

# Quercetin inhibits okadaic acid-induced tau protein hyperphosphorylation through the Ca<sup>2+</sup>-calpain-p25-CDK5 pathway in HT22 cells

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**Abstract.** Alzheimer's disease (AD) is a common neurodegenerative disorder characterized by aberrant tau protein hyperphosphorylation, which eventually leads to the formation of neurofibrillary tangles. Hyperphosphorylated tau protein is considered as a vital factor in the development of AD and is highly associated with cognitive impairment. Therefore, it is recognized to be a potential therapeutic target. Quercetin (QUE) is a naturally occurring flavonoid compound. In the present study, the inhibitory effect of QUE on okadaic acid (OA)-induced tau protein hyperphosphorylation in HT22 cells was explored. Western blotting results indicated that QUE significantly attenuated OA-induced tau protein hyperphosphorylation at the Ser396, Ser199, Thr231 and Thr205 sites. Further experiments demonstrated that QUE inhibited the activity of cyclin-dependent kinase 5 (CDK5), a key enzyme in the regulation of tau protein, and blocked the Ca<sup>2+</sup>-calpain-p25-CDK5 signaling pathway. These observations indicate the ability of QUE to decrease tau protein hyperphosphorylation and thereby attenuate the associated neuropathology. In conclusion, these results support the

potential of QUE as a therapeutic agent for AD and other neurodegenerative tauopathies.

## Introduction

The incidence of Alzheimer's disease (AD) is gradually increasing, and AD has become a major threat to human health (1). Unfortunately, an effective treatment method has not yet been discovered (2,3). AD is a degenerative disease of the central nervous system. The deposition of extracellular amyloid plaques and the formation of intracellular neurofibrillary tangles (NFTs) are its primary pathological features.  $\beta$ -amyloid (A $\beta$ ), a cleavage product of the amyloid precursor protein, is the main component of amyloid plaques (4-6). Tau protein is a microtubule-associated protein closely involved in the maintenance of microtubule stability (7). Tau protein has numerous potential phosphorylation sites, which are mainly serine and threonine residues. The abnormal phosphorylation of tau protein reduces its affinity for microtubules and damages its microtubule assembly capacity (8,9). Furthermore, tau hyperphosphorylation is the dominating cause of the formation of NFTs (7,10). Although A $\beta$  has been the principal focus of AD treatments, since tau phosphorylation has been indicated to be a consequence of A $\beta$  pathology (11), the focus of attention has shifted from A $\beta$  to tau protein (12).

The hyperphosphorylation of tau protein is mainly due to an increase in kinase activity and reduction of phosphatase activity (13,14). Among various kinases associated with this process, cyclin-dependent kinase 5 (CDK5) is considered to be particularly relevant (15). Abnormal CDK5 activity leads to the hyperphosphorylation of tau protein, which contributes to the formation of NFTs (16). A previous study indicated that CDK5 silencing decreased the number of NFTs in transgenic Alzheimer's mice (17). Notably, CDK5 is activated via subunits p35 or p39, and the cleavage of p35 to form p25 may occur due to the action of calpain, the activity of which is dependent upon calcium (18-21). Compared with p35, p25 has a longer half-life; p25/CDK5-binding prolongs the activity of CDK5 and further promotes tau protein hyperphosphorylation, which serves an important role in the development of AD (22). Therefore, blocking the Ca<sup>2+</sup>-calpain-p25-CDK5 pathway has

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**Abbreviations:** AD, Alzheimer's disease; NFTs, neurofibrillary tangles; QUE, quercetin; CDK5, cyclin-dependent kinase 5; A $\beta$ ,  $\beta$ -amyloid; OA, okadaic acid; CALP, calpeptin; ROS, roscovitine

**Key words:** quercetin, Alzheimer's disease, tau protein hyperphosphorylation, cyclin-dependent kinase 5

considerable significance for AD. A previous study has shown that A-705253, a calpain inhibitor, blocks stress-induced tau hyperphosphorylation (23). The restriction of CDK5 activity has an inhibitory effect on the aggregation of NFTs (17,23). In addition, a specific calpain inhibition, calpastatin, has demonstrated the ability to prevent tauopathy and neurodegeneration and restore a normal lifespan in tau P301L mice (24).

Quercetin (QUE) is a natural flavonoid compound that has been shown to exert extensive pharmacological effects, including antioxidant, antitumor, anti-inflammatory (25-27), anti-chemotherapy-induced fatigue (28) and anti-aging effects (29). QUE has been demonstrated to cross the blood-brain barrier and prevent the progression of neurodegenerative diseases (30-33). Numerous traditional Chinese medicines contain QUE, including Japanese pagoda tree flower, *Apocynum venetum* and cattail pollen (34,35). A previous study has indicated that QUE has the ability to reduce A $\beta$ -induced cytotoxicity (36). Additionally, QUE has been revealed to attenuate tauopathy, although the mechanism has not been elucidated (37). Furthermore, QUE has been demonstrated to ameliorate AD pathology and protect cognitive and emotional functions *in vivo* (38,39).

The hippocampus, an important brain structure, is responsible for the strengthening of short-term memories into long-term memories and is closely relevant to AD (40). Okadaic acid (OA) is widely used to block protein phosphatase 2A (PP2A) activity (41,42). PP2A serves a vital role in the development of neurodegenerative disorders via the hyperphosphorylation of tau protein (43,44). Therefore, OA-induced HT22 mouse hippocampal neuronal cells were selected for use in the present study as a model of AD. The effect of QUE pretreatment on tau protein hyperphosphorylation in OA-induced HT22 cells was investigated and the involvement of the Ca<sup>2+</sup>-calpain-p25-CDK5 signaling pathway in the underlying mechanism was evaluated. Natural compounds with fewer side effects are increasingly favored, which have lower toxicity and higher efficacy (45). The present study continues previous studies conducted by the current research team concerning the neuroprotective effects of other natural compounds (46,47).

