6 h before and through the OGD injury. Cells were first divided randomly into three groups being the high-glucose DMEM treatment control, the OGD injury and the inhibitor-treated groups. Each group was further divided into five subgroups based on tilianin concentration (0 μM, 1 μM, 3 μM, 10 μM and 30 μM). Additionally, the inhibitor KN93 was pre-incubated as 5 μM for 1 h prior to treatment with tilianin.

2.2. Cell viability and lactate dehydrogenase release assay

Cell viability and lactate dehydrogenase (LDH) released from cell membranes were detected by [3-(4,5-dimethylthiazol-2-yl) -5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] MTS and the CytoTox-ONE homogeneous membrane integrity assays (Promega, Madison, WI, USA), respectively, following the manufacturers' instructions.

2.3. Hoechst 33342 and $DCFH_2$-DA staining assay

Nuclear staining changes and intracellular reactive oxygen species (ROS) levels of SH-SY5Y cells were evaluated by Hoechst 33342 (Dojindo Laboratory, Kumamoto, Japan) and 2′,7′-dihydrodichlorofluorescein diacetate ($DCFH_2$-DA, Sigma-Aldrich, St Louis, MO, USA) staining, respectively [19]. The degree of staining was measured by fluorescence at 386 nm excitation/460 nm emission and 485 nm excitation/535 nm emission, and then the values were quantified as an average fluorescent intensity of cells (Mean_TargetAvgInten).

2.4. Measurement of mitochondrial membrane potential and superoxide levels

Changes in intracellular mitochondrial function were evaluated using two molecular probes, Rhodamine 123 (Rh123) (Dojindo Laboratory, Kumamoto, Japan) and MitoSOX Red (Invitrogen, Carlsbad, CA, USA), as we described previously [20]. Rh123 and MitoSOX Red at final concentrations of 10 μM and 5 μM, respectively, were added to the treated SH-SY5Y cells and incubated at 37 °C for 30 min. Fluorescent images and intensity values were acquired and analyzed by a Cellomics ArrayScan ^{VTI} HCS Reader (Thermo Fisher Scientific Cellomics, Pittsburgh, PA, USA). Detection conditions for Rh123 and MitoSOX Red were 488 nm excitation/535 nm emission and 510 nm excitation/580 nm emission, respectively. Values of mean average fluorescent intensities were recorded as the experimental data.

2.5. Cellular immunofluorescence to assess activation of apoptosis and inflammatory signaling pathways

Cellular immunofluorescence assays were presented on a high-content imaging platform as we have described previously [21]. Briefly, after the treatments described above, SH-SY5Y cells were fixed in 4% paraformaldehyde solution, permeabilized with 0.25% Triton X-100, blocked in 3% bovine serum albumin (BSA) and incubated with the appropriate primary antibody (for specifics, see below) at 4 °C overnight. Cells were then washed with phosphate buffered saline (PBS) and incubated with secondary antibody (goat anti-rabbit conjugated with Alexa Fluor 488 or Alexa Fluor 546, 1:1000, Invitrogen) at room

temperature for 2 h. The cells were again washed with phosphate buffered saline (PBS), fluorescence level detected using a Cellomics ArrayScan^{VTI} HCS Reader (Thermo Fisher Scientific Cellomics, Waltham, MA, USA) and images of the cells saved. The primary antibodies used were: rabbit anti-ox-CaMKII (Met281/282) (1:50, GeneTex, Irvine, CA, USA), rabbit anti-p-CaMKII (Thr286) (1:200, Abcam, Cambridge, MA, USA), rabbit anti-p-JNK1/2 (T183/Y185) (1:100, Abcam), rabbit anti-p-ERK1/2(T202/Y204) (1:200, Cell Signaling Technology, Danvers, MA, USA), rabbit anti-p38 MAPK(T180/Y182) (1:200, CST), rabbit anti-p-c-Jun (Ser63) (1:100, Abcam), rabbit anti-p-MK2 (Thr334) (1:200, CST), rabbit anti-p-NF-κB p65 (Ser536) (1:100, Abcam), rabbit anti-Bcl-2 (1:200, Abcam), rabbit anti-Bax (1:100, Abcam) and rabbit anti-cytochrome *c* (1:100, Abcam). Translocation of p-JNK1/2, p-ERK1/2, p38 MAPK, p-p65 and p- MAPKAP kinase-2 (MK2) were represented by the Mean_CircRingAvgIntenDiff value (difference in average fluorescence intensity between nucleus and cytoplasm). The nuclear fluorescence intensity for p-c-Jun, and the cell fluorescence intensity for CaMKII, Bcl-2, Bax and cytochrome *c* was calculated.

2.6. Detection of caspase-3 and caspase-9 activity

The activity of caspase-3 and caspase-9 was measured in SH-SY5Y cell culture using the Caspase-Glo 3 and Caspase-Glo 9 assay kits (Promega) as per the manufacturer's protocols.

2.7. ELISA assay for TNF-α and IL-6

Concentrations of tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) in SH-SY5Y cell culture medium following different treatments were assessed using enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen), following the manufacturer's instructions. Data are represented as pg/mL.

2.8. Molecule docking analysis

The crystal structure of recombinant human CaMKIIα complexed with indirubin E804 at a resolution of 2.3 Å was downloaded from the PDB database (PDB code: 2VZ6) and used as the initial three-dimensional (3D) model. The 3D structure of tilianin was obtained from the PubChem database (Pubchem CID: 5321954) The valid, single 3D conformations were then generated through addition of hydrogen atoms (protonation using strong bases) after washing and energy minimization, using the molecular modeling software MOE 2010 (Chemical Computing Group. Inc., Canada). MOE 2010 was also used to generate binding models for CaMKIIα in complexes with tilianin. Crystallographic water molecules and inorganic molecules were preliminarily removed from 2VZ6, while the active site pocket was defined as the ligand of indirubin E804. The specific parameters were set as follows: placement method, first scoring function rescoring 1 and saved poses were set to Triangle Matcher, London dG and 20, respectively. Additionally, refinement, second refinement scoring function rescoring 2 and saved poses were set to force field, none and 10, respectively.

2.9. CaMKII activity assay

Recombinant CaMKIIα (Life Technologies, Carlsbad, CA, USA, 50 ng) was incorporated into autocamtide-2 peptide (KKALRRQETVDAL, 5 μg) as the substrate in an assay buffer (40 mM Tris/HCl, 0.1 mg/mL BSA, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mg/mL calmodulin and 0.5 mM dithiothreitol, pH 7.5). Kinase reactions were initiated by the addition of adenosine triphosphate (ATP) and incubated at 30 °C for 15 min. Reactions were terminated by the addition of ADP-Glo™ reagent (Promega, Madison, WI, USA) and incubated at room temperature for a further 40 min. The detection reagent containing luciferase was then added and incubated for 30 min at room temperature. Luminescence was measured on a Spark 20 M multimode microplate reader (Tecan Group Ltd., Mannedorf, Switzerland) that quantified relative adenosine diphosphate (ADP) levels and kinase activity. Tilianin was pre-incubated with CaMKIIα before the addition of calmodulin and peptide substrates.

