

Rapamycin inhibits lipopolysaccharide-induced neuroinflammation *in vitro* and *in vivo*

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Abstract. Alzheimer's disease (AD) is the most common type of progressive neurodegenerative disorder, and is responsible for the most common form of dementia in the elderly. Inflammation occurs in the brains of patients with AD, and is critical for disease progression. In the present study, the effects of rapamycin (RAPA) on neuroinflammation lipopolysaccharide (LPS)-induced were investigated. SH-SY5Y human neuroblastoma cells were treated with 20 $\mu\text{g/ml}$ LPS and 0.1, 1 or 10 nmol/l RAPA, and were analyzed at various time points (6, 12 and 24 h). The mRNA expression levels of interleukin (IL) 1 β , IL6 and hypoxia-inducible factor 1 α (HIF1 α) were determined using reverse transcription-quantitative polymerase chain reaction. The protein expression levels of phosphorylated (p-)S6, p-nuclear factor κB (NF κB), p-inhibitor of NF κB kinase subunit β (IKK β) and p-tau protein were measured by western blot analysis. p-IKK β , p-NF κB , p-S6 and p-tau were significantly decreased at 6, 12 and 24 h when cells were treated with ≥ 0.1 nmol/ml RAPA. In addition, female Sprague Dawley rats were intracranially injected with a single dose of 100 $\mu\text{g/kg}$ LPS in the absence or presence of 1 mg/kg RAPA pretreatment. Brain tissues were subjected to immunohistochemical analysis 6-24 h later, which revealed that the expression levels of HIF1 α and p-S6 in rat cerebral cortex were increased following LPS injection; however, this increase was abrogated by RAPA treatment. RAPA may therefore be considered a potential

therapeutic agent for the early or emergency treatment of neuroinflammation.

Introduction

Alzheimer's disease (AD) is characterized clinically by progressive memory loss and cognitive decline (1). In the United States, ~5 million people have AD and with an aging population, ~16 million people are predicted to be diagnosed with AD by middle of the century in the US alone (2). Neuropathological features of AD include extracellular aggregation of amyloid- β (A β) and intraneuronal neurofibrillary tangles (aggregates of tau composed primarily of hyperphosphorylated tau) (3). In addition, inflammation occurs in the brains of patients with AD. In primary microglial and astrocyte cell cultures obtained from postmortem AD patients and nondemented elderly (ND) controls, and in neuroblastoma cell lines, the plaque-associated cytokines interleukin (IL) 1 β , IL6 and hypoxia-inducible factor 1 α (HIF1 α) stimulated the production of complement component (C) 1 s and C1r as a result of direct binding and activation of C1 by A β (4). Furthermore, patients with no history of dementia who exhibit sufficient limbic A β deposits and neurofibrillary tangles at autopsy to qualify for a diagnosis of AD, reveal levels of inflammatory markers that are significantly greater than in typical ND controls but markedly reduced compared with AD patients.

The mammalian target of rapamycin (mTOR) is a phosphatidylinositol kinase-related serine-threonine kinase (5) that modulates the activity of ribosomal S6, nuclear factor κB (NF κB) and inhibitor of NF κB kinase subunit β (IKK β) via phosphorylation. As a consequence, mTOR initiates translation and affects cell growth and proliferation. Rapamycin (RAPA) is a macrolide antibiotic that was initially developed as an antifungal agent, which has potent immunosuppressive and antiproliferative properties (5), and is currently used to prevent rejection in organ transplantation, particularly lung and kidney (6). Lipopolysaccharide (LPS), which is a component of the outer membrane of Gram-negative bacteria, activates glial cells, inducing inflammation in the periphery and in the central nervous

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Key words: rapamycin, lipopolysaccharide, neuroinflammatory, mammalian target of rapamycin

Table I. Primer sequences.