## Materials and methods

**Materials.** QUE (molecular formula, C<sub>15</sub>H<sub>10</sub>O<sub>7</sub>; molecular weight, 302.24 g/mol; purity, >98.5%; CAS no., 117-39-5) was obtained from Aladdin Industrial Corporation (Shanghai, China). Calpeptin (CALP; CAS no., 117591-20-5), roscovitine (ROS; CAS no., 186692-46-6) and OA (molecular formula, C<sub>44</sub>H<sub>68</sub>O<sub>13</sub>; molecular weight, 805.00 g/mol; purity, >90%; CAS no., 78111-17-8) were all from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Fluo-3 AM (S1056), Super ECL Plus (P1010) and a bicinchoninic acid (BCA) protein assay kit (P0010) were obtained from Beyotime Institute of Biotechnology (Jiangsu, China). Primary antibodies targeting CDK5 (ab40773), calpain-1 (ab28258), tau-5 (ab80579), tau [pS396] (ab32057) and tau [pT231] (ab15559) were from Abcam (Cambridge, UK). Tau-1 primary antibody (MAB3420) was from EMD Millipore (Billerica, MA, USA). Primary antibodies for tau [pS199] (44734G) and tau [pT205] (44738G) were from Invitrogen (Thermo Fisher Scientific,

Inc., Waltham, MA, USA). Primary antibodies for p35/p25 (C64B10) and  $\beta$ -actin (13E5) were from Cell Signaling Technology, Inc. (Danvers, MA, USA). The primary antibody for p-CDK5 (Tyr<sup>15</sup>) (sc-12918) was from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The horseradish peroxidase (HRP)-conjugated secondary antibody was from Wuhan Boster Biological Technology, Ltd. (BA1088; Wuhan, China).

**Cell culture.** The HT22 cells were a generous gift from Dr Jun Liu of the Memorial Hospital of Sun Yat-sen University (Guangzhou, China) (48). The HT22 cells were grown in a humidified incubator with 5% CO<sub>2</sub> and 95% air at 37°C in Dulbecco's modified Eagle's medium (Hyclone DMEM; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (all Gibco; Thermo Fisher Scientific, Inc.).

**Cell treatment.** The HT22 cells were grown in 6-well plate. OA was dissolved in DMSO to a concentration of 1  $\mu$ M as a stock solution. The stock solution was diluted with DMEM to 80 nM prior to use. When the cell density reached 80%, the cells were incubated with QUE (5 or 10  $\mu$ M), CALP (10  $\mu$ M) or ROS (0.16  $\mu$ M) for 24 h prior to exposure to OA (80 nM) for 12 h at 37°C.

**Western blotting.** HT22 cells were harvested following the treatments described above and lysed in ice-cold lysis buffer [1X PBS, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 5 mM EDTA, 0.5% sodium deoxycholate and 1% phenylmethane sulfonyl fluoride] supplemented with phosphatase inhibitor for 30 min. The lysate was centrifuged at 14,000 x g for 20 min at 4°C. The supernatant was collected and its protein content was quantified using the BCA protein assay kit. Samples with equal amounts of protein (40  $\mu$ g) were separated using 10% SDS-PAGE. The proteins were then transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% bovine serum albumin (Sigma-Aldrich) dissolved in 20 ml TBS with 1 ml Tween-20 buffer for 1 h at room temperature. Subsequently, the membranes were incubated overnight at 4°C with primary antibodies against CDK5 (1:2,000), p-CDK5 (1:1,000), tau-5 (1:1,000), tau-1 (1:10,000), tau [pS199] (1:1,000), tau [pT205] (1:1,000), tau [pS396] (1:1,000), tau [pT231] (1:1,000), calpain-1 (1:1,000), p35/p25 (1:1,000) and  $\beta$ -actin (1:1,000). Following this, the membranes were incubated with secondary HRP-conjugated antibody (1:10,000) at room temperature for 1 h. Immunoreactive proteins were detected using Super ECL Plus and exposed to X-ray films. ImageJ 1.410 software (National Institutes of Health, Bethesda, MD, USA) was used to quantitatively analyze the expression levels of the target proteins.

**Reverse transcription-polymerase chain reaction (RT-PCR).** Total RNA was extracted from the HT22 cells in 6-well plates using TRIzol reagent (Thermo Fisher Scientific, Inc.). Spectrophotometry at 260 nm was conducted to determine the amount of RNA extracted. The primers used were as follows: p35 forward, 5'-GCAACGGTCCCAAAGGCTT-3' and reverse, 5'-ACAGCAAGAACGCCAAGGACA-3'; CDK5 forward, 5'-GCATTGAGTTTGGGCACGACA-3' and reverse, 5'-AAAACCGGGAAACCCATGAGA-3';  $\beta$ -actin forward,