2.10. Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Inc., La Jolla, CA, USA). Comparisons were made using Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. All assays were repeated at least six times. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Tilianin treatment protects SH-SY5Y cells against OGD-induced cytotoxicity

SH-SY5Y cells injured by $Na_2S_2O_4$, which triggers anoxic conditions, served as an OGD model in vitro. Three cytotoxicity assays were used to examine the protective effects of tilianin on OGD-treated SH-SY5Y cells. The MTS assay demonstrated that OGD-induced SH-SY5Y cell viability decreased significantly compared with the control group (Fig. 2A, $P < 0.05$). Further, the quantity of LDH leakage was much higher in the OGD group than in the control group (Fig. 2B, $P < 0.05$). OGD-treated SH-SY5Y cells had condensed nuclei with strong fluorescence intensity (Fig. 2C, F, $P < 0.05$). However, tilianin at 1 μM, 3 μM, 10 μM and 30 μM concentration dependently increased SH-SY5Y cell viability following OGD injury (Fig. 2A, all $P < 0.05$). Similarly, the same concentrations of tilianin reduced LDH release from OGD-injured cells dose-dependently (Fig. 2B, all $P < 0.05$). Tilianin also mitigated the cytotoxic effects, as detecting nuclear morphological condensation and fluorescence intensity (Fig. 2C, F, all $P < 0.05$). Hence, these results indicate that tilianin decreased the impact of OGD-induced injury on SH-SY5Y cells.

We also investigated the effective treatment conditions for tilianin using both control and OGD-injured cells. The increase in cell viability, decrease in LDH release and reduction in nuclear injury indicated that tilianin provided protective effects in the environment of ischemia-like injury. In addition, there were no significant differences in cell viability and LDH release between control cells and tilianin-treated cells. This showed that these concentrations of tilianin were nontoxic under basic conditions.

3.2. Tilianin ameliorates mitochondrial damage and attenuates mitochondrion-mediated apoptotic signaling transduction in OGD-injured SH-SY5Y cells

To evaluate mitochondrial function, we quantified the membrane potential of mitochondria, superoxide concentration and intracellular ROS. After OGD injury, the fluorescence intensity of Rh123, MitoSOX and $DCFH_2$-DA in SH-SY5Y cells increased significantly (Fig. 3A–D, all $P < 0.05$). This indicated mitochondrial dysfunction, as characterized by a reduction in MMP, excessive generation of mtROS and overproduction of intracellular ROS in response to OGD injury. Treatment with tilianin 1 μM, 3 μM, 10 μM and 30 μM resulted in concentration-dependent decreases in Rh123 and MitoSOX fluorescence intensity levels (all $P < 0.05$). Similarly, reduced levels of excessive intracellular ROS were observed ($P < 0.05$). These results suggested that maintenance of mitochondrial homeostasis and preservation of redox balance were mediated by tilianin following OGD injury.

Mitochondrial dysfunction is closely related to nerve cell apoptosis induced by ischemia, which leads to dysregulation of apoptosis-related proteins in a highly programmed process. Using immunocytochemistry and luminescence assays, we evaluated mitochondrion-mediated

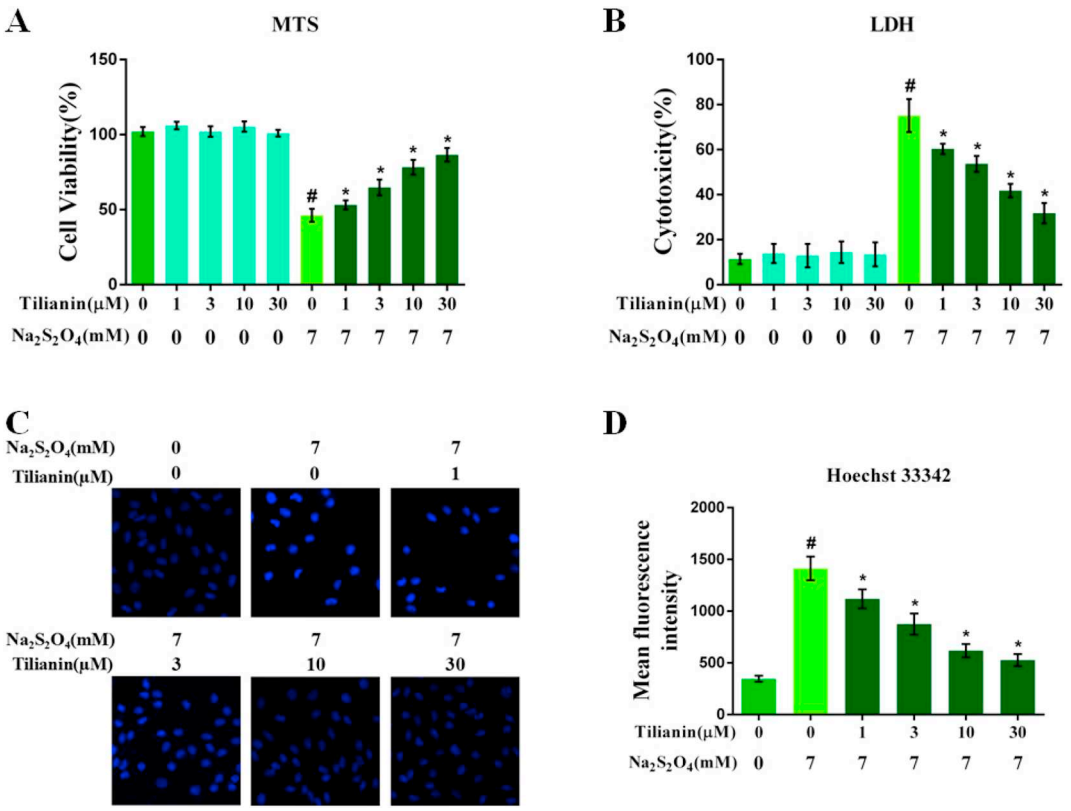

Fig. 2. Tilianin treatment protected SH-SY5Y cells against OGD-induced cytotoxicity. (A) Tilianin increased cell viability as evaluated by MTS assay. (B) Tilianin decreased LDH release after OGD injury. (C) Representative images of nuclei stained by Hoechst 33342 (×20). (D) Tilianin decreased mean fluorescence intensity of nuclei stained with Hoechst 33342 in SH-SY5Y cells after OGD injury. Data are presented as mean ± SD, $n = 6$. $^{\#}P < 0.05$ vs. control, $^{*}P < 0.05$ vs. OGD.

apoptotic transduction after OGD injury. The quantities of cytochrome *c* and Bax increased significantly in SH-SY5Y cells subjected to OGD. This was accompanied by a decrease in Bcl-2, and activation of caspase-3 and caspase-9 (Fig. 3A, F–I, $P < 0.05$). Tilianin treatment at concentrations of 1 μM, 3 μM, 10 μM and 30 μM reversed these deleterious changes in a concentration-dependent manner (all $P < 0.05$). Consistent with preservation of mitochondrial function, these results suggested that mitochondrion-targeted neuroprotection plays an important role in the therapeutic effects of tilianin against OGD injury.

3.3. Tilianin decreases p38 MAPK/MK2, JNK/c-Jun and NF-κB inflammatory signaling in OGD-injured SH-SY5Y cells

The pathways responsible for apoptosis are also involved in activation of the MAPK family of enzymes and in triggering NF-κB-mediated inflammatory reactions. OGD injury promoted phosphorylation of MAPKs. This was demonstrated by a significant increase in Mean_CircRingAvgIntenDiff values indicating translocation of phosphorylated p38 (p-p38), extracellular signal-regulated kinase 1 and 2 (p-ERK1/2), and c-Jun N-terminal protein kinase (p-JNK) from the cytoplasm to the nucleus (Fig. 4A–D, all $P < 0.05$). The changing levels of p-MK2 and p-c-Jun (p-p38 and p-JNK downstream substrates, respectively) in the nucleus was shown by an increased variation in translocation from nucleus to cytoplasm and an elevated mean fluorescent intensity in the nucleus (Fig. 4A, F–G, all $P < 0.05$). Tilianin 1 μM to 30 μM inhibited translocation of cytosolic p-p38 and p-JNK to the nucleus as well as down-regulation of p-MK2 and p-c-Jun in OGD-injured SH-SY5Y cells (all $P < 0.05$). However, there was no significant effect on nuclear translocation of p-ERK1/2.