Target gene	GenBank accession no.	Sequence (5'-3')		Product size (bp)
		Forward	Reverse	
GAPDH	NM_017008.4	CAGGGCTGCCTTCTCTTGTG	GATGGTGATGGGTTTCCCGT	172
IL6	NM_012589.2	AGAGACTTCCAGCCAGTTGC	AGTCTCCTCTCCGGACTTGT	85
IL1 β	NM_031512.2	CCTTGTCGAGAATGGGCAGT	TTCTGTGCGACAATGCTGCCT	222
HIF1 α	NM_024359.1	AGCAATTCTCCAAGCCCTCC	TTCATCAGTGGTGGCAGTTG	111

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; HIF1 α , hypoxia-inducible factor 1 α ; bp, base pairs.

system (CNS) (7). A local injection of LPS has been demonstrated to induce inflammation in numerous tissue types (8,9).

The present study examined the effects of RAPA on the LPS-stimulated inflammatory response in SH-SY5Y human neuroblastoma cells. In addition, the effects of RAPA treatment, acting via mTOR signaling pathways on traumatic brain injury recovery in a rat model of neuroinflammation, were investigated.

Materials and methods

RAPA preparation. RAPA (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in deionized water (10 mg/ml) and stored at -20°C. For *in vivo* experiments, this stock solution was diluted to 1 mg/ml immediately prior to intragastric administration. Rats in the treatment group received RAPA (1 mg/kg) and control animals received normal saline (NS), 30 min prior to surgery (10).

Cell culture and drug treatment. SH-SY5Y human neuroblastoma cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured at 37°C in a humidified incubator containing 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 15% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 2 mM L-glutamine. The cells were maintained in the logarithmic phase of growth and subcultured every 3-4 days. Prior to experimentation, the medium was removed and replaced with 0.01% trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco; Thermo Fisher Scientific, Inc.), which had been warmed to 37°C. The cell layer was completely covered and incubated at 37°C for ~3 min to allow detachment of the cells. Subsequently, complete medium (containing 0.02-0.03% trypsin) was added and the cell suspension was transferred to a clean tube. The cells were centrifuged at 200 x g for 5 min at room temperature, the supernatant was discarded and the cells were resuspended in fresh medium. Experiments were performed 24 h later. The medium was removed and replaced with medium supplemented with FBS containing LPS (20 μ g/ml) or LPS (20 μ g/ml) and RAPA (0.1, 1.0 or 10 nmol/ml). Cells were incubated for 6, 12 or 24 h, and were

subsequently digested with trypsin for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis.

RT-qPCR. Total RNA samples were isolated from SH-SY5Y cells using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The total RNA products (1 μ g) were immediately transcribed into cDNA using a PrimeScript[™] RT Reagent kit with Genomic DNA Eraser (Takara Biotechnology Co., Ltd.). qPCR was performed on a Chromo4 Four-Color Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using the SYBR[®] Premix Ex Taq[™] II (Tli RNaseH Plus) kit (Takara Biotechnology Co., Ltd.). Cycling conditions were as follows: Initial denaturation at 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec, 60°C for 20 sec and 72°C for 15 sec. Data was normalized using the 2^{- $\Delta\Delta$ C_q} method (11). Primers were synthesized by Thermo Fisher Scientific, Inc. (Table I).

Western blot analysis. Cells were trypsinized, washed twice with ice-cold phosphate-buffered saline, lysed in radioimmunoprecipitation assay buffer (20 mmol/l Tris-HCl, 150 mmol/l NaCl, 1 mmol/l EDTA, 1% NP-40, 1 mmol/l Na₃VO₄, 1 mmol/l phenylmethylsulfonyl fluoride, 5 μ g/ml leupeptin, 5 μ g/ml aprotinin and 5 μ g/ml chymostatin; pH 7.4) and were centrifuged at 3,000 x g for 5 min at room temperature. Lysates were clarified and the protein concentrations were determined according to the method of Lowry *et al* (12), using bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc.) as the standard. Cellular proteins (20 μ g) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST) for 2 h at room temperature. Subsequently, membranes were probed with the following primary antibodies, diluted 1:1,000, overnight at 4°C: rabbit anti-NF- κ B (catalog no. 8242), rabbit anti-phosphorylated (p)-NF- κ B (catalog no. 3033), rabbit anti-IKK β (catalog no. 8943), rabbit anti-p-IKK β (catalog no. 2694), rabbit anti-p-tau (catalog no. 15013), rabbit anti-p-S6 (catalog no. 4858) and rabbit anti- β -actin (catalog