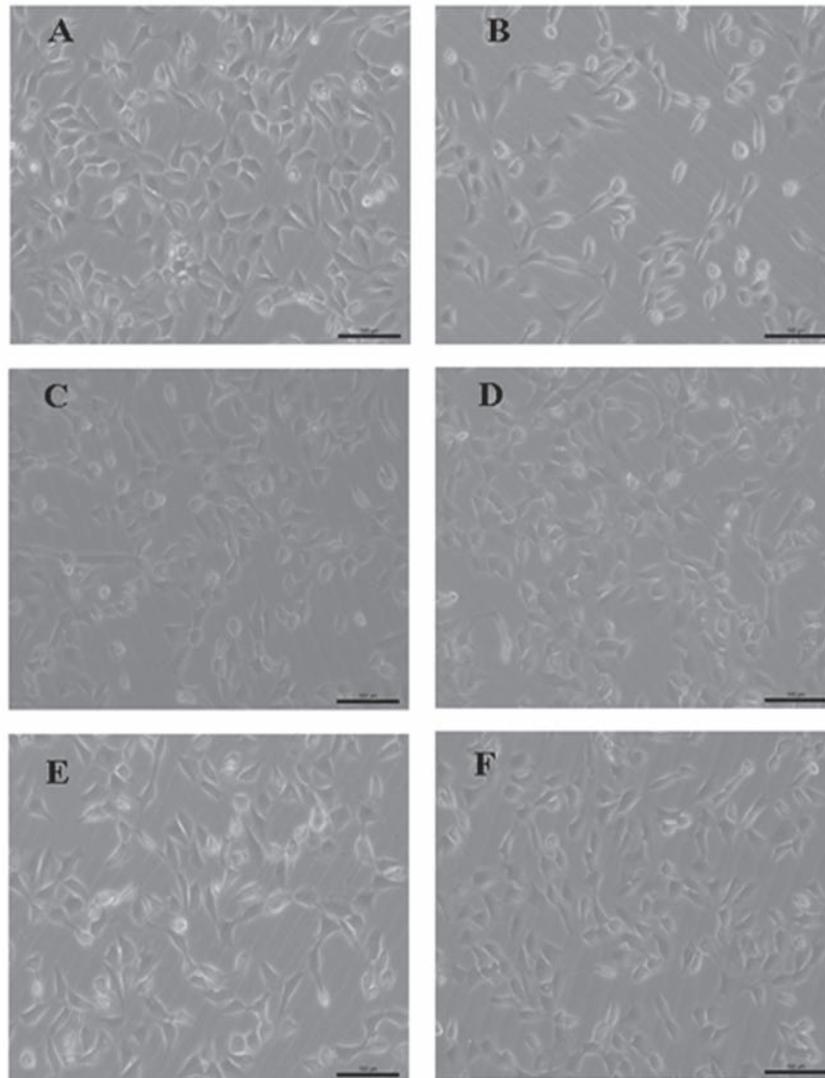


Figure 1. Effects of QUE on OA-induced toxicity in HT22 cells. The morphology of HT22 cells treated with OA and QUE revealed that QUE attenuated the OA-induced HT22 cell injury. (A) Control group; (B) 80 nM OA; (C) 5  $\mu$ M QUE + 80 nM OA; (D) 10  $\mu$ M QUE + 80 nM OA; (E) 10  $\mu$ M calpeptin + 80 nM OA; and (F) 0.16  $\mu$ M roscovitine + 80 nM OA. Scale bar, 100  $\mu$ m (magnification, x200). QUE, quercetin; OA, okadaic acid.

5'-ATGCCATCCTGCGTCTGGACCTGGC-3' and reverse, 5'-AGCATTGCGGTGCACGATGGAGGG-3'. These primers were described previously by Chen *et al* (49). Samples were reverse-transcribed in RT reaction buffer using 400 ng total RNA according to the manufacturer's protocol. The PCR was conducted with template, primers, The RT kit was from Takara Biotechnology Co., Ltd. (Dalian, China). GoTaq Green Master mix and RNase-free dH<sub>2</sub>O added to a volume of 50  $\mu$ l. Amplification was carried out under the following conditions: Initial denaturation at 95°C for 2 min, denaturation at 95°C for 45 sec, annealing at 59°C for 45 sec, extension at 72°C for 1 min, and a final extension step of 72°C for 10 min. The number of PCR cycles was 35. The products were detected in 2% agarose/Tris-acetate-EDTA gels and stained with ethidium bromide for visualization by Syngene G:BOX F2 and GeneTools software.

**Intracellular calcium measurement.** Intracellular calcium was determined by means of flow cytometry following the staining of the cells with the calcium indicator Fluo-3 AM.

Following the various treatments, the cells were collected and cultured with 5  $\mu$ M Fluo-3 AM at 37°C in the absence of light for 45 min. Cell fluorescence was then measured by flow cytometry. When the cell density reached 80%, the cells were incubated with QUE (5 or 10  $\mu$ M), for 24 h prior to exposure to OA (80 nM) for 12 h at 37°C.

**Statistical analysis.** Data are presented as the mean  $\pm$  standard error of the mean. Statistical significance was determined by one-way analysis of variance followed by Duncan's multiple range test using a computerized statistical package (SPSS 16.0).  $P < 0.05$  was considered to indicate a statistically significant difference. All experiments were repeated at least three times.