The extent of NF-κB-mediated inflammatory response was quantified by assessment of cytosolic p65 translocation to the nucleus and release of pro-inflammatory cytokines from SH-SY5Y cells. The phosphorylated p65 subunit (p-p65) also translocated from the cytoplasm to the nucleus, and the levels of TNF-α and IL-6 in the culture supernatant increased (Fig. 4A, E, H–I, all $P < 0.05$). Tilianin blocked p-p65 translocation and attenuated TNF-α and IL-6 secretion (all $P < 0.05$). These results indicated that in SH-SY5Y cells subjected to OGD-triggered toxicity, tilianin provided an anti-inflammatory effect via inhibition of the p38/MK2, JNK/c-Jun pathway and NF-κB activation.

3.4. Prediction of the binding mode of CaMKIIα/tilianin

Persistent CaMKII activation contributes to the pathogenesis of cerebral ischemia, evoking mitochondrion-mediated programmed cell death that acts as a nodal signal for promoting cellular apoptosis [8]. Therefore, of the multiple molecular targets for tilianin within the mitochondrion-related signaling pathways, we selected the possible interaction between tilianin and CaMKII protein as a potential mechanism. Molecular docking revealed that the most part of tilianin (except the glucoside group) could fit the active binding site with a weak binding score of −11.56 kcal/mol. Specifically, none of hydrogen bond interactions were found between tilianin and CaMKIIα. The major of weak binding force was owing to van der Waals/hydrophobic interactions formed by residues Leu142 and Ala155 (Fig. 5A).

3.5. Tilianin inhibits CaMKII kinase activity in a limited capacity, and decreases expression of CaMKII against OGD-induced toxicity in SH-SY5Y cells

Results from the in vitro kinase assay demonstrated that higher concentrations of tilianin (125 μM to 625 μM) inhibited CaMKIIα kinase activity to a certain extent (Fig. 5B, all $P < 0.05$) and subsequent double-reciprocal analysis of these data did not yield straight lines

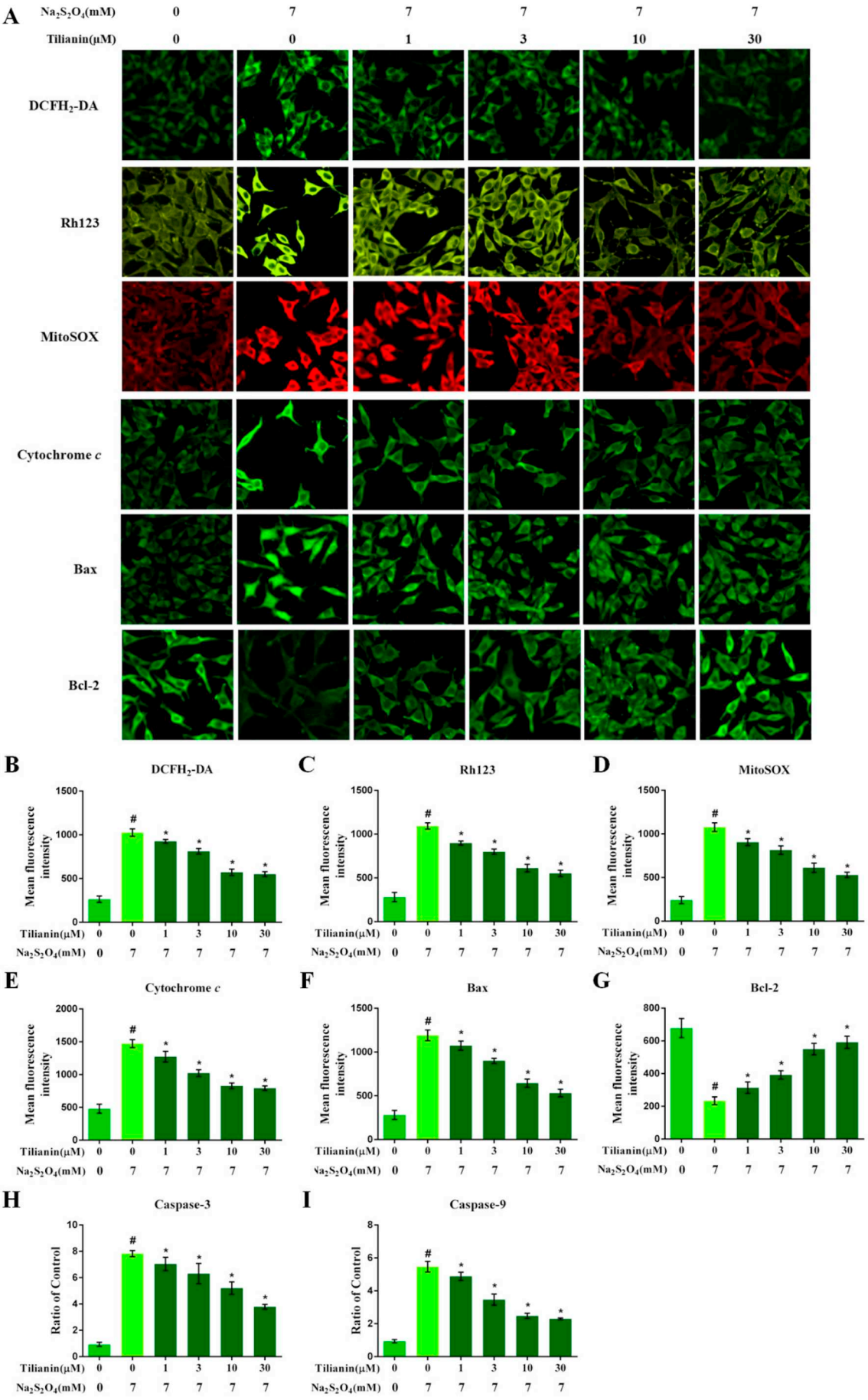

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Fig. 3. Tilianin ameliorated mitochondrial damage and attenuated mitochondrion-mediated apoptotic signaling transduction in OGD-injured SH-SY5Y cells. (A) Representative images of intracellular ROS stained by $DCFH_2$-DA (×20), superoxides in mitochondria stained by MitoSOX (×20), MMP stained by Rh123 (×20) and cytochrome *c*, Bax and Bcl-2 expression (×20). (B–G) Mean fluorescence intensity of images stained with $DCFH_2$-DA (B), Rh123 (C), MitoSOX (D), cytochrome *c* (E), Bax (F) and Bcl-2 (G). (H, I) The activities of caspase-3 and caspase-9. Data are expressed as mean ± SD, $n = 6$. $^{\#}P < 0.05$ vs. control, $^{*}P < 0.05$ vs. OGD.

intersecting on the *y*-axis (Fig. 5C). In accordance with the in silico molecular docking analysis, tilianin may not specifically interact with CaMKIIα with respect to the Ca^{2+}/CaM complex.

Except activating CaMKII by combining with Ca^{2+}/CaM, the enzyme can activate via oxidation and phosphorylation in downstream signaling pathways that contribute to deterioration of mitochondrion-linked apoptosis during acute ischemia. Therefore, expression of these modifications, including oxidized (ox-CaMKII) and phosphorylated (p-CaMKII) forms, was examined via visual immunofluorescence by high-content analysis in SH-SY5Y cells. As shown in Fig. 5D–F, exposure to OGD conditions elevated expression of ox-CaMKII and p-CaMKII in the cytoplasm of SH-SY5Y cells with stronger fluorescence intensity compared with control cells (both $P < 0.05$). Treatment with tilianin from 1 μM to 30 μM lowered the enhanced expression of ox-CaMKII and p-CaMKII in response to OGD injury in a concentration-dependent manner (all $P < 0.05$). Combined with the study of the interaction between tilianin and CaMKII, these results suggested that neuroprotection of tilianin against cerebral ischemic injury is largely due to the inhibitory effects on ox-CaMKII and p-CaMKII, and their signaling transduction.

