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ORIGINAL PAPER

Magnesium Lithospermate B Protects Neurons Against Amyloid β (1–42)-Induced Neurotoxicity Through the NF-κB Pathway

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Abstract Magnesium lithospermate B (MLB) is one of the major bioactive components of *Radix Salviae miltiorrhizae*, a Chinese traditional herbal medicine colloquially known as Dan Shen. In this study, we investigated the neuroprotective effect of MLB against oligomeric amyloid β (Aβ) (1–42)-induced neurotoxicity in cultured FVB mouse hippocampal neurons. We found that pretreatment with MLB not only prevents a loss in neuronal cell viability following exposure to Aβ (1–42), but also attenuates Aβ (1–42)-induced release of pro-inflammatory cytokines and neuronal apoptosis in a dose-dependent manner. Mechanistic studies show that MLB counteracts Aβ (1–42)-induced activation of the nuclear factor kappa B (NF-κB) pathway, evidenced by the suppression of NF-κB luciferase reporters, decreased expression of phosphorylated Inhibitor κB α and IκB kinase α, and reduced nuclear translocation of p65 in response to pre-treatment with 50 μg/ml MLB prior to Aβ (1–42) exposure. MLB was able to reverse the increase in phosphorylated c-Jun N-terminal kinase (JNK) levels as well as the decrease in phosphorylated Akt levels that are induced by Aβ (1–42), although this finding did not extend to extracellular signal-regulated kinase or p38 kinases. Furthermore, combining MLB with the JNK inhibitor SP600125 synergistically counteracts the Aβ (1–42)-induced reduction in cell viability and neurite growth, and the neuroprotective effects of MLB could be attenuated by the Akt inhibitor triciribine. In conclusion, these results suggest that MLB can protect against Aβ (1–42)-induced neuronal damage, which is most likely to be mediated by the NK-κB pathway.

Keywords Magnesium lithospermate B · Neuron · Amyloid β (1–42) · Neurotoxicity · Nuclear factor kappa B

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease that progressively destroys memory and other important mental functions [1]. Although there is no absolute certainty, the accumulation of amyloid β (Aβ) into senile plaques in the brain is hypothesized as a leading cause of AD, as these plaques have been found to cause progressive neuronal death [2, 3]. Multiple pathogenic processes, including oxidative stress, inflammatory responses, and apoptosis, are implicated in Aβ-induced neurotoxicity. Numerous in vitro and in vivo studies indicate that Aβ oligomers can induce a cascade of oxidative damage to neurons [4], and in AD patients, inflammation usually occurs in pathologically vulnerable regions of the brain associated with increased pro-inflammatory cytokine production and neuronal apoptosis [5].

Many signaling pathways have been implicated in Aβ toxicity, of which the nuclear factor kappa B (NF-κB) pathway plays a key role [6]. A large number of genes involved in the pathogenesis of AD have been reported to

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be under immediate-early transcriptional control of NF-κB [7]. Researchers also found that activated NF-κB can be detected in neuronal nuclei proximal to early stage senile plaques in AD brains, and that Aβ is able to activate NF-κB in primary neurons [8]. It has been hypothesized that Aβ may interact with its putative receptor(s), subsequently activating a series of mitogen-activated protein kinases (MAPKs) including JNK, ERK, and p38 [9]. These activated MAPKs can function as upstream activators of NF-κB to induce phosphorylation at serine residues of the Rel homology domain (RHD) of NF-κB p65 subunit, stimulating transcriptional activity of p65 by promoting an interaction with CBP (CREB binding protein) and p300 co-activators [6]. Although there is debate over the effect of NF-κB activation on neuronal survival and development, substantial evidence demonstrates that it is responsible, at least in part, for Aβ-induced pathogenic processes, and that suppression of NF-κB activation may be a potential therapeutic approach to control the progression of AD [10].

Treatment of AD remains a great challenge for clinicians in neurology. In recent years, more and more researchers have focused on natural products to provide lead compounds with potential neuroprotective effects. Magnesium lithospermate B (MLB) is one of the major bioactive components of *Radix Salviae miltiorrhizae* (Dan Shen), a Chinese traditional herbal medicine that is widely used for the treatment of cardiovascular diseases [11]. MLB is a water-soluble compound composed of salvianolic acid B (Sal B) anions and magnesium ions (structure shown in Fig. 1). Prior research has demonstrated a role for MLB in protecting primary neurons against *N*-methyl-D-aspartic acid (NMDA) injury and attenuating kainic acid (KA)-induced neurodegeneration in the brain of FVB mice [12]. Additionally, MLB is able to reduce the production of cytokines and the activation of the NF-κB and octamer binding transcription factor (Oct-1) signaling pathways in human peripheral T cells treated by phorbol 12-myristate 13-acetate (PMA) and ionomycin [13]. Based on these findings, we hypothesize that MLB has a protective effect against Aβ-induced neurotoxicity through the NF-κB pathway. In order to test this hypothesis, in the current study we investigate the neuroprotective effects of MLB and the underlying mechanism using mouse primary neurons injured by oligomeric Aβ (1–42). We reveal that MLB can protect against Aβ (1–42)-induced neuronal damage and provide evidence that this is most likely mediated by the NK-κB pathway.

Materials and Methods

Reagents and Animals

MLB (purity = 99.7 %) was purchased from the Research Center of Traditional Chinese Medicine Modernization, Shanghai Institute of Materia Medica (Shanghai, China). FVB mice (wild-type) were from Shanghai Laboratory Animal Center (Shanghai, China). Synthetic Aβ (1–42) was purchased from Shanghai Apeptide Co., Ltd (Shanghai, China). 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT), SP600125, and triciribine were from Sigma-Aldrich (St. Louis, MO, USA). The protocol of animal study