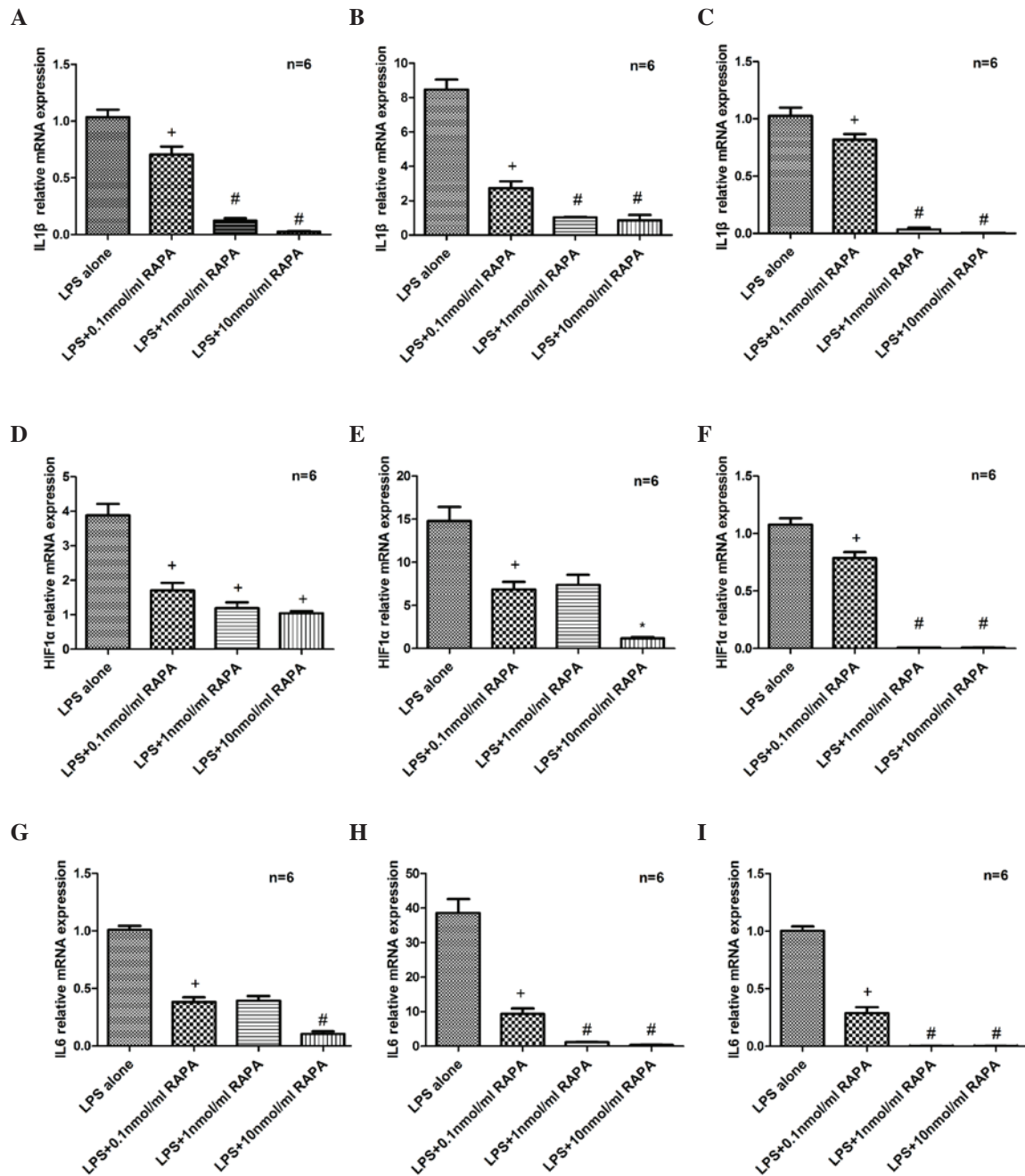


Figure 1. Effects of RAPA on IL1 β , HIF1 α and IL6 mRNA expression. Total RNA was prepared from SH-SY5Y cells pretreated with LPS (20 μ g/ml) for 30 min and exposed to RAPA (0-10 nmol/ml) for 6, 12 and 24 h. RT-qPCR analysis of IL1 β following (A) 6, (B) 12 and (C) 24 h of RAPA treatment. RT-qPCR analysis of HIF1 α following (D) 6, (E) 12 and (F) 24 h of RAPA treatment RT-qPCR analysis of IL6 following (G), (H) 12 and (I) 24 h of RAPA treatment. Data are presented as fold-change vs. the LPS alone group. Each sample was measured in triplicate and the experiment was repeated twice with similar results. Data are expressed as the mean \pm standard error from independent experiments. * P <0.05 vs. LPS alone; # P <0.05 vs. 0.1 nmol/ml RAPA; and $^{\#}P$ <0.05 vs. 1 nmol/ml RAPA. RAPA, rapamycin; IL, interleukin; HIF1 α , hypoxia-inducible factor 1 α ; LPS, lipopolysaccharide; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

no. 8457), purchased from Cell Signaling Technology, Inc., (Danvers, MA, USA). The membranes were then washed three times for 7 min with TBST and incubated with an anti-rabbit IgG conjugated to horseradish peroxidase (1:5,000; catalog no. 7074; Cell Signaling Technology, Inc.) for 1 h at room temperature. Protein bands were visualized using SignalFire™ Elite Enhanced Chemiluminescence reagent (Cell Signaling Technology, Inc.). The results were

normalized to β -actin using Image J software version 1.48 (National Institutes of Health, Bethesda, MD, USA).

Animals and drug treatment. Ethical approval was provided by the Medical Ethics Committee of Southern Medical University (Guangzhou, China). Female Sprague Dawley (SD) rats (n=18; age, 6 weeks; weight, 170-180 g), were purchased from Southern Medical University. Rats were

maintained at a constant humidity and temperature under a 12-h light/dark cycle with *ad libitum* access to food and water. The rats were randomly assigned to sham, LPS and LPS+RAPA groups (n=6/group). In the LPS + RAPA group, RAPA (1 mg/kg) was administered by gavage 30 min prior to surgery. Neuroinflammation was induced as described by Espinosa-Oliva *et al.