3.6. CaMKII mediates the cytoprotective effects of tilianin against OGD injury involving mitochondrion dysfunction, apoptosis and inflammation

To determine whether the potentiated activation of CaMKII contributed to tilianin-mediated neuroprotection against OGD-induced cytotoxicity, we used a specific CaMKII inhibitor, KN93. As shown in Fig. 6A and B, pharmacological inhibition of CaMKII with KN93 blocked the ability of tilianin at all concentrations to rescue the decreased cell viability and increased membrane leakage after exposure to OGD (Fig. 6A–B, $P < 0.05$).

In addition to its role in nerve cell viability and membrane integrity, CaMKII was found to be involved in mitochondrion-associated apoptotic signaling based on prior measurements. However, after pretreatment with KN93, the effects of tilianin treatment on mitochondrial protection involving increased MMPs, decreased mtROS and restoration of apoptotic-related proteins such as cytochrome *c*, Bax, Bcl-2, caspase-3 and caspase-9 were diminished significantly (Figs. 6C–E and 7A–D, F, all $P < 0.05$). Additionally, Hoechst staining revealed similar results in the nuclei. This illustrated that KN93 caused tilianin-mediated alleviation to be more aggregated in nuclear brightness and condensation (Fig. 6C, F, all $P < 0.05$). These results indicated that tilianin may specifically ameliorate mitochondrion-targeted apoptotic pathways following CaMKII inhibition of rescuing deficits from OGD injury.

Considering that MAPK signaling plays a role in the coupling transduction with CaMKII, we also determined changes in CaMKII-associated inflammatory reactions after tilianin treatment. After KN93 inhibition, tilianin 1 μM to 30 μM could not reverse the effects of p-p38, p-JNK and p-MK2 on translocation blockage, down-regulation of p-c-Jun expression, inhibition of p-p65 activation or reduced secretion of pro-inflammatory cytokines such as TNF-α and IL-6 (Fig. 8A–G, all $P < 0.05$). Thus, these results suggested a specific role for p38/MK2, JNK/c-Jun and NF-κB in causing inflammation, and CaMKII signaling in reducing OGD-generated deficits by treatment with tilianin.

4. Discussion

We investigated the mechanisms for the neuroprotective effect of tilianin in the OGD-induced toxicity model. This was done by evaluating the role of CaMKII, and though determining its action on mitochondrion-mediated apoptosis and MAPK/NF-κB-triggered inflammation. This study has three major contributions to the science of tilianin action. First, to the best of our knowledge this is the first study to establish participation of CaMKII in tilianin-mediated therapeutic effect in OGD-injury nerve cells. Second, after OGD-induced cerebral injury, tilianin was found to interfere with cellular signaling including mitochondrion-mediated apoptosis and p38 MAPK/JNK/NF-κB-induced inflammation via CaMKII inhibition. Third, our evidence showed that two active isoforms of CaMKII, ox-CaMKII and p-CaMKII, could be prospective therapeutic targets for treatment of cerebral ischemia. These findings provide novel evidence and insights into the possible mechanisms of tilianin's neuroprotective effects against cerebral ischemia injury.

Brain ischemia results when blood flow to the brain is insufficient to meet metabolic demand. A critical reduction in blood flow causes inadequate glucose supply and/or cerebral hypoxia with resultant brain cell or tissue death cerebral infarction or ischemic stroke, or irreversible brain damage [22]. In the present study, we submitted SH-SY5Y cells to OGD-induced toxicity to approximate brain ischemia *in vivo* [23]. In this *in vitro* OGD model, we observed significant damage to SH-SY5Y cells with a reduction in cell viability, LDH leakage and nuclear condensation. These observations indicated that our *in vitro* model replicated cerebral ischemia injury *in vivo*.

The effect of tilianin treatment on recovery of OGD-injured SH-SY5Y cells was significant during the OGD process. The protective effects of tilianin included preservation of cell viability, membrane integrity and nuclear uniformity. This is in line with previous studies that demonstrated that tilianin exhibited anti-ischemic effects in ischemia-reperfusion (I/R) rats [15]. Thus, we concluded that tilianin has an overall protective effect on neuronal cells in response to OGD.

Increasing evidence shows that mitochondria play a major role in apoptotic neuronal cell death after cerebral ischemia, and rescue of mitochondrion dysfunction is the most important target for neuroprotective therapies [24]. During cerebral ischemia, mitochondrial dysfunction (e.g. mitochondrial swelling, opening of mitochondrial permeability transition pores) results in overproduction of ROS in mitochondria and cytoplasm. This facilitates cytosolic translocation of Bax and cytochrome *c* from the mitochondria, with subsequent activation of caspases that initiate and execute apoptosis. The anti-apoptotic protein, Bcl-2, maintains mitochondrial membrane integrity and prevents release of cytochrome *c* from mitochondria. Conversely, the pro-apoptotic protein, Bax, induces mitochondrial injury that leads to cell death [25,26]. After tilianin treatment, we showed that SH-SY5Y cells subjected to OGD had significant improvements in mitochondrial state involving MMP and mtROS production. Furthermore, we proved that treatment with tilianin inhibited OGD-induced expression of pro-apoptotic cytochrome *c*, maintained the balance between Bcl-2 and Bax, and decreased subsequent activation of caspase-3 and caspase-9 *in vitro*. These results demonstrated that tilianin inhibited OGD-induced mitochondrial dysfunction effectively and played a vital role in restraining mitochondrion-mediated apoptosis.

We evaluated the inhibitory effects of tilianin on the sensitization of p38 MAPK/JNK/NF-κB signaling and subsequent release of inflammatory cytokines. Activation of MAPKs can further activate NF-κB, inducing synthesis of pro-inflammatory mediators including cytokines, which subsequently triggers an inflammatory response [27]. In our study, we found that tilianin-mediated neuroprotection occurred with a significant reduction in p38 MAPK/MK2 and JNK/c-Jun signaling and further NF-κB activation. Several studies have shown that the NF-κB subunit p65 is activated during OGD injury and that neuro-protectants