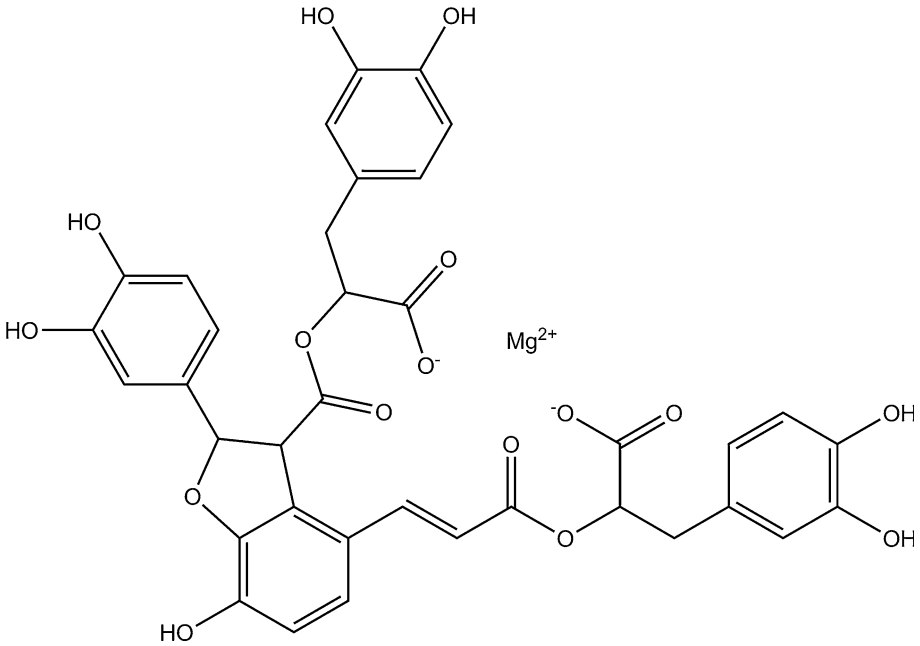

Fig. 1 Structure of MLB

was approved by the Animal Ethics Committee of Soochow University. All animal care and use was performed in accordance with institutional guidelines.

Preparation of Oligomeric Aβ (1–42)

Oligomeric Aβ (1–42) used in this study was prepared and characterized as described previously [14, 15]. Briefly, lyophilized Aβ (1–42) was firstly monomerized by dissolving the peptide in 100 % 1,1,1,3,3,3 hexafluoro-2-propanol (HFIP, Sigma-Aldrich) to 1 mM. Then, the prepared monomer solution was divided into aliquots in sterile microcentrifuge tubes, and HFIP was evaporated under vacuum using a Savant Speed Vac system (GMI, Ramsey, MN). Afterwards, the dried Aβ (1–42) was re-suspended in dimethylsulfoxide (DMSO) to make a 5 mM stock solution by sonication. The resulting stock solution was then diluted using phosphate buffer saline (PBS) to a final concentration of 100 μM and incubated at 37 °C for 24 hours (h) before use.

Culture of Primary Neurons

Primary neurons were prepared from the hippocampi of embryonic day 17 (E17) FVB mice, and were cultured according to the procedures described in previous literature [12]. After being cultured for 10 days, neurons were pre-incubated with varying concentrations of MLB (0, 5, 10, 20, and 50 μg/ml) for 4 h, and then exposed to 10 μM oligomeric Aβ (1–42) for 24 h. The doses of MLB were chosen based on the studies described previously [12, 13].

MTT Assay

Primary neurons were seeded in a 96-well plate at a density of 1×10^4 cells per well. Before being exposed to 10 μM Aβ (1–42) for 24 h, cells were pre-treated with different concentrations of MLB for 4 h. Then, MTT assay was performed to evaluate cell viability according to the manufacturer's instructions and previous literature [15].

Immunocytochemical Staining

Neurite growth of primary neurons under different treatment conditions was assessed by immunocytochemical staining as previously described [12, 16]. In brief, neurons were fixed in 4 % paraformaldehyde (PFA) in PBS (pH 7.4) for 20 min (min) at room temperature (RT). Then, the PFA-fixed neurons were blocked using 5 % normal goat serum in PBS for 2 h at RT, followed by incubation with a mouse anti-MAP2 antibody (1:1000, Sigma, Product No.: M9942) at 4 °C over night. Afterwards, cells were incubated with goat anti-mouse Alexa Fluor® 488 conjugate (1:500; Life Technologies, Grand Island, NY, USA) for 1 h, and then were observed by confocal microscopy (LSM 700, Carl Zeiss, Oberkochen, Germany). Images were taken and morphology analysis (counting of neuronal branches and neurite tracing) was performed in over 100 randomly selected neurons using ImageJ software (version 1.42, http://imagej.nih.gov/ij/).

Measurement of Cytokine Levels

Production of three main cytokines, interleukin (IL)-1β, IL-10, and tumor necrosis factor-α (TNF-α), in neurons under various treatment conditions were measured using enzyme-linked immunosorbent assay (ELISA). Briefly, the supernatants of neurons exposed to different treatment conditions were collected and stored at −80 °C until assays were performed. The levels of the three cytokines were detected by the corresponding ELISA kits (Life Technologies) according to the manufacturer's instructions and previous literature [17].

Annexin V Assay

Annexin V assay was used to identify the cells at the early stage of apoptosis according to Schutte et al. [18]. In brief, $0.5–1.0 \times 10^5$ neurons subjected to different treatment conditions were harvested, washed twice with a cold PBS, and re-suspended in 500 μl of Annexin-V binding buffer. Then, the cells were stained with 5 μl of Alexa Fluor® 488-conjugated Annexin V (Life Technologies) and 5 μl of propidium iodide (PI) in the dark at RT for 15 min. The percentage of Annexin V positive cells was analyzed using a FACS Calibur Flow Cytometry System (BD Bioscience, San Jose, CA, USA).

Luciferase Assay

Luciferase assay was used to detect NF-κB activation in neurons in response to various treatments. Specifically, a Cignal NF-κB reporter (luc) kit (Qiagen, Venlo, Netherlands) was used to monitor trans-activation activity of NF-κB. A Dual-luciferase Reporter Assay System (Promega, Madison, WI, USA) was used to measure NF-κB-responsive luciferase activity according to the manufacturer's protocol and previous literature [19].