* (13). Rats were anesthetized with an intraperitoneal injection of 250 mg/kg ketamine (Zoetis, Inc., Florham Park, NJ, USA). Sterile techniques were used and the left lateral ventricle was reached by stereotaxic surgery. With the incisor bar placed 3.3 mm below the interaural line (horizontal zero), the coordinates with respect to bregma for the guide cannula were 1.0 mm anteroposterior, 1.5 mm lateral and 3.8 mm dorsoventral. LPS (100 µg/kg) was then injected intracranially in the LPS and LPS+RAPA groups. The sham group underwent surgery but received no treatment.

Immunohistochemistry. Rats were anesthetized using 250 mg/kg ketamine and 2% xylazine (10 mg/kg, Sigma-Aldrich) for a duration of 6, 12 or 24 h following surgery, and were subsequently sacrificed by rapid decapitation. The collected brain tissues were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 8 h at 0°C. Fixed brains were paraffin embedded and cut into 2 µm sections using a microtome (Leica Microsystems GmbH, Wetzlar, Germany). Sections were briefly washed with TBS and incubated for 30 min in 3% H₂O₂ to quench endogenous peroxidase activity. The following primary antibodies: Rabbit anti-HIF1α (1:100; catalog no. sc-10790; Santa Cruz Biotechnology, Inc., Dallas; Texas; U.S.A) and rabbit anti-p-S6 (1:100; catalog no. 4858; Cell Signaling Technology, Inc.) were applied overnight at 4°C. Subsequently, sections were washed three times with TBS and then incubated with SignalStain® Boost Immunohistochemistry Detection reagent (1:50; catalog no. 8114; Cell Signaling Technology, Inc.) for 1 h at room temperature. Sections were then incubated with 3,3'-diaminobenzidine (ZSGB-BIO, Beijing, China). Finally, the sections were dehydrated, mounted and examined under a light microscope (Leica Microsystems GmbH). Positive cells were counted using Image-Pro Plus software version 7.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Histological assays. Paraffin sections were stained with hematoxylin and eosin (H&E) according to the manufacturer's protocol. The cells which were swollen or were abnormally circular cells were defined as positive cells, and these cells were counted using Image-Pro Plus software version 7.0.