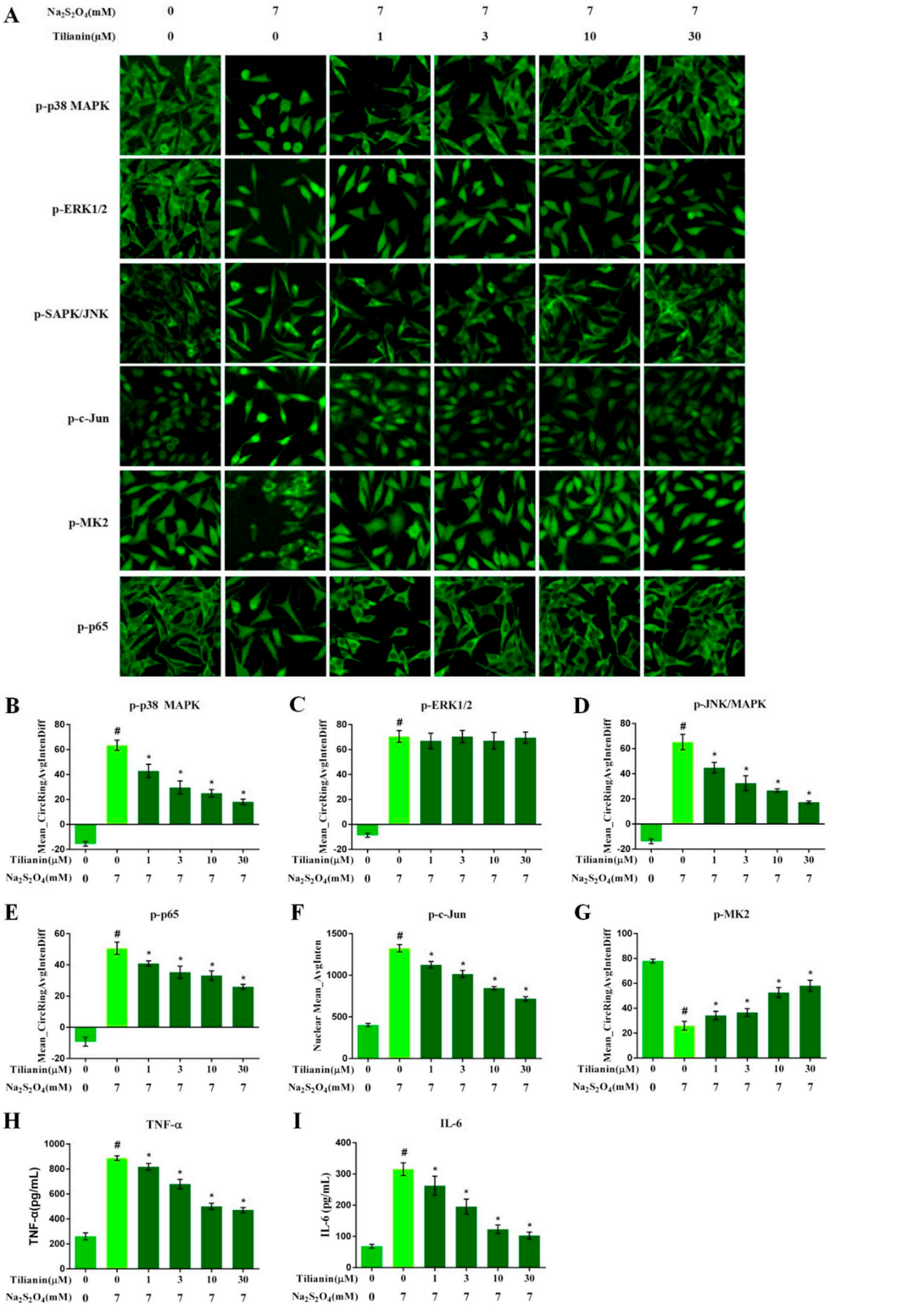

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Fig. 4. Tilianin decreased p38/MK2, JNK/c-Jun and NF-κB inflammatory signaling in OGD-injured SH-SY5Y cells. (A) Representative images of p-p38, p-ERK1/2, p-JNK, p-c-Jun, p-MK2 and p-p65 expression (×20). (B–E) Mean_CircRingAvgIntenDiff values that describe translocation of cytosolic p-p38 (B), p-ERK1/2 (C), p-JNK (D) and p-p65 (E) to the nucleus. (F) Mean fluorescence intensity of p-c-Jun expression in nuclei. (G) Mean_CircRingAvgIntenDiff values that describe translocation of nucleus phospho-MK2 to the cytoplasm. (H–I) Release of TNF-α and IL-6 as detected by ELISA. Data are expressed as the mean ± SD, $n = 6$. $^{\#}P < 0.05$ vs. control, $^{*}P < 0.05$ vs. OGD.

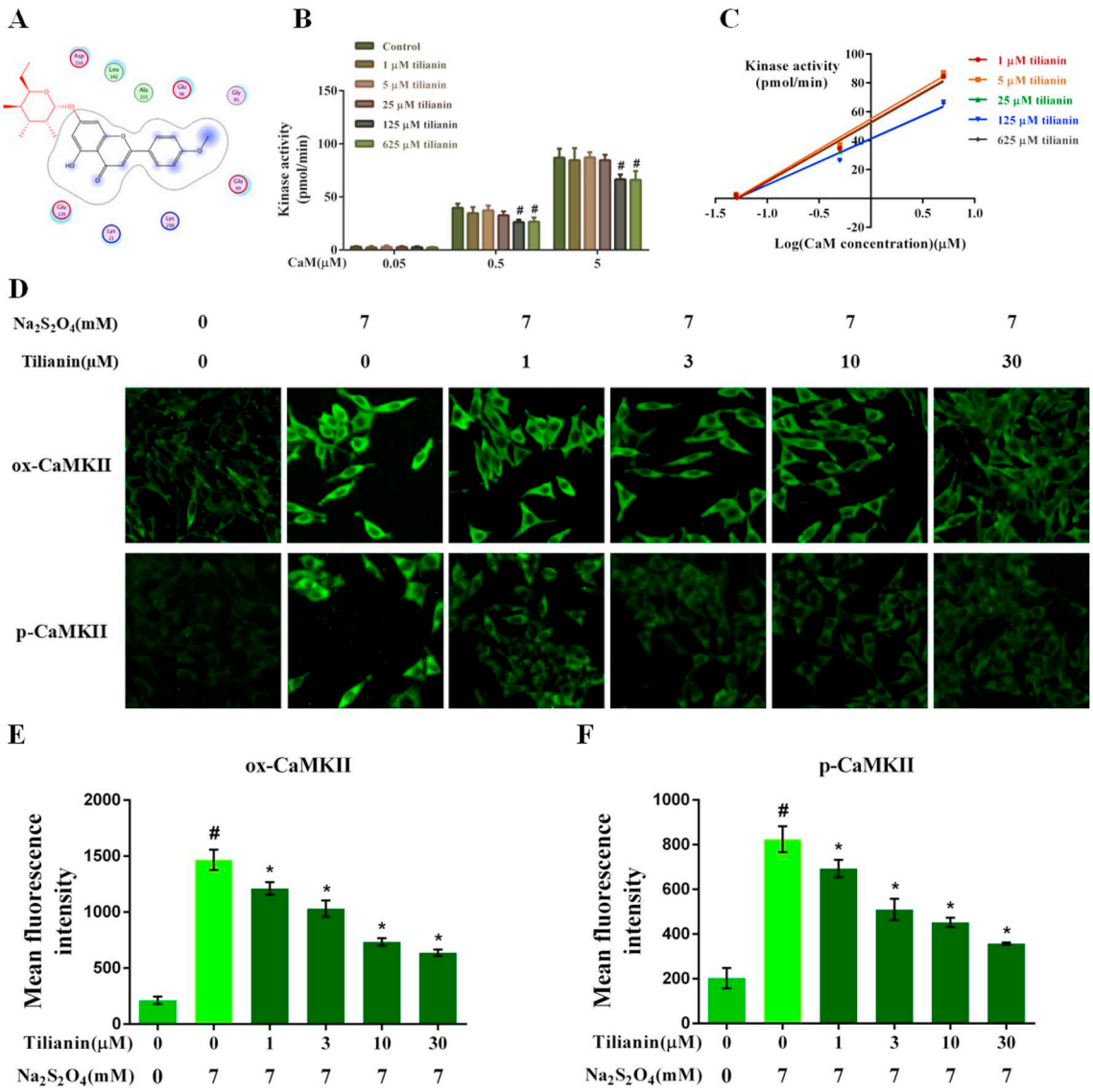

Fig. 5. Prediction of the binding mode of CaMKIIα/tilianin, and the inhibition of tilianin on CaMKII. (A) Three-dimensional model of tilianin within the binding domain of CaMKIIα. (B) Inhibitory effect of tilianin on CaMKII kinase activity in vitro ($n = 5$). (C) Double-reciprocal analysis of the inhibitory effect. (D) Representative images of p-CaMKII and ox-CaMKII (×20). (E, F) Mean fluorescence intensity of p-CaMKII and ox-CaMKII ($n = 6$). Data are expressed as mean ± SD, $^{\#}P < 0.05$ vs. control, $^{*}P < 0.05$ vs. OGD.

reduced NF-κB expression in cerebral ischemia injury. This suggested that NF-κB contributed greatly to ischemic brain injury [28,29]. Our study showed tilianin suppressed the increased phosphorylated NF-κB p65 nuclear translocation that occurred in response to OGD, and also inhibited the increased secretion of TNF-α and IL-6. Thus, tilianin contributed to anti-inflammatory effects with protective activity against.