Western Blot

Primary neurons under various treatment conditions were harvested by centrifugation at 1500 rpm for 5 min and lysed with RIPA buffer. The homogenates were centrifuged at 10,000*g* for 10 min. The supernatant was then subject to Western blot analysis according to routine

procedures. The preparation of nuclear and cytoplasmic extracts was carried out according to the previously described methods [15, 20]. Immunoblots were performed with the following antibodies: cleaved caspase 3 (p17 fragment, ab2302), Inhibitor κB α (IκBα, ab32518), phosphorylated IκBα (p-IκBα at Ser32/36, ab12135), IκB kinase α (IKKα, ab54628), phosphorylated IKKα (p-IKKα at Ser176, ab138426), (Abcam, Cambridge, MA, USA); Bcl-2 (sc-7382), Bax (sc-493), JNK (sc-7345), phosphorylated JNK (p-JNK at Thr 183/Tyr 185, sc-12882), ERK (sc-94), phosphorylated ERK (p-ERK at Thr 202/Tyr 204, sc-16982), p38 (sc-535), and phosphorylated p38 (p-p38 at Tyr 182, sc-7973) (Santa Cruz Biotechnology, Dallas, TX, USA); NF-κB P65 (#8242), Akt (#9272), and phosphorylated Akt (p-Akt at Ser473, #9271) (Cell Signaling; Danvers, MA, USA). The antibodies against β-actin (Santa Cruz Biotechnology, sc-47778) and histone H3 (Abcam, ab1791) were used as loading controls. Proteins were detected using an Amersham Enhanced Chemiluminescence (ECL) Western Blotting Detection Kit (GE Healthcare Life Sciences, Pittsburgh, PA, USA). The bands were quantified using ImageJ software.

Statistical Analysis

Statistical analysis was performed using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). The Shapiro–Wilk test was used to test the normality of data. Data with normal distribution are presented as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM). Data with skewed distributions are presented as median and range. Statistical comparisons between means were performed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc multiple comparison tests. Statistical differences between medians were assessed with the nonparametric Kruskal–Wallis test. A P value <0.05 was considered statistically significant.

Results

MLB Protects Primary Neurons Against Aβ (1–42)-Induced Cytotoxicity

We first investigated the neuroprotective effects of MLB against Aβ (1–42) in primary neurons. Primary hippocampal neurons isolated from E17 embryos of pregnant FVB mice were incubated with varying doses of MLB prior to exposure to 10 μM oligomeric Aβ (1–42). Cell viability was measured by MTT assay and neuronal morphology was examined by confocal microscopy. As shown in Fig. 2a, treatment with Aβ (1–42) alone induced a decrease of approximately 70 % in cell viability; while pre-incubation with MLB significantly attenuated the Aβ (1–42)-induced loss of cell viability in a dose-dependent manner. Fifty micromolar MLB exhibited the most significant protective effect against the decrease of neuronal viability caused by Aβ (1–42).

Next, we validated the protective effect of MLB based on neuronal morphology. Figure 2b, c, d show that neurons treated with Aβ (1–42) were fewer in number and had fewer branches and shorter neurite length compared neurons treated with the vehicle. However, pre-incubation with varying concentrations of MLB suppressed the Aβ (1–42)-induced injury to neuronal morphology, which was evidenced by the fact that MLB pre-incubation prevented a decrease in branch number and neurite length caused by Aβ (1–42). Taken together, these results demonstrate the protective effects of MLB against Aβ (1–42)-induced cytotoxicity in primary neurons.

MLB Attenuates the Release of Pro-inflammatory Cytokines and Neuronal Apoptosis Induced by Aβ (1–42)

Pro-inflammatory conditions promote neuronal damage mediated by Aβ [21]. Hence, we examined whether MLB has anti-inflammatory effects on Aβ (1–42)-treated neurons. As shown in Fig. 3a, neurons exposed to 10 μM Aβ (1–42) for 24 h had a significant increase in the production of IL-1β, IL-10, and TNF-α when compared to vehicle-treated cells (4.2-, 3.2-, and 5.3-fold, respectively). When cells are first pretreated with MLB, we observed a dose dependent effect of MLB inhibiting the production of proinflammatory cytokines induced by Aβ (1–42). These data support the anti-inflammatory effects of MLB on Aβ (1–42)-treated neurons.

To investigate whether MLB prevents neuronal apoptosis induced by Aβ (1–42), we measured apoptotic events and markers of apoptosis by annexin V assay and Western blot, respectively. Neurons were pretreated with varying doses of MLB for 4 h before Aβ (1–42) treatment for another 24 h. We then stained cells with fluorescein-conjugated annexin V, which binds to phosphatidylserine, an early cell-surface apoptosis marker. Figure 3b shows that in the absence of MLB, Aβ (1–42)-treated neurons had almost five times more apoptotic cells than those treated with vehicle. Nevertheless, the number of apoptotic cells was notably decreased in a dose-dependent manner by addition of MLB. Additionally, in the Western blot analysis, neurons treated with Aβ (1–42) showed significantly higher levels of pro-apoptotic protein Bax and cleaved caspase 3, and a considerably lower level of anti-apoptotic protein Bcl-2 than the vehicle group (Fig. 3c, d). However, these changes were prevented following pretreatment of neurons with varying doses of MLB. Notably, in neurons