Statistical analysis. Data are representative of a minimum of three independent experiments, each of which were performed in triplicate. Statistical analyses were conducted using GraphPad Prism software, version 3.0 (GraphPad Software, Inc., La Jolla, CA, USA). Data were analyzed using a one-way analysis of variance followed by Tukey's *post hoc* test. Data are expressed as the mean ± standard error. P<0.05 was considered to indicate a statistically significant difference.

Results

RAPA attenuates LPS-induced increases in *IL1β*, *HIF1α* and *IL6* mRNA expression levels. SH-SY5Y cells were cultured with 20 µg/ml LPS and 0.1-10 nmol/ml RAPA for 6, 12 or 24 h. RT-qPCR revealed that *IL1β* mRNA expression was significantly reduced following treatment with RAPA compared with the LPS only group, at 6 (P=0.029; Fig. 1A), 12 (P=0.0011; Fig. 1B) and 24 h (P=0.003; Fig. 1C). In addition, *HIF1α* (P=0.0229 at 6 h, P=0.0005 at 12 h and P=0.0001 at 24 h vs. LPS alone; Fig. 1D-F) and *IL6* (P=0.015 at 6 h, P=0.005 at 12 h and P=0.0001 at 24 h vs. LPS alone; Fig. 1G-I) mRNA expression levels were significantly reduced in the RAPA groups compared with the LPS only group.

RAPA attenuate LPS-induced increases in p-S6, p-tau, p-NFκB and p-IKKβ. SH-SY5Y cells were treated with 20 µg/ml LPS and 0.1-10.0 nmol/ml RAPA for 6, 12 or 24 h. Western blot analysis (Fig. 2A and B) revealed that expression levels of p-NFκB decreased gradually with increasing concentrations of RAPA at 6 (P=0.0025; Fig. 2C), 12 (P=0.0011; Fig. 2D) and 24 h (P=0.0014; Fig. 2E). A similar pattern was observed for p-IKKβ (P=0.0125 at 6 h, P=0.0115 at 12 h and P=0.001 at 24 h vs. LPS alone; Fig. 2F-H), p-tau (P=0.019 at 6 h, P=0.025 at 12 h and P=0.003 at 24 h vs. LPS alone; Fig. 2I-K) and p-S6 (P=0.005 at 6 h, P=0.001 at 12 h and P=0.0005 at 24 h vs. LPS alone; Fig. 2L-N). At 6 h, suppression of p-NFκB (Fig. 2C), p-IKKβ (Fig. 2F) and p-S6 (Fig. 2L) began and appeared to be dose-dependent. In addition, the inhibitory effect on p-NFκB lasted for 12 h (Fig. 2D), and even 24 h (Fig. 2E). Similarly, expression of p-IKKβ was decreased at 12 (Fig. 2G) and 24 h (Fig. 2H). Therefore, critical components of the NFκB signaling pathway, p-NFκB and p-IKKβ, were suppressed by RAPA and the neuroinflammation protein, p-tau was decreased. In addition, the downstream protein of the mTOR signaling pathway, p-S6 was downregulated by RAPA.

RAPA increases the anti-inflammatory properties of neurons. Female SD rats were randomly divided into sham, LPS and LPS + RAPA groups. Cerebral cortex sections stained with H&E revealed that treatment with LPS caused cells to become swollen and abnormally circular, in a time-dependent manner (P=0.029 at 6 h, P=0.018 at 12 h and P=0.003 at 24 h vs. sham; Fig. 3A-D); this damage was abrogated by RAPA (P=0.009 at 6 h, P=0.001 at 12 h and P=0.0002 at 24 h vs. LPS; Fig. 3A-D). The swollen and abnormally circular cells within sections were counted to quantify the LPS-induced inflammation. The inflammatory response in the neurons increased in the LPS group; however, this increase was reduced over time in the LPS + RAPA group [6 (Fig. 3B), 12 (Fig. 3C) and 24 h (Fig. 3D)]. Detection of HIF1α (Fig. 4A) and p-S6 (Fig. 4B) by immunohistochemistry determined that expression levels of HIF1α in rat cerebral cortex increased following LPS injection (P=0.0021 at 6 h, P=0.0011 at 12 h and P=0.00022 at 24 h vs. sham; Fig. 4C-E); however, this increase was abrogated over time by RAPA treatment, at 6 (P=0.001 vs. LPS; Fig. 4C), 12 (P=0.005 vs. LPS; Fig. 4D) and 24 h (P=0.003 vs. LPS; Fig. 4E). A similar pattern was