Hence, the effects of tilianin on mitochondrion-induced apoptosis and p38 MAPK/JNK/NF-κB-mediated inflammatory activation provided molecular insights into the critical signaling mechanisms modulated by cerebral ischemia. Collectively, our findings suggested that tilianin is a promising neuroprotective compound.

Natural flavonoids have been shown to modulate a wide range of neural intracellular signaling pathways. Thus, we used bioinformatics and mechanistic verification to predict rationally a potential target that may have a strong association with the pharmacologic effects.

CaMKII is a crucial brain mediator for functions such as learning and memory. It is regulated intermediate in multiple cellular pathways involving mitochondrion-mediated programmed cell death and MAPK-associated cascades. Under basic conditions, CaMKII is squint toward inactivation. When intracellular Ca^{2+} rises, CaMKII is activated by the binding of Ca^{2+}/CaM at regulatory domains, which triggers a conformational change allowing access of the substrate to catalytic sites. Active forms of both threonine-287 (autophosphorylation) and

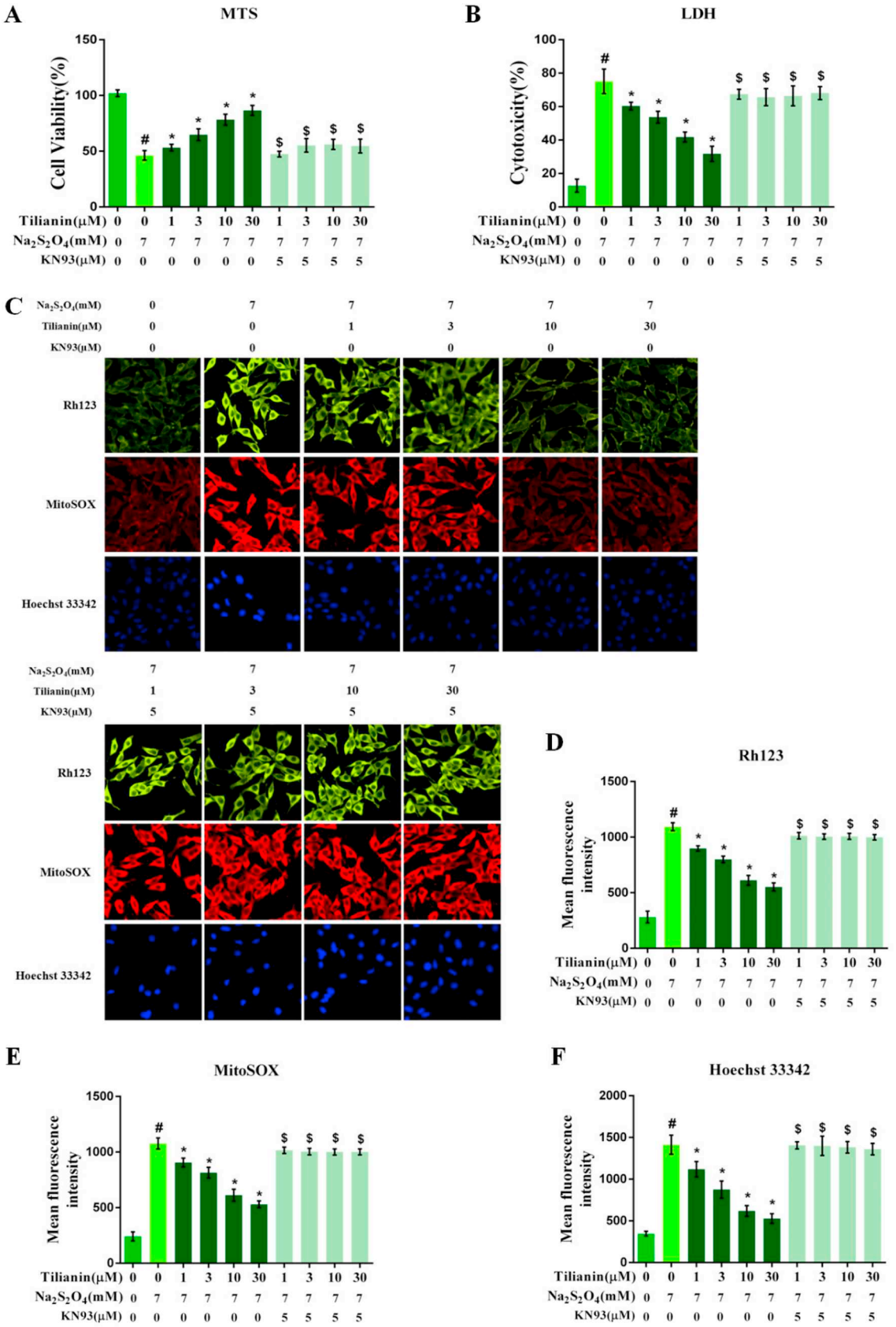

Fig. 6. CaMKII contributed to the cytoprotective effects of tilianin against OGD injury caused by mitochondrion dysfunction. (A) Tilianin treatment did not rescue decreased cell viability against OGD-induced toxicity when CaMKII was pharmacologically inhibited by KN93. (B) Tilianin treatment did not decrease LDH release when OGD-injured SH-SY5Y cells were pre-treated with KN93. (C) Representative images of MMP stained by Rh123 (×20), superoxide in mitochondria stained by MitoSOX (×20) and nuclear changes stained by Hoechst 33342(×20). (D–F) Protective effects of mitochondrial function and nuclear morphology, indicated by staining with MitoSOX (D), Rh123 (E) and Hoechst 33342 (F), were abolished when OGD-injured SH-SY5Y cells were pre-treated with KN93. Data are expressed as mean ± SD, $n = 6$. $^{\#}P < 0.05$ vs. control, $^{*}P < 0.05$ vs. OGD, $^{\$}P < 0.05$ vs.OGD + tilianin.