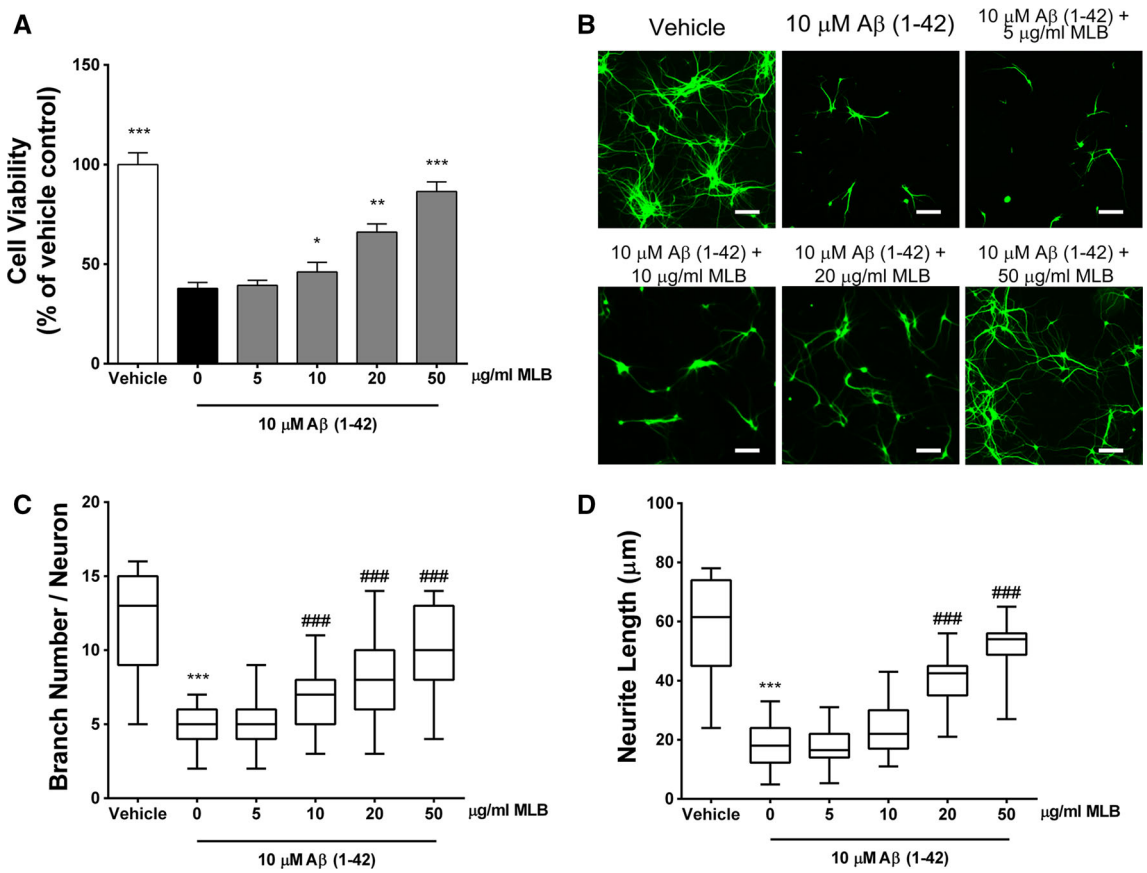

Fig. 2 MLB attenuates Aβ (1–42)-induced neurotoxicity in primary neurons. Neurons were incubated with different doses of MLB for 4 h before exposure to 10 μM oligomeric Aβ (1–42) for an additional 24 h. **a** Neuronal viability was detected by MTT assay. **b** Representative confocal images of MAP2 staining showing neuronal morphology upon oligomeric Aβ (1–42) treatment in the presence or absence of MLB (*scale bar* = 10 μm). The number of neuronal branches (**c**) and neurite length (**d**) were also measured under different treatment conditions. Data of neuronal viability (histograms) were from three independent experiments performed in sextuplicate and are presented as mean ± SEM. Data of neuronal branch number and neurite length (*box-and-whisker plots*) were from over 100 randomly selected neurons and are presented as median and range. $^{***}P < 0.001$, as compared with the vehicle group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, as compared with the group treated with Aβ (1–42) alone; ANOVA followed by Tukey's post hoc test or nonparametric Kruskal–Wallis test

pretreated with 50 μg/ml MLB, the expression profile of the three apoptotic markers appeared similar to that of vehicle-treated cells. Collectively, these findings indicate that MLB is able to attenuate Aβ (1–42)-induced apoptosis in primary neurons.

MLB Suppresses Aβ (1–42)-Induced Activation of the NF-κB Pathway

MLB has been reported to suppress the NF-κB pathway in activated T cells [13]. To test whether MLB is able to prevent Aβ (1–42)-induced activation of the NF-κB pathway in primary neurons, we measured this pathway using an NF-κB luciferase reporter construct and detected the levels of cytoplasmic and nuclear NF-κB p65 subunit by Western blot. Primary neurons transfected with an NF-κB luciferase reporter construct and then treated with Aβ (1–42) exhibited an increase of over threefold increase in luciferase activity over that observed in cells treated with vehicle or 50 μg/ml MLB alone (Fig. 4a). This result indicates that treatment with Aβ (1–42) could induce activation of the NF-κB pathway in primary neurons, which is consistent with previous reports. In MLB-pretreated neurons, however, this Aβ (1–42)-mediated increase in luciferase activity was significantly suppressed, suggesting MLB prevents NF-κB pathway activation in response to Aβ (1–42). In the Western blot analysis, we observed that treatment with Aβ (1–42) resulted in a considerably higher level of nuclear p65 and a dramatically lower level of cytoplasmic p65 than vehicle or MLB alone did (Fig. 4b, c). However, this effect was reversed by pre-incubation with 50 μg/ml MLB, further demonstrating the inhibitory effects of MLB on Aβ (1–42)-induced NF-κB pathway activation.

In addition, activation of the NF-κB pathway involves phosphorylation of IKK, which subsequently phosphorylates