## Results

**Effects of QUE on OA-induced toxicity in HT22 cells.** The control HT22 cells grew in a healthy condition, with the cell bodies exhibiting good translucency and clear boundaries (Fig. 1A). However, following the exposure of the

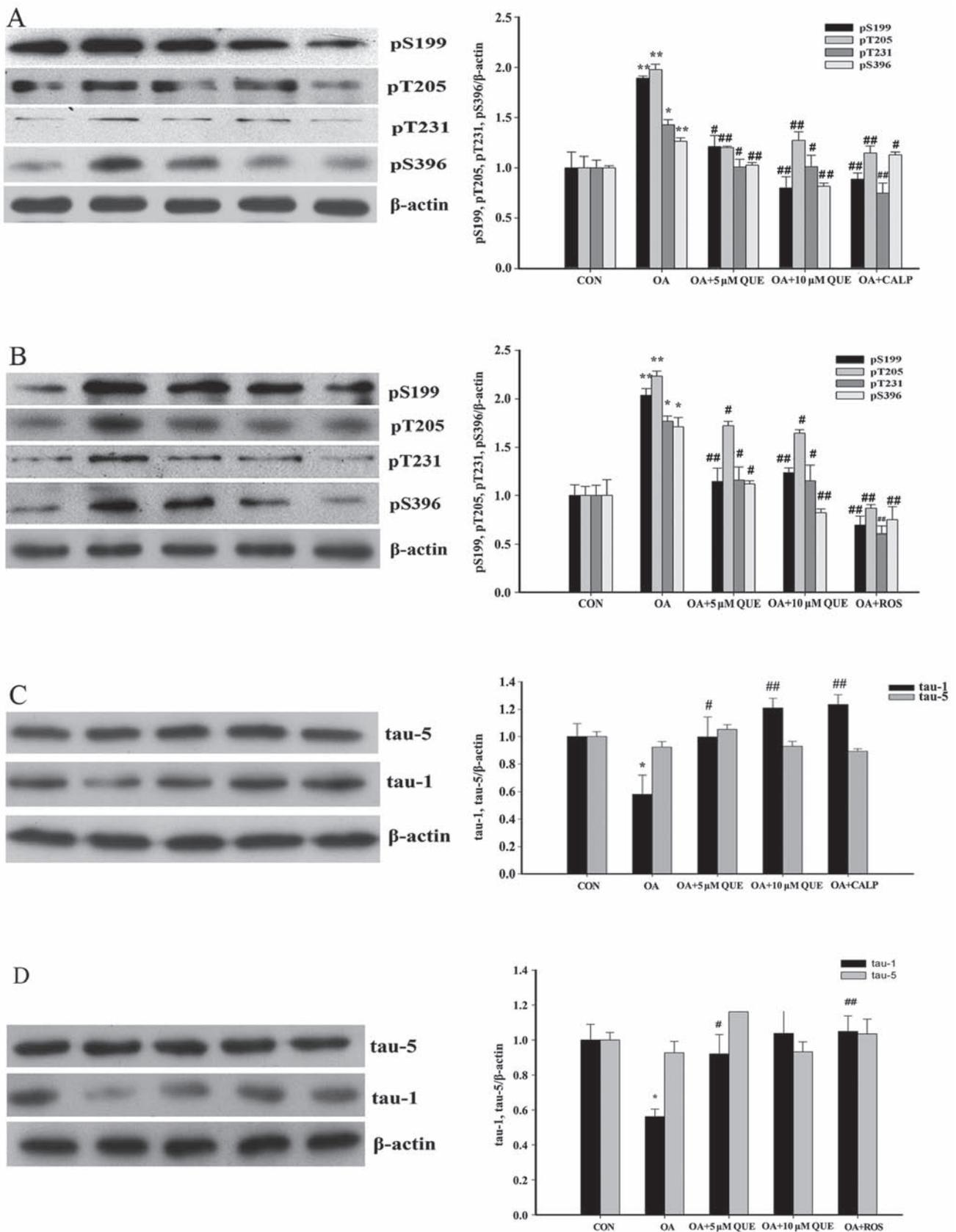


Figure 2. Effects of QUE on OA-induced tau protein hyperphosphorylation in HT22 cells. QUE decreased the OA-induced hyperphosphorylation of tau. HT22 cells were pretreated with QUE (5 and 10 μM), CALP (10 μM) or ROS (0.16 μM) for 24 h prior to OA (80 nM) exposure for 12 h. Western blotting revealed that treatment with OA alone augmented tau hyperphosphorylation at Ser396, Ser199, Thr231 and Thr205 sites. However, pretreatment with QUE, (A) CALP and (B) ROS significantly decreased tau hyperphosphorylation. Total tau protein (tau-5) was not altered among the various groups, whereas the changes in non-phosphorylated tau protein (tau-1) expression induced by OA, QUE, (C) CALP and (D) ROS were the opposite of those on tau hyperphosphorylation. Western blotting data were quantified as densitometry values, normalized using β-actin. Data are expressed as the mean ± standard error of the mean. <sup>a-d,##</sup>P<0.05 and <sup>A-D,##</sup>P<0.01 vs. the OA-treated group; \*P<0.05 and \*\*P<0.01 vs. the CON group. CON, control; QUE, quercetin; OA, okadaic acid; CALP, calpeptin; ROS, roscovitine.