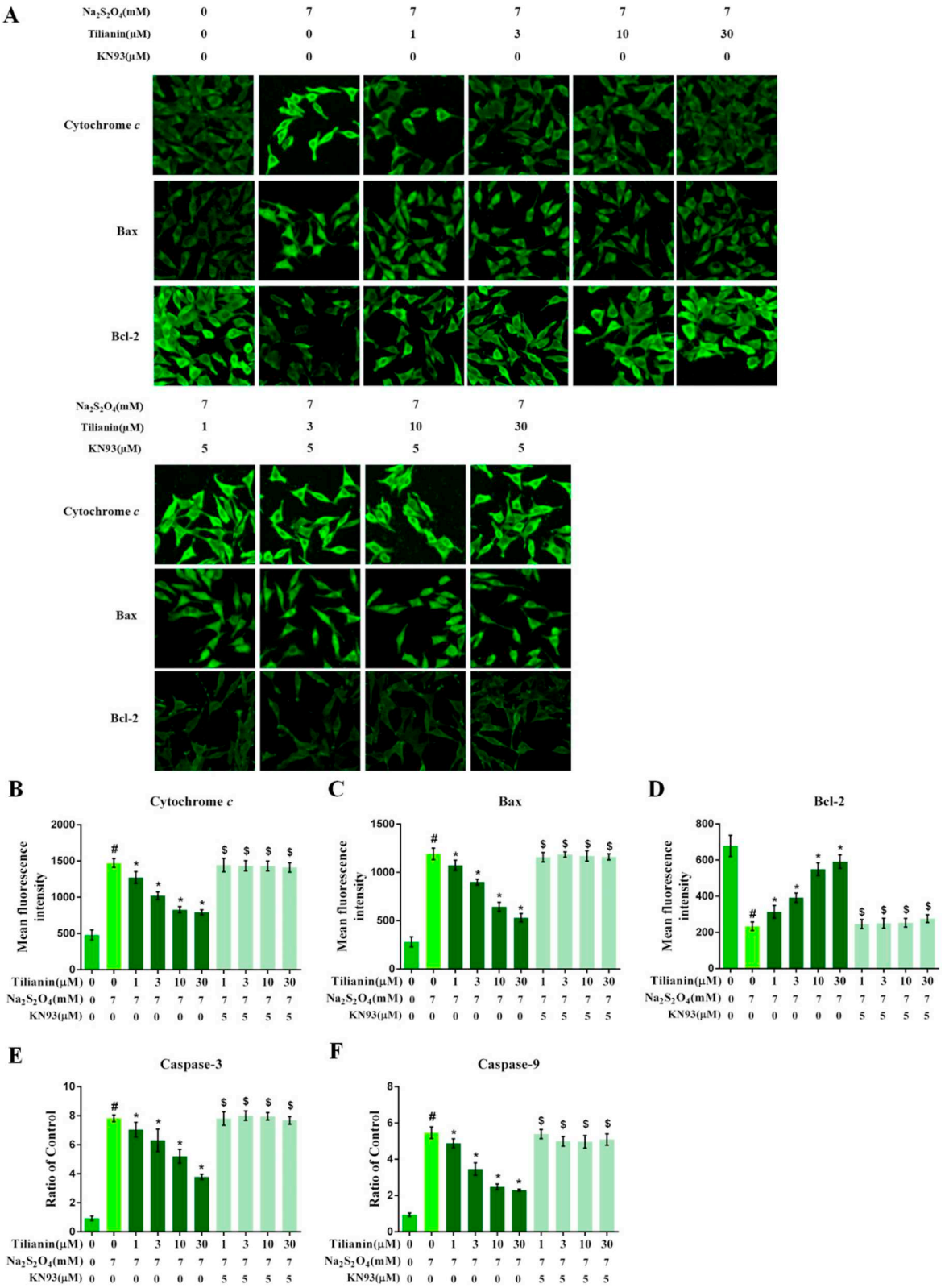

Fig. 7. Tilianin protected SH-SY5Y cells against OGD injury through the CaMKII-dependent mitochondrion-mediated apoptotic signaling pathway. (A) Representative images of immunocytochemical expression of cytochrome *c*, Bax and Bcl-2 (×20). (B–D) Ameliorative effects of tilianin treatment on the expression of cytochrome *c*, Bax and Bcl-2, caused by OGD-induced toxicity, were diminished when pre-treated with KN93. (E, F) Inhibition of caspase-3 and caspase-9 activity by tilianin was decreased by KN93. Data are expressed as mean ± SD, $^{\#}P < 0.05$ vs. control, $^{*}P < 0.05$ vs. OGD, $^{\$}P < 0.05$ vs.OGD + tilianin.

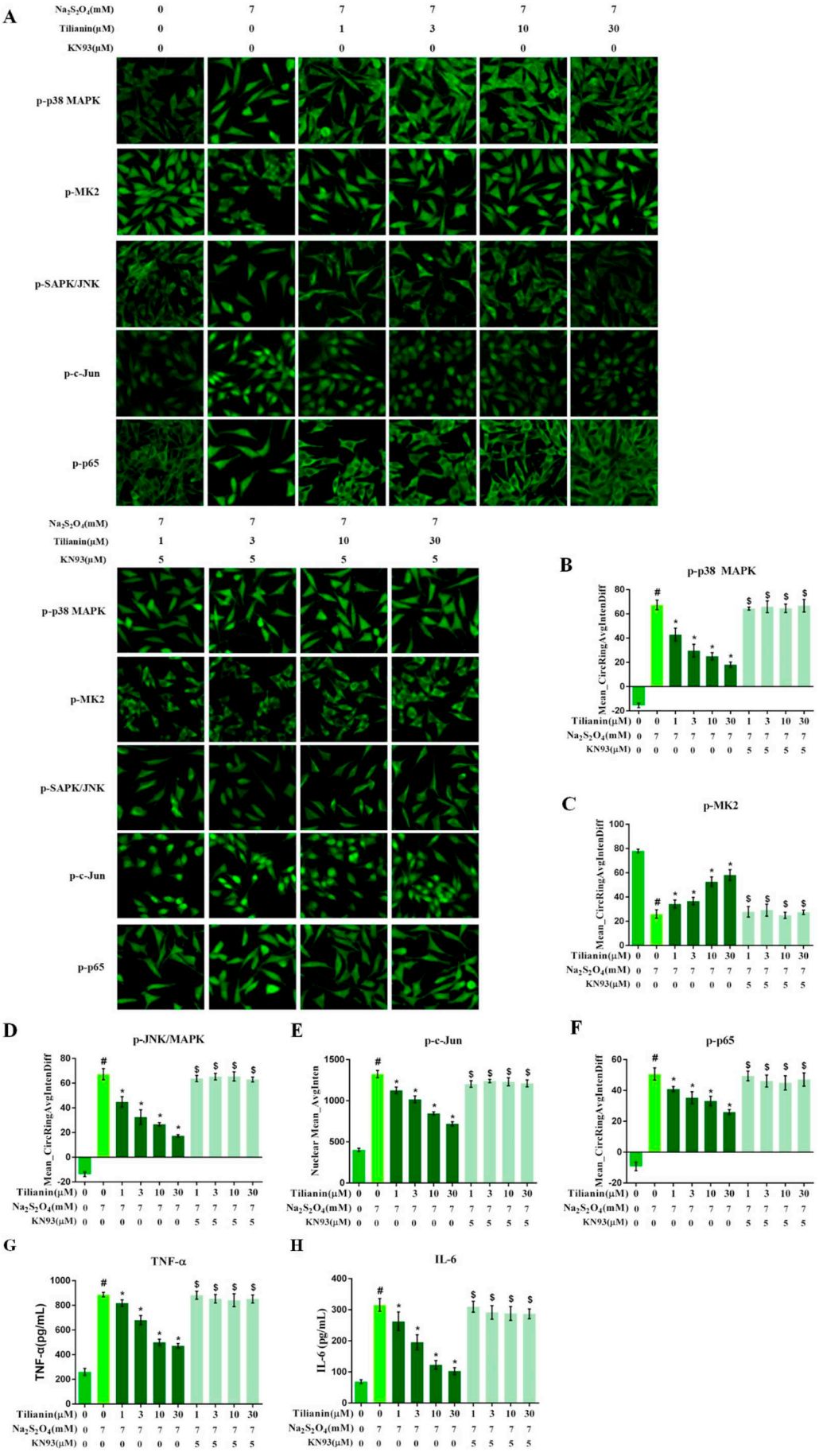

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Fig. 8. Tilianin protected SH-SY5Y cells against OGD injury through the CaMKII-dependent p38/JNK/NF-κB inflammatory signaling pathway. (A) Representative immunocytochemical images of p-p38, p-MK2, p-JNK, p-c-Jun and p-p65 (×20). (B–F) Suppressive effects of tilianin on p38/MK2, JNK/c-Jun and p-p65 activation were weakened by treatment with KN93. (G–H) Inhibition of TNF-α and IL-6 release by tilianin was prevented when cells were treated with KN93. Data are expressed as mean ± SD, $^{\#}P < 0.05$ vs. control, $^{*}P < 0.05$ vs. OGD, $^{\$}P < 0.05$ vs. OGD + tilianin.