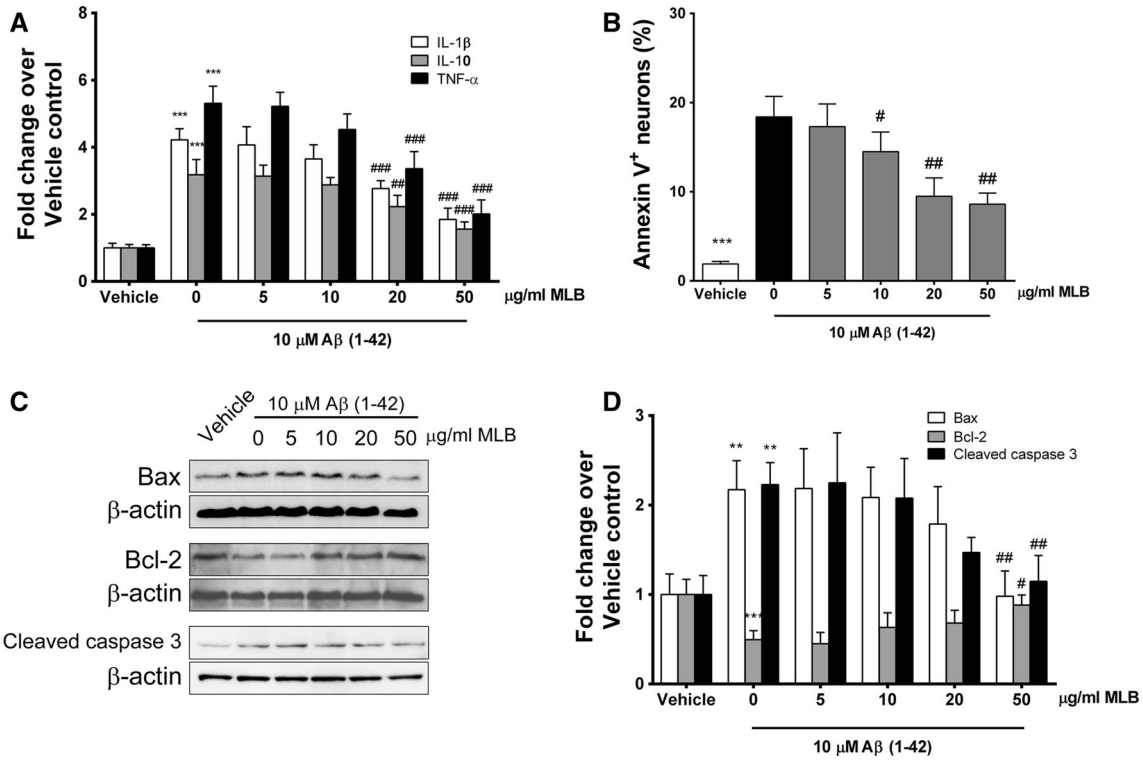

Fig. 3 MLB attenuates the release of pro-inflammatory cytokines and neuronal apoptosis induced by Aβ (1–42). Neurons were incubated with different doses of MLB for 4 h before exposure to 10 μM oligomeric Aβ (1–42) for an additional 24 h. **a** The production of IL-1β, IL-10, and TNF-α were measured by ELISA kits. **b** The percentage of Annexin V positive cells was analyzed by flow cytometry. **c**, **d** Expression levels of Bax, Bcl-2, and cleaved caspase 3 were detected by Western blot and quantified with ImageJ software. All data (histograms) were from three independent experiments, and the *bars* indicate mean ± SD. $^{**}P < 0.01$ and $^{***}P < 0.001$, as compared with the vehicle group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, as compared with the group treated with Aβ (1–42) alone; ANOVA followed by Tukey's post hoc test

inhibitory IκBα, leading to its proteasomal degradation and NF-κB nuclear localization [6]. We therefore examined the expression of phosphorylated forms of IKKα and IκBα in primary neurons exposed to different treatments. As presented in Fig. 5a, b, Western blot analysis showed that exposure to Aβ (1–42) for 1 h induced significantly higher levels of phosphorylated IKKα and IκBα than vehicle did; while these effects could be counteracted by pretreatment with 50 μg/ml MLB before exposure to Aβ (1–42). These results are consistent with data presented in Fig. 4, further confirming that MLB suppresses Aβ (1–42)-induced activation of the NF-κB pathway. Taken together, our findings suggest that the neuroprotective effects of MLB against Aβ (1–42)-induced cytotoxicity are associated with the NF-κB pathway.

MLB Inhibits Aβ (1–42)-Induced Activation of JNK but Reverses Aβ (1–42)-Induced Inactivation of Akt

Several kinases, including JNK, ERK, p38, and Akt, have been demonstrated to be involved in mediating the neuronal toxicity of Aβ [4, 22]. We investigated the regulatory effects of MLB on these kinases in combination with 1-h Aβ (1–42) stimulation. Figure 6 shows that exposure to Aβ (1–42) induced significantly higher phosphorylation of JNK, ERK, and p38 (2.4, 2.0, and 1.6 fold of the vehicle control, respectively), and considerably lower phosphorylation of Akt (42 % decrease compared with the vehicle control) in primary neurons than vehicle and MLB alone did. Nevertheless, pre-incubation with 50 μg/ml MLB could suppress the Aβ (1–42)-induced effects on JNK and Akt, which was evidenced by the decreased phosphorylation of JNK and increased phosphorylation of Akt in MLB-pretreated cells. For ERK and p38, however, MLB did not show any notable effects on their activation in response to Aβ (1–42) exposure. These data indicate that MLB is able to inhibit Aβ (1–42)-induced activation of JNK but reverse Aβ (1–42)-induced inactivation of Akt.

To further verify that the protective effects of MLB against Aβ (1–42) are mediated by the NF-κB pathway, we utilized the JNK inhibitor SP600125 (20 μM) and the Akt inhibitor triciribine (10 μM) to treat primary neurons for 4 h prior to MLB and Aβ (1–42) treatments. We found that the combined pretreatment of SP600125 and MLB

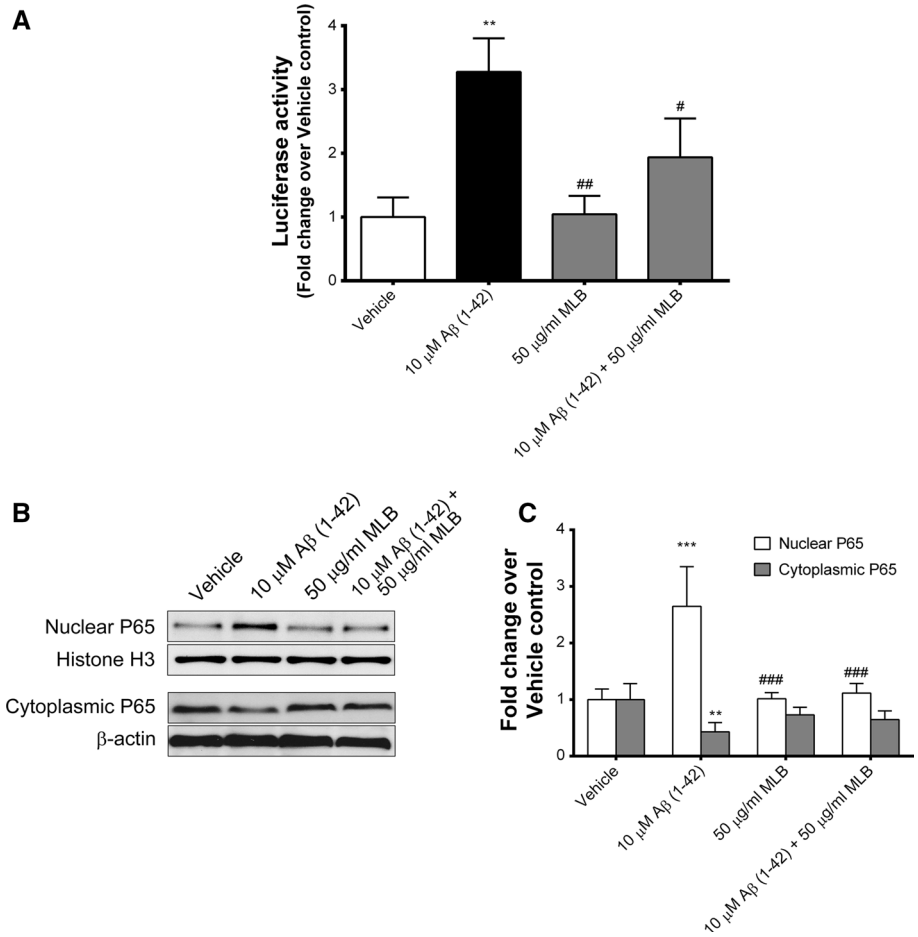

Fig. 4 MLB suppresses Aβ (1–42)-induced activation of NF-κB. Primary neurons were pre-incubated with 50 μg/ml of MLB for 4 h prior to Aβ (1–42) treatment for another 1 h. **a** Luciferase activity was measured using a Cignal NF-κB reporter (luc) kit. **b**, **c** The levels of cytoplasmic and nuclear NF-κB p65 subunit were detected by Western blot and quantified with ImageJ software. All data (histograms) were from three independent experiments, and the *bars* indicate mean ± SD. $^{**}P < 0.01$ and $^{***}P < 0.001$, as compared with the vehicle group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, as compared with the group treated with Aβ (1–42) alone; ANOVA followed by Tukey's post hoc test