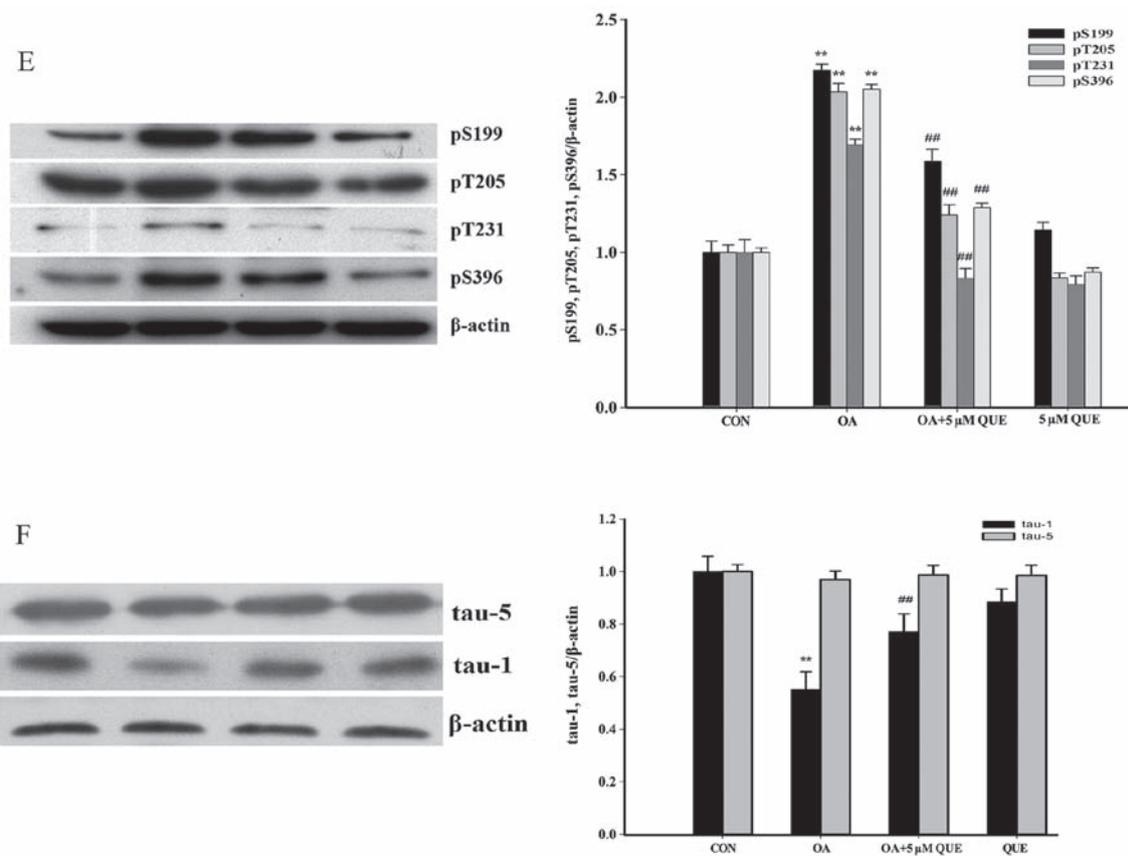


Figure 2. Continued. (E and F) Treatment with QUE alone had no effect on tau phosphorylation. Western blotting data were quantified as densitometry values, normalized using  $\beta$ -actin. Data are expressed as the mean  $\pm$  standard error of the mean. \* $P < 0.01$  vs. CON group; ## $P < 0.01$  vs. OA-treated group. CON, control; QUE, quercetin; OA, okadaic acid.

cells to 80 nM OA for 12 h, cell growth was inhibited, the number of cells was markedly reduced, and the intercellular spaces appeared to be widened (Fig. 1B). Pretreatment with QUE (5 or 10  $\mu$ M), the calpain inhibitor CALP (10  $\mu$ M) or the CDK5 inhibitor ROS (0.16  $\mu$ M) improved the cell morphology and increase the numbers of the OA-treated HT22 cells (Fig. 1C-F).

**Effects of QUE on OA-induced tau protein hyperphosphorylation in HT22 cells.** Tau protein hyperphosphorylation serves a very important role in AD and is a typical pathological feature of this disease (50). Therefore, the effects of QUE on OA-induced tau protein hyperphosphorylation were investigated. Western blotting demonstrated that tau protein hyperphosphorylation at four sites (S199, T205, T231 and S396) was significantly increased following the exposure of HT22 cells to OA (80 nM) for 12 h. However, these increases in phosphorylation were significantly attenuated to varying degrees by pretreatment with 5 or 10  $\mu$ M QUE for 24 h (Fig. 2A and B). In addition, total tau protein and non-phosphorylated tau protein were also investigated. As shown in Fig. 2C and D, total tau protein (tau-5) did not vary significantly among the control, OA and OA plus QUE treatment groups, and the changes in the levels of non-phosphorylated tau (tau-1) were the converse of those for phosphorylated tau, which confirms the consistency of the results.

In order to study the effect of QUE on baseline tau protein phosphorylation, tau phosphorylation levels in HT22 cells

cultured with QUE alone were detected (Fig. 2E and F). However, treatment with QUE culture exhibited no significant effect on tau phosphorylation compared with that in the untreated control group.

CDK5 is vital for tau protein hyperphosphorylation; the augmentation of intracellular  $Ca^{2+}$  levels leads to increased CDK5 activity, which consequently results in tau protein hyperphosphorylation (51). As shown in Fig. 2A and B, tau protein hyperphosphorylation was significantly reduced by pretreatment with 10  $\mu$ M CALP or 0.16  $\mu$ M ROS for 24 h. Furthermore, as shown in Fig. 2C and D, the effects of CALP and ROS on unphosphorylated tau protein in OA-treated HT22 cells were the converse of those on phosphorylated tau, and no significant changes in total tau protein were detected.

These results indicate that QUE markedly attenuated tau protein hyperphosphorylation in OA-induced HT22 cells via the  $Ca^{2+}$ -calpain-p25-CDK5 signaling pathway, and indicate that QUE may exhibit a neuroprotective effect. However, QUE exhibited no effect on normal HT22 cells.