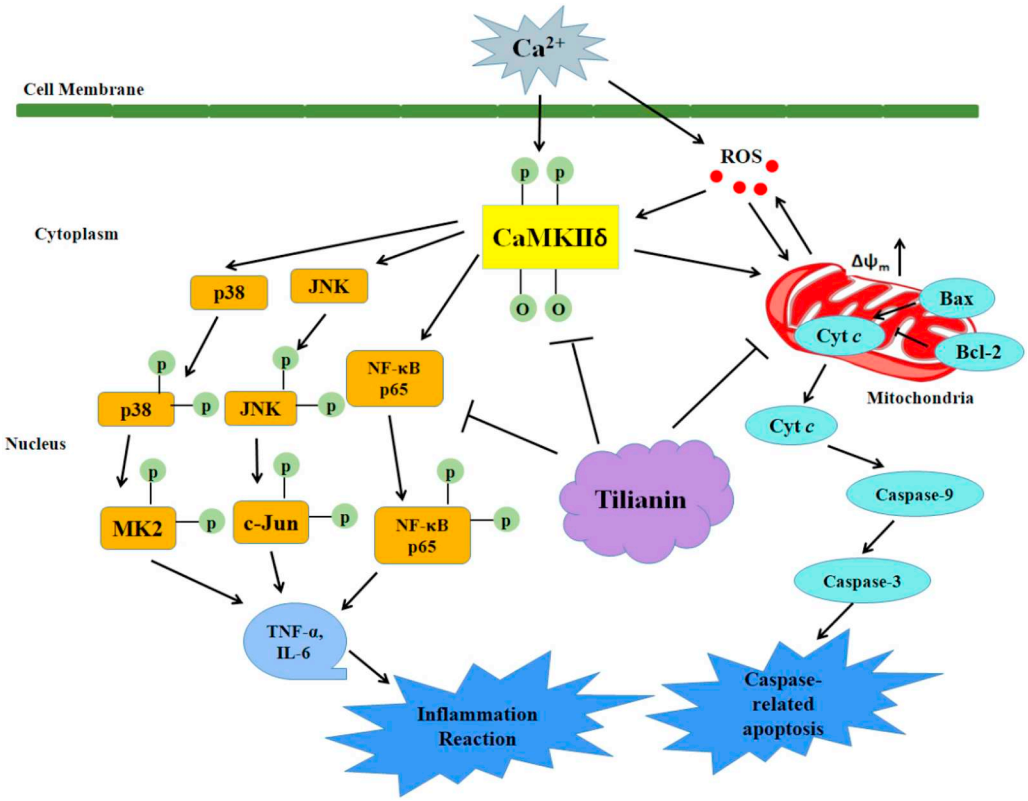

Fig. 9. Proposed neuroprotective mechanisms of tilianin against OGD injury via CaMKII-dependent mitochondrion-mediated apoptotic and MAPK/NF-κB signaling pathways. Tilianin relieved CaMKII-dependent mitochondrion-mediated apoptosis, including the expressions of Bax, Bcl-2, cyt *c* and caspase-3/9, as well as MAPK/NF-κB inflammatory activation, including the activation of p38/MK2, JNK/c-Jun and p65, following cellular OGD injury. Bax, Bcl-2 associated X protein; Bcl-2, B cell lymphoma-2; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; cyt *c*, cytochrome *c*; ERK1/2, extracellular signal-regulated kinase 1 and 2; IL-6, interleukin-6; JNK, c-Jun N-terminal protein kinase; MAPK, mitogen-activated protein kinase; MK2, MAPKAP kinase-2; NF-κB, nuclear factor kappa-B; TNF-α, tumor necrosis factor alpha.

methionine-281/282 (oxidation) are linked to OGD-induced nerve cell death. In the pathology of cerebral ischemia, activation of cytosolic CaMKII at phosphorylation (Thr287) and oxidation (Met281/282) sites leads to mitochondrial dysfunction that causes a wide range of mitochondrial-mediated apoptotic events. Additionally, it has been demonstrated that CaMKII mediates NF-κB brain activation after cerebral ischemia in mice. This serves to trigger changes in expression of multiple inflammatory genes that contribute to cerebral ischemic damage [30–32]. Therefore, we assumed that restoration of excessive activation of CaMKII by tilianin may rescue the damage from mitochondrion-mediated apoptosis and multiple protein kinase activating MAPK signaling pathways.

To explore this hypothesis, we utilized an *in vitro* kinase assay and *in silico* docking analysis and for the interaction between tilianin and CaMKII, but found that tilianin only had a slight inhibitory effect (IC50 over 625 μM) on CaMKII, and that only part of its structure extended into the active binding site of CaMKII with a weak binding force due to van der Waals/hydrophobic interactions formed by a few residues. However, tilianin treatment decreased OGD-boosted expression of ox-CaMKII and p-CaMKII significantly. Critically, inhibition of CaMKII with KN93, a specific CaMKII inhibitor, blocked the effects of tilianin-mediated neuroprotection against OGD *in vitro* including the loss of MMP and mtROS overproduction. After collating these results, we considered that tilianin neuroprotection against OGD may also result from modulating the oxidative and phosphorylated activated forms of CaMKII, and intervening in their interrelated signaling.

Overexpression of CaMKII in cultured nerve cells led to increased apoptosis due to oxidative stress. This was accompanied by elevated cytosolic Ca^{2+} and enhanced mitochondrial cytochrome *c* release [33,34]. Thereafter, the balance between pro-apoptotic and anti-apoptotic family proteins determines the fate of cells to undergo apoptosis or to survive pathophysiologically. In this study, our data indicated that tilianin reduced cell apoptosis in OGD-injury SH-SY5Y nerve cells markedly. This was manifested as a down-regulation of the expression of cytochrome *c*/Bax/caspase-3/caspase-9 and up-regulation of Bcl-2 expression. However, this signaling equilibrium initiated by tilianin was blocked by the CaMKII inhibitor, KN93. This indicated that tilianin potentiated the beneficial anti-apoptotic effects against OGD by specifically targeting CaMKII.

Tilianin was also found to decrease p38 MAPK/MK2, JNK/c-Jun and NF-κB-associated inflammatory signaling pathways via inhibition of CaMKII in OGD-injured SH-SY5Y cells. Activation of p38 MAPK/MK2, JNK/c-Jun and NF-κB is caused by CaMKII-mediated cerebral ischemia. CaMKII is recognized as an important upstream kinase that transfers signals to JNK and/or p38MAPK isoforms. These initiate subsequent NF-κB activation that is also recognized as a direct downstream signal in response to CaMKII-mediated phosphorylation via elevation of NF-κB targeted gene expression, such as TNF-α and IL-6 [35]. Thus, during OGD, tilianin treatment in the presence of KN93 could not bring about significant inhibition of p38, MK2, JNK or c-Jun activation, nuclear translocation of the p65 subunit, or the release of TNF-α and IL-6 in SH-SY5Y cells. These results indicate clearly that tilianin-mediated neuroprotection was associated with inhibition of p38/JNK/NF-κB signaling via the CaMKII pathway.

5. Conclusion

Our study identified the potential mechanisms for tilianin against OGD. We revealed that tilianin-induced inhibition of CaMKII was

involved in cerebral neurological protection via effects on mitochondrial function and inflammation. Importantly, our results suggested that tilianin relieved CaMKII-dependent mitochondrion-mediated apoptosis and MAPK/NF-κB inflammatory activation following cellular OGD injury (Fig. 9). These findings highlighted the potential therapeutic benefits of tilianin through attenuating CaMKII signaling during cerebral ischemia.

Conflict of interest

The authors declare no conflict of interest. All authors agree with the publication in this journal.

Author contribution to study

Zhuorong Li and Rui Liu designed the research, wrote the manuscript and had primary responsibility for final content. Hailun Jiang and Jianguo Xing conducted the in vitro cellular experiments and analyzed the data. Jiansong Fang conducted all the in silico experiments. Jiansong Fang, Linlin Wang, Qian Wang and Yu Wang conducted the in vitro kinase experiments and analyzed the data.

Acknowledgments

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