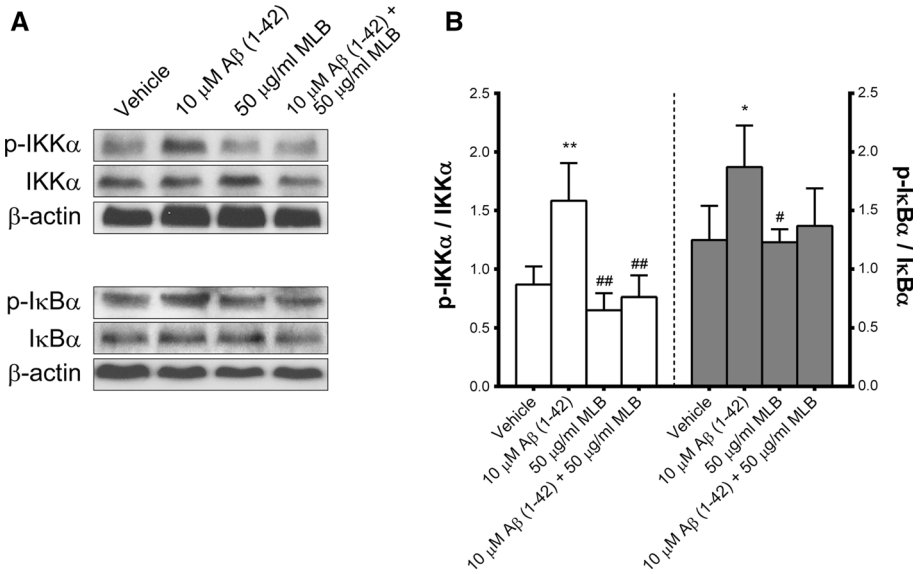

Fig. 5 MLB suppresses Aβ (1–42)-induced phosphorylation of IKK and IκBα. Primary neurons were pre-incubated with 50 μg/ml of MLB for 4 h prior to Aβ (1–42) treatment for another 1 h. The expression of phosphorylated IKKα and IκBα was detected by Western blot (**a**) and quantified with ImageJ software (**b**). All data (histograms) were from three independent experiments, and the *bars* indicate mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$, as compared with the vehicle group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, as compared with the group treated with Aβ (1–42) alone; ANOVA followed by Tukey's post hoc test

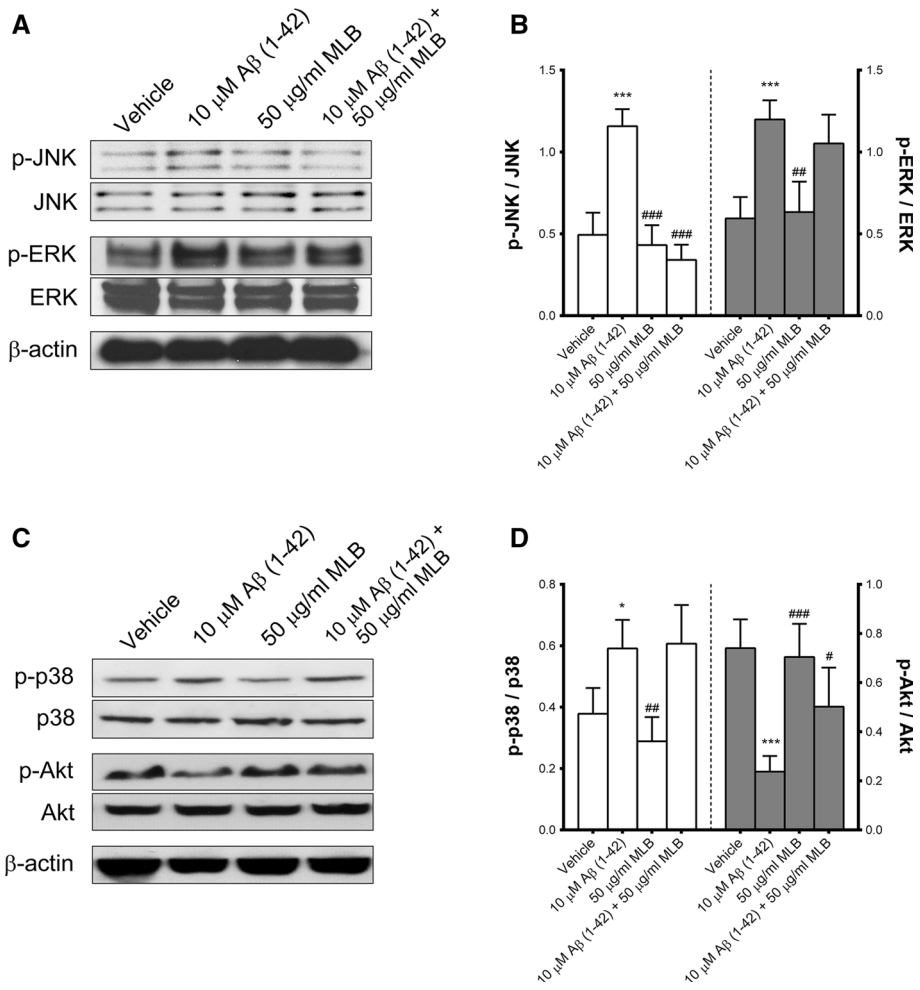

Fig. 6 MLB inhibits Aβ (1–42)-induced activation of JNK and reverses Aβ (1–42)-induced inactivation of Akt. Primary neurons were pre-incubated with 50 μg/ml of MLB for 4 h prior to Aβ (1–42) treatment for another 1 h. The expression of phosphorylated JNK, ERK, p38, and Akt was detected by Western blot (**a**, **c**) and quantified with ImageJ software (**b**, **d**). All data (histograms) were from three independent experiments, and the *bars* indicate mean ± SD. $^{*}P < 0.05$ and $^{***}P < 0.001$, as compared with the vehicle group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, as compared with the group treated with Aβ (1–42) alone; ANOVA followed by Tukey's post hoc test