**Effects of QUE on the OA-induced cleavage of p35.** To investigate whether the  $Ca^{2+}$ -calpain-p25-CDK5 signaling pathway is associated with the effects of QUE on OA-induced tau protein hyperphosphorylation, the changes of p25, p35 and CDK5 in OA-induced HT22 cells were explored. As shown in Fig. 3, p35 and CDK5 levels exhibited no significant difference among the groups. However, p25 was significantly increased following treatment with OA for 12 h, and this effect was significantly

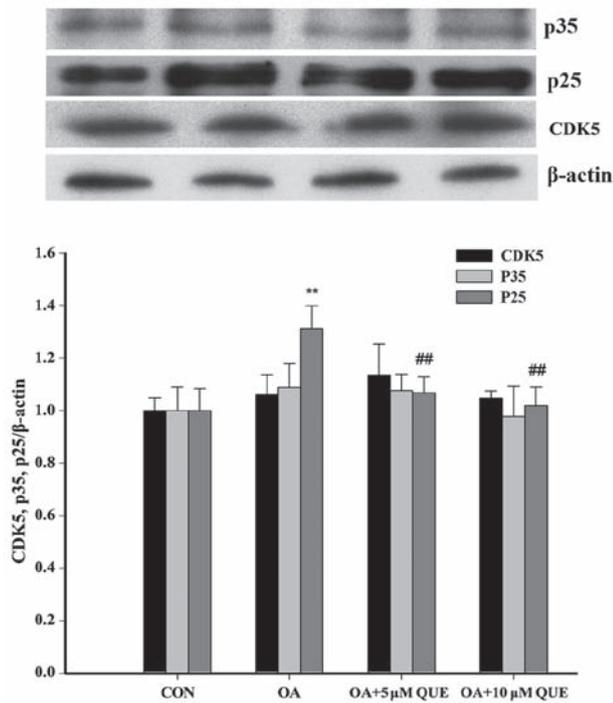


Figure 3. Effects of QUE on the OA-induced cleavage of p35. HT22 cells were pretreated with QUE (5 and 10  $\mu$ M) for 24 h prior to OA (80 nM) exposure for 12 h. The levels of p25 were increased following OA challenge. However, pretreatment with QUE attenuated the increase. Neither p35 nor CDK5 were altered following treatment with QUE and OA. Protein levels were quantified as densitometry values normalized to  $\beta$ -actin. Data are expressed as the mean  $\pm$  standard error of the mean. \*\* $P$ <0.01 vs. CON group; ## $P$ <0.01 vs. OA-treated group. CON, control; QUE, quercetin; OA, okadaic acid; CDK5, cyclin-dependent kinase 5.

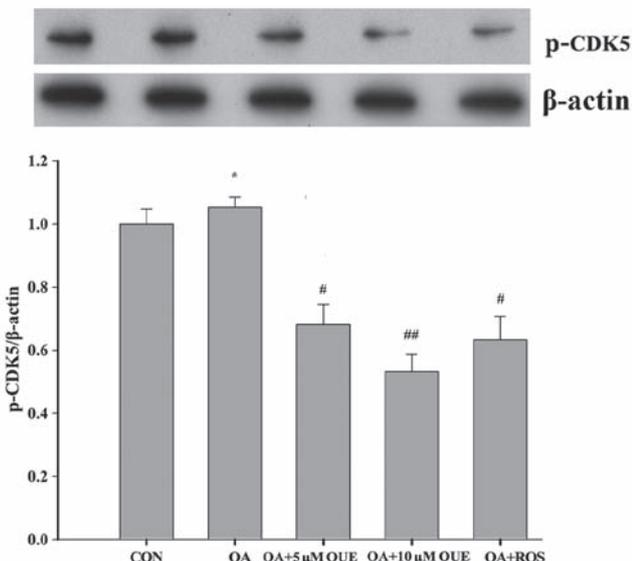


Figure 4. Effects of QUE on OA-induced p-CDK5 expression. QUE inhibited the OA-induced expression of p-CDK5. HT22 cells were pretreated with QUE (5 and 10  $\mu$ M) or ROS (0.16  $\mu$ M) for 24 h prior to OA (80 nM) exposure for 12 h. The expression of p-CDK5 was increased following OA challenge. However, pretreatment with QUE or ROS blocked p-CDK5 expression. The protein level was quantified as densitometry values normalized to  $\beta$ -actin. Data are expressed as the mean  $\pm$  standard error of the mean. \* $P$ <0.05 vs. CON group; # $P$ <0.05 and ## $P$ <0.01 vs. OA-treated group. CON, control; QUE, quercetin; OA, okadaic acid; ROS, roscovitine; p-CDK5, phosphorylated cyclin-dependent kinase 5.

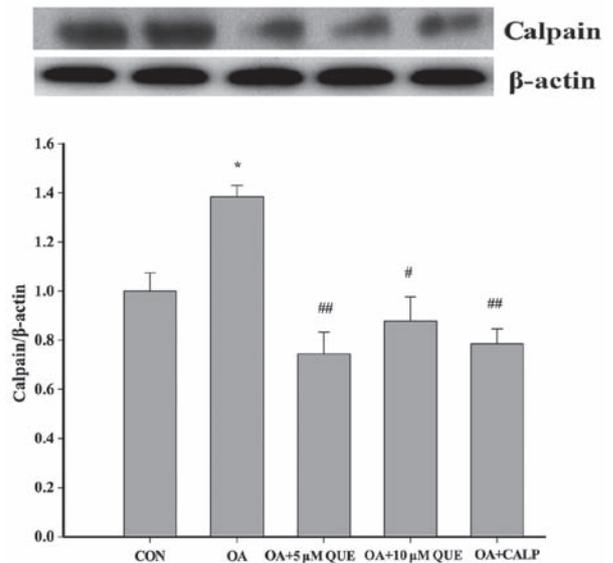


Figure 5. Effects of QUE on OA-induced calpain expression. QUE attenuated OA-induced calpain expression. HT22 cells were pretreated with QUE (5 and 10  $\mu$ M) or CALP (10  $\mu$ M) for 24 h prior to OA (80 nM) exposure for 12 h. Calpain was increased following OA exposure. However, pretreatment with QUE or CALP reduced the protein level of calpain. Protein levels were quantified as densitometry values normalized to  $\beta$ -actin. Data are expressed as the mean  $\pm$  standard error of the mean. \* $P$ <0.05 vs. CON group; # $P$ <0.05 and ## $P$ <0.01 vs. OA-treated group. CON, control; QUE, quercetin; OA, okadaic acid; CALP, calpeptin.

attenuated via pretreatment with QUE. These results indicate that QUE decreased the conversion of p35 into p25.