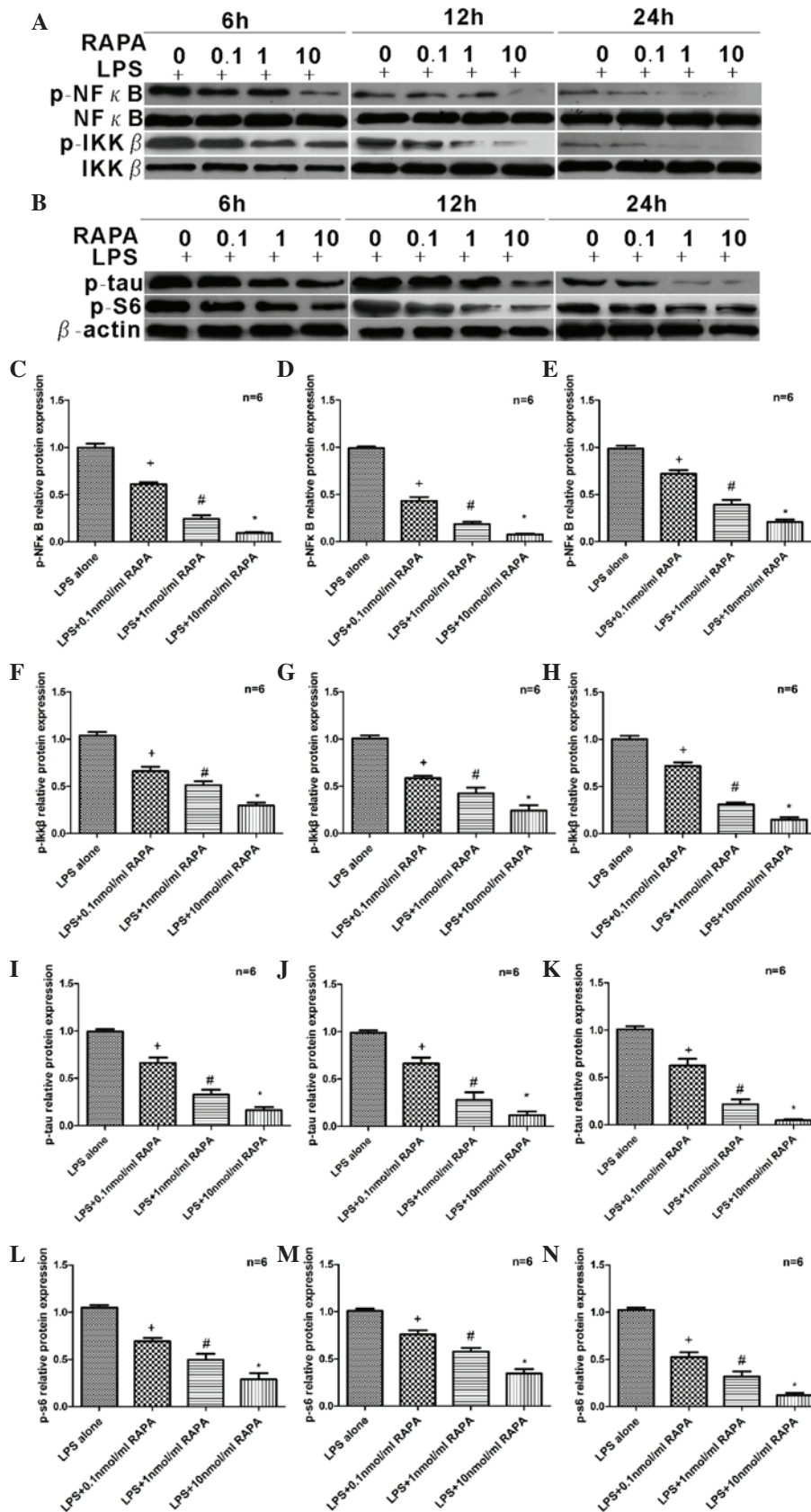


Figure 2. Effects of RAPA on protein expression levels of p-NFκB, p-IKKβ, p-tau and p-S6. (A and B) Proteins extracted from SHSY5Y cells pretreated with 20 μg/ml LPS for 30 min and exposed to 0-10 nmol/ml RAPA for 6, 12 and 24 h were subjected to western blot analysis. Protein expression levels of p-NFκB were analyzed following (C) 6, (D) 12 and (E) 24 h of RAPA treatment. Protein expression levels of p-IKKβ were analyzed following (F) 6, (G) 12 and (H) 24 h of RAPA treatment. Protein expression levels of p-tau were analyzed following (I) 6, (J) 12 and (K) 24 h of RAPA treatment. Protein expression levels of p-S6 were analyzed following (L) 6, (M) 12 and (N) 24 h of RAPA treatment. Protein expression levels of all proteins examined decreased following RAPA treatment, in a dose-dependent manner, at all time points. Data are presented as fold-change vs. the LPS alone group. Each sample was measured in triplicate and the experiment was repeated twice with similar results. Data are expressed as the mean ± standard error from independent experiments. *P<0.05 vs. LPS alone; #P<0.05 vs. 0.1 nmol/ml RAPA; and +P<0.05 vs. 1 nmol/ml RAPA. RAPA, rapamycin; p-, phosphorylated; NFκB, nuclear factor κB; IKKβ, inhibitor of NFκB kinase subunit β; LPS, lipopolysaccharide.