synergistically counteracted the Aβ (1–42)-induced decrease in cell viability (Fig. 7a), but triciribine significantly abolished the protective effects of MLB on cell viability. Additionally, neurons pretreated with SP600125 and MLB showed a significant increase in the number of neuronal branches and neurite length compared to those pretreated with MLB alone (Fig. 7b, c, d). However, neurons pre-incubated with triciribine prior to MLB and Aβ (1–42) treatments had considerably lower neuronal branch number and neurite length than MLB-pre-treated cells. Furthermore, pretreatment with the combination of SP600125 and MLB resulted in a dramatically lower level of nuclear p65 and a significantly higher level of cytoplasmic p65 than pretreatment with MLB alone did; whereas pre-incubation with triciribine showed the opposite effect (Fig. 7e, f). Taken together, these results further demonstrate that the neuroprotective effects of MLB against Aβ (1–42)-induced toxicity in primary neurons is mediated by the NF-κB pathway.

Discussion

In the current study, we investigated the neuroprotective effects of MLB against Aβ (1–42)-induced toxicity in mouse primary neurons. We found that pretreatment with MLB not only prevented a decrease in neuronal cell viability following exposure to Aβ (1–42), but attenuated Aβ (1–42)-induced release of pro-inflammatory cytokines and neuronal apoptosis in a dose-dependent manner. Mechanistic studies showed that MLB is able to suppress Aβ (1–42)-induced activation of the NF-κB pathway, attributed to the inhibition of JNK activation and the prevention of decreased Akt phosphorylation. These results demonstrate that the neuroprotective effects of MLB are most likely mediated by the NF-κB pathway.

Senile plaques formed by the accumulation of Aβ are a key feature of AD [2]. As the major component of senile plaques, Aβ is reported to be neurotoxic to primary neurons due to the induction of oxidative stress and inflammatory

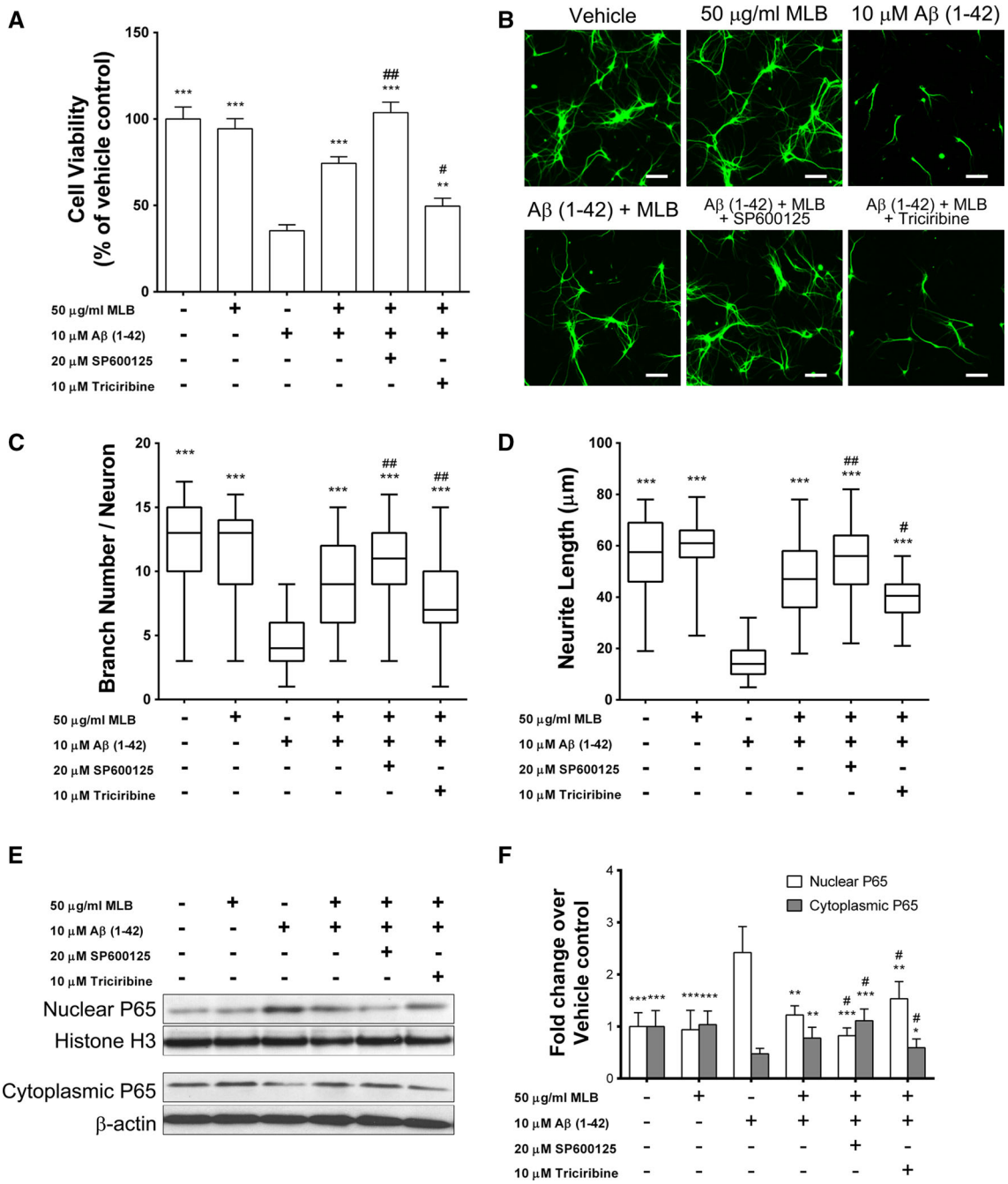

Fig. 7 MLB protects neurons against Aβ (1–42)-induced neurotoxicity through the NF-κB pathway. The JNK inhibitor SP600125 (20 μM) and the Akt inhibitor triciribine (10 μM) were used to treat primary neurons for 4 h prior to 50 μg/ml MLB and 10 μM oligomeric Aβ (1–42) treatments. **a** Neuronal viability was detected by MTT assay. **b** Representative confocal images of MAP2 staining showing neuronal morphology under various treatment conditions (*scale bar* = 10 μm). The number of neuronal branches (**c**) and neurite length (**d**) were also measured using ImageJ software. **e**, **f** The levels of cytoplasmic and nuclear NF-κB p65 subunit were detected by Western blot and quantified with ImageJ software. Data of neuronal viability (histograms) were from six independent experiments performed in sextuplicate and are presented as mean ± SEM. Data of neuronal branch number and neurite length (*box-and-whisker plots*) were from over 100 randomly selected neurons and are presented as median and range. Data of Western blot (histograms) were from three independent experiments, and the *bars* indicate mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{**}P < 0.001$, as compared with the group treated with Aβ (1–42) alone; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, as compared with the group treated with MLB plus Aβ (1–42); ANOVA followed by Tukey's post hoc test or nonparametric Kruskal–Wallis test