**Effects of QUE on OA-induced p-CDK5 expression.** p-CDK5, the active form of CDK5, is involved in the aggregation of phosphorylated tau protein (52). Therefore, the expression of p-CDK5 in the OA-induced HT22 cells was investigated. As shown in Fig. 4, p-CDK5 expression was increased significantly following exposure to OA for 12 h. However, this increase was significantly attenuated by pretreatment with 5 or 10  $\mu$ M QUE, or 0.16  $\mu$ M ROS for 24 h. These results are in accordance with the variations in p25 expression, and suggest that QUE reduced tau protein hyperphosphorylation by attenuating the cleavage of p35 and downregulating CDK5 activity.

**Effects of QUE on OA-induced calpain expression.** As p35 is converted to p25 by calpain, a calcium-dependent protease, the possibility that QUE may affect calpain expression was investigated. As shown in Fig. 5, calpain expression was significantly augmented following treatment with OA for 12 h. However, this increase was significantly attenuated by pretreatment with 5 or 10  $\mu$ M QUE or 10  $\mu$ M CALP for 24 h. These results indicate that QUE inhibited tau protein hyperphosphorylation, which was associated with a reduction of calpain expression.

**Effects of QUE on OA-induced p35 and CDK5 mRNA.** To further study the mechanism by which QUE decreased tau protein hyperphosphorylation via the CDK5 signaling pathway, the expression of p35 and CDK5 mRNA was examined. The exposure of HT22 cells to OA caused a significant increase in p35 mRNA, which was significantly blocked by pretreatment with 5 or 10  $\mu$ M QUE. However, treatment with

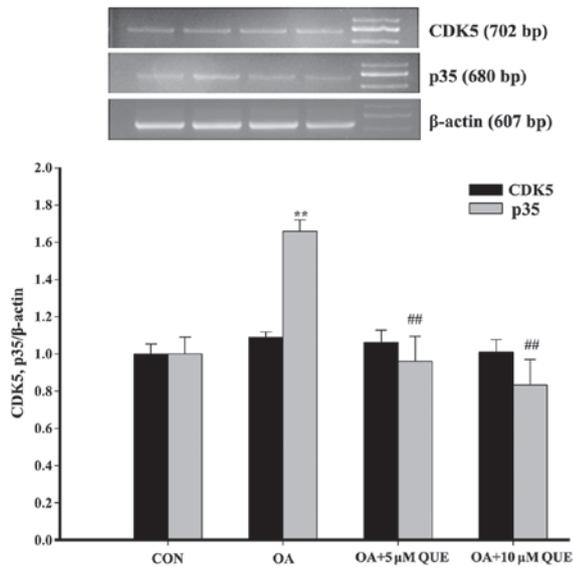


Figure 6. Effects of QUE on OA-induced p35 and CDK5 mRNA. QUE affected the mRNA levels of p35 but not those of CDK5. HT22 cells were pretreated with QUE (5 and 10  $\mu$ M) for 24 h prior to OA (80 nM) exposure for 12 h. The mRNA level of p35 was elevated following OA challenge, and this increase was reversed by pretreatment with QUE. The mRNA levels are densitometry values normalized to  $\beta$ -actin. Data are expressed as the mean  $\pm$  standard error of the mean. \*\* $P$ <0.01 vs. CON group; ## $P$ <0.01 vs. OA-treated group. CON, control; QUE, quercetin; OA, okadaic acid; CDK5, cyclin-dependent kinase 5.

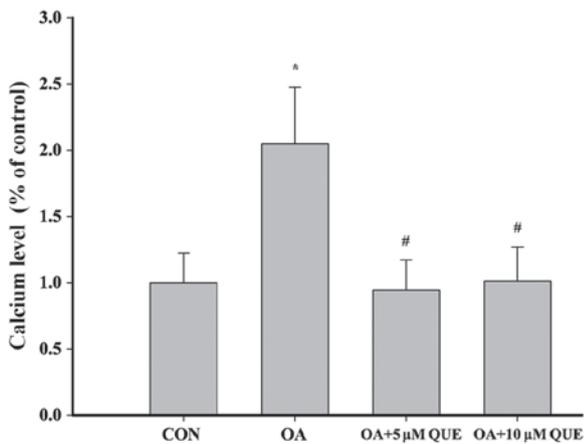


Figure 7. Effects of QUE on OA-induced intracellular calcium level. HT22 cells were pretreated with QUE (5 and 10  $\mu$ M) for 24 h prior to OA (80 nM) exposure for 12 h. The calcium level was enhanced following treatment with OA. Pretreatment with QUE attenuated the increase in intracellular calcium. Data are expressed as mean  $\pm$  standard error of the mean. \* $P$ <0.05 vs. CON group; # $P$ <0.05 vs. OA-treated group. CON, control; QUE, quercetin; OA, okadaic acid.