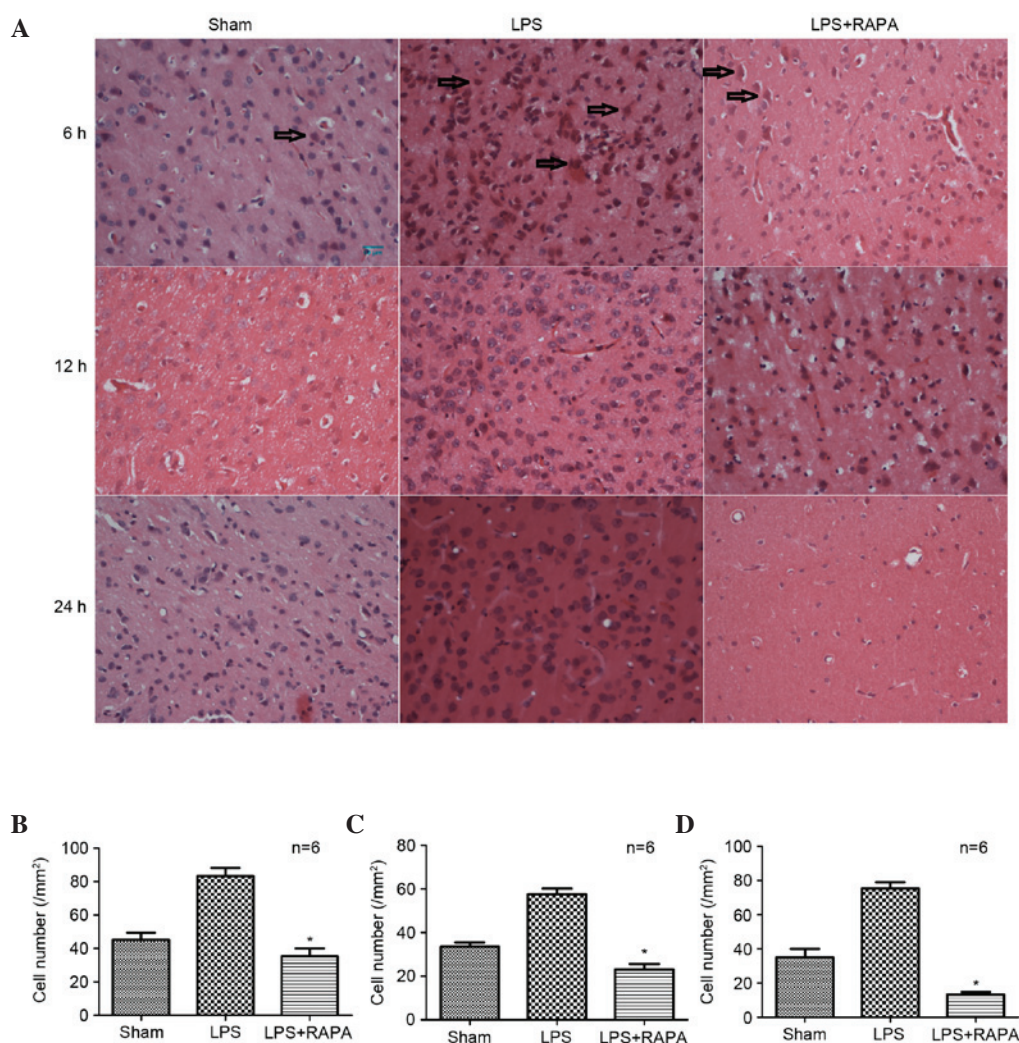


Figure 3. H&E staining of rat cerebral cortex following LPS injection in the absence or presence of RAPA. Cerebral cortex sections of the sham, 100 $\mu\text{g}/\text{kg}$ LPS and 100 $\mu\text{g}/\text{kg}$ LPS + 1 mg/kg RAPA groups were stained with H&E. (A) Representative images of H&E staining revealed swollen and abnormally circular cells induced by LPS injection; this was abrogated by pretreatment with RAPA. Magnification, $\times 100$. Arrows indicate swollen and abnormally circular cells. The number of swollen and abnormally circular cells increased in the LPS groups but was reduced over time following RAPA treatment at (B) 6, (C) 12 and (D) 24 h. Each section was measured in triplicate and the experiment was repeated twice with similar results. Data are expressed as the mean \pm standard error from independent experiments. * $P < 0.05$ vs. LPS alone. H&E, hematoxylin and eosin; LPS, lipopolysaccharide; RAPA, rapamycin.

observed for p-S6 expression levels, with increased levels following LPS injection ($P = 0.021$ at 6 h, $P = 0.011$ at 12 h and $P = 0.013$ at 24 h vs. sham), abrogated by RAPA treatment ($P = 0.0029$ at 6 h, $P = 0.0021$ at 12 h and $P = 0.0022$ at 24 h vs. LPS; Fig. 4F-H).

Discussion

The present study demonstrated that, following an LPS-induced neuroinflammatory response, RAPA significantly altered the expression of key proteins and cytokines, and modulated the neuronal inflammatory response. It has previously been demonstrated that RAPA inhibits the mTOR complex 1 (mTORC1) signaling pathway and prevents phosphorylation of proteins involved in transcription, translation and cell cycle control (14).

Although inflammation has long been implicated in AD pathogenesis, its importance has recently become more

widely recognized. Increasing evidence has suggested that inflammation is critical for the initiation and progression of AD; it has therefore been identified as a potential therapeutic target. AD has been associated with polymorphisms in genes involved in inflammation (15). IL1 is an immunoregulatory cytokine that has been shown to be overexpressed in affected regions of the cerebral cortex in patients with AD, as determined by quantitative assays of tissue IL1 concentrations and analysis of IL1-immunoreactive microglia (16,17). IL6 is a pleiotropic cytokine that mediates inflammation affecting the growth and differentiation of cells in the CNS (18,19). Although IL6 is widely accepted to be involved in various CNS disorders, there is evidence to suggest that, in certain circumstances, IL6 may have anti-inflammatory effects as well as other beneficial properties. For example, IL6 has been demonstrated to be critical in the regulation of neuronal survival and function (20-24). In the present study, RT-qPCR analyses revealed that IL1 β and IL6 mRNA expression levels