responses [23]. According to Varadarajan et al. [24], soluble and aggregated Aβ interacts with neuronal membranes, consequently causing lipid peroxidation and protein oxidation, as well as generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Several lines of evidence indicate that there is increased oxidative stress in the AD brain [25]. In addition, Aβ-induced oxidative stress is an initiator of inflammatory response from activated microglia in the brain, which subsequently promotes the release of pro-inflammatory cytokines and augments cell apoptosis, ultimately resulting in the loss of neuronal function [26].

While multiple cellular pathways are known to be involved in Aβ-induced neurotoxicity, the NF-κB pathway is considered to be highly important and has thus been well studied. Although activation of the NF-κB pathway has been found to exert a protective effect in neurons [27], many studies indicate that it contributes to the up-regulation of AD-inducing pro-inflammatory and cytotoxic genes during the degenerative process of disease [28–30]. Taking into consideration these findings, NF-κB can be considered as potential pharmacological targets for Aβ-associated neurotoxicity. In this study, we found that exposure to Aβ (1–42) decreased cell viability and neurite growth, induced apoptosis in FVB mouse primary neurons, and activated the NF-κB pathway. However, these effects were counteracted by pretreatment with MLB in a dose-dependent manner. These results demonstrate the protective activity of MLB against Aβ (1–42)-induced neuronal injury, which may be attributed to its inhibitory effect on the NF-κB signaling cascade.

MLB is the major aqueous compound in Dan Shen. It possesses strong anti-oxidative, anti-apoptotic, and anti-inflammatory effects, which are mediated through multiple signaling pathways including the NF-κB cascade. Cheng et al. [13] reported that in human peripheral T lymphocytes, MLB suppresses IκBα degradation and nuclear translocation of p65 and p50 subunits, as well as it reduces IKK activity, thus inhibiting activation of T lymphocytes upon exposure to PMA plus ionomycin. Jung et al. [31] found that MLB has anti-wrinkle effect and is able to reverse both age- and ultraviolet B-related skin pro-collagen reduction by suppressing the expression and activities of NF-κB and AP-1-dependent matrix-metalloproteinases (MMPs). In line with these findings, in our study, MLB counteracts Aβ (1–42)-induced activation of the NF-κB pathway, which is evidenced by suppressed luciferase activity, decreased expression of phosphorylated IKKα and IκBα, and reduced nuclear translocation of p65 in response to pre-treatment with 50 μg/ml MLB prior to Aβ (1–42) exposure. In addition, we found that MLB was able to reverse the increase in phosphorylated JNK expression induced by Aβ (1–42), but this was not the case for ERK or p38 kinases. Furthermore, the combination of JNK inhibitor SP600125 and MLB could synergistically counteract Aβ (1–42)-induced decrease in cell viability and neurite growth. These results were expected based on previous literature, and provide further evidence for the suppressive effect of MLB on JNK activation in response to Aβ (1–42).

According to recent studies, activation of JNK is likely to be only one of several contributing components of Aβ-related neuronal death [32], and down-regulation of some regulators of neuron survival induced by Aβ may also be involved. Akt is the major neuronal survival promoter that has been implicated in neurite growth and synaptic plasticity. Recent studies suggest that activation of Akt cascade attenuates Aβ-induced apoptosis through the inhibition of glycogen synthase kinase-3 beta (GSK-3β), consequently inhibiting hyperphosphorylation of tau proteins and neurofibrillary formation [33–35]. Magrané et al. [36] reveal that intraneuronal Aβ expression impairs Akt signaling and blunts the stress response. For MLB, Xiao et al. [12] reported that it can prevent the decrease in phosphorylated Akt both in NMDA-injured neurons and KA-injured mouse brain. In line with this finding, we also found that MLB restored the decrease in phosphorylated Akt in Aβ (1–42)-treated neurons and its neuroprotective effects could be attenuated by Akt inhibitor triciribine. These results implicate Akt signaling as a molecular pathway involved in the protective activity of MLB against Aβ (1–42)-induced toxicity.

Interestingly, it should be noted that MLB alone neither has any direct stimulatory effect on survival and neurite growth of cultured neurons, nor directly influences the NF-κB pathway and its upstream regulators. Hence, we assume that there might be a mechanism by which MLB interferes

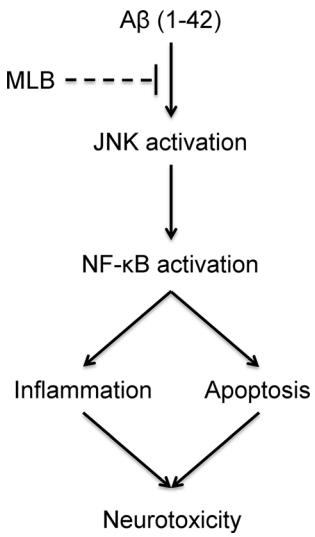

Fig. 8 Hypothetical scheme of the proposed mechanism for the neuroprotective effects of MLB against Aβ (1–42)-induced toxicity

with the interaction between Aβ (1–42) and their putative cellular receptors and/or cell surface neurotransmitters. Nevertheless, further extensive studies are required to examine this assumption in more detail.

In summary, this study shows that MLB has neuroprotective effects against Aβ (1–42)-induced toxicity in primary mouse neurons. Mechanistic studies indicate that the NF-κB pathway is implicated in mediating the neuroprotective effects of MLB (the hypothetical scheme is shown in Fig. 8). These findings demonstrate the efficacy of MLB against neuronal injury caused by Aβ, thus providing supporting evidence for its clinical application in the treatment of AD.

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