OA alone or with QUE pretreatment exhibited no significant effect on CDK5 mRNA (Fig. 6).

*Effects of QUE on the OA-induced intracellular  $Ca^{2+}$  level.* Calcium influx activates calpain, which has been reported as a factor in AD pathogenesis (18). Fig. 7 indicates that exposing HT22 cells to OA (80 nM) for 12 h caused a significant increase in intracellular  $Ca^{2+}$  levels. This increase was significantly attenuated by pretreatment with 5 or 10  $\mu$ M QUE.

## Discussion

Hyperphosphorylated tau protein is an essential component of NFTs, which are a major pathological factor in AD (12). Thus, tau protein hyperphosphorylation is a potential therapeutic target. Furthermore, it has been reported that tau pathology has a greater effect than amyloid burden on the clinical symptoms associated with AD; magnetic resonance imaging and electroencephalography indicate that  $A\beta$  deposition is relevant to functional network destruction whereas hyperphosphorylated tau directly affects memory deficits and cognition (53). Therefore, the inhibition of tau protein hyperphosphorylation is an important aim in AD. The present study provides evidence that QUE defended HT22 cells from OA-induced neurotoxicity by reducing tau protein hyperphosphorylation. During the study, it was observed that QUE reduced OA-induced tau protein hyperphosphorylation at Ser396, Ser199, Thr231 and Thr205 sites. However, no evident difference between 5 and 10  $\mu$ M QUE was observed, with the exception of the Thr231 site, where QUE (10  $\mu$ M) exhibited a stronger effect. Additionally, CALP and ROS, which are specific inhibitors of calpain and CDK5, respectively, decreased OA-induced tau protein hyperphosphorylation. Furthermore, none of the treatments affected the total tau levels. However, unphosphorylated tau levels were reduced by treatment with OA alone, and the reduction was attenuated by pretreatment with QUE, CALP or ROS. These results indicate that QUE effectively reduced tau hyperphosphorylation without changing the levels of total tau protein.

The aberrant phosphorylation of tau due to overactivated CDK5 activity is considered a major pathological mechanism in the development of AD (54). CDK5 serves as an upstream signaling molecule in the regulation of tau protein hyperphosphorylation, and is an important determinant of the state of tau protein (20). Therefore, downregulating CDK5 kinase activity is a potential target for AD. In the present study, whether the effect of QUE on hyperphosphorylated tau is attributable to downregulated CDK5 kinase activity was investigated. CDK5 activation was further examined by detecting the phosphorylation of CDK5 at Tyr<sup>15</sup>, which represents the activity of CDK5. Western blotting results demonstrated that the phosphorylation of CDK5-Tyr<sup>15</sup> was attenuated by pretreatment with QUE or ROS. This indicates that QUE affected the activity of CDK5, and demonstrates the potential of QUE as a CDK5 inhibitor for use in AD.

As mentioned above, CDK5 activity is dependent on the p35 or p39 subunits of the enzyme, and the former can be cleaved to form p25. Therefore, CDK5 activity may be blocked by decreasing p35 or p25, which should consequently reduce tau protein hyperphosphorylation. Thus, the expression of p35, p25 and CDK5 was investigated in HT22 cells following exposure to OA. OA caused a significant increase in p25 levels, and pretreatment with QUE blocked the conversion of p35 to p25, leading to a reduction of hyperphosphorylated tau protein levels. Notably, the expression of CDK5 exhibited no difference among the groups. Based on these results, it may be concluded that QUE restrained OA-induced tau hyperphosphorylation by inhibiting the cleavage of p35 to p25 without affecting CDK5 expression. Additionally, the cleavage of p35 to p25 requires calpain, which is activated by intracellular calcium (55,56).

To better explain the molecular mechanism by which QUE attenuated tau protein hyperphosphorylation, calpain expression was explored in the present study. Calpain expression was significantly increased following exposure to OA. However, this increase was attenuated by pretreatment with QUE. The positive control, CALP, also reduced calpain expression. Furthermore, intracellular calcium levels were evaluated, and the changes observed were in accordance with those for calpain. These findings demonstrate that QUE inhibited the OA-induced increases in calpain expression and intracellular calcium levels. Furthermore, the results indicate that QUE blocked the Ca<sup>2+</sup>-calpain-p25-CDK5 signaling pathway, and thus may be an effective treatment for AD.

To further elucidate the molecular mechanism by which QUE modulates the Ca<sup>2+</sup>-calpain-p25-CDK5 signaling pathway, p35 and CDK5 mRNA levels were detected. Notably, only p35 mRNA exhibited any changes when different treatments were applied. Thus, it appears that QUE reduced CDK5 activity by inhibiting the expression of p35, thereby decreasing the conversion of p35 to p25. This demonstrates that p35 mRNA is an effective target for QUE. Together, the results indicate that QUE acted on various targets in the Ca<sup>2+</sup>-calpain-p25-CDK5 signaling pathway to downregulate tau protein hyperphosphorylation.

In conclusion, the results of the present study suggest that QUE exhibited a marked neuroprotective effect on OA-induced HT22 cells by inhibiting the hyperphosphorylation of tau protein, and this effect may have been mediated via inhibition of the Ca<sup>2+</sup>-calpain-p25-CDK5 signaling pathway. This demonstrates that QUE is a potential therapeutic candidate for the prevention of tau protein hyperphosphorylation. Collectively, these findings expand our knowledge of the neuroprotective mechanism of QUE. However, the present study was restricted to *in vitro* experiments, and future studies to investigate the effect of QUE on transgenic mouse are planned. These may support the use of QUE as an effective therapeutic agent for AD and other tauopathies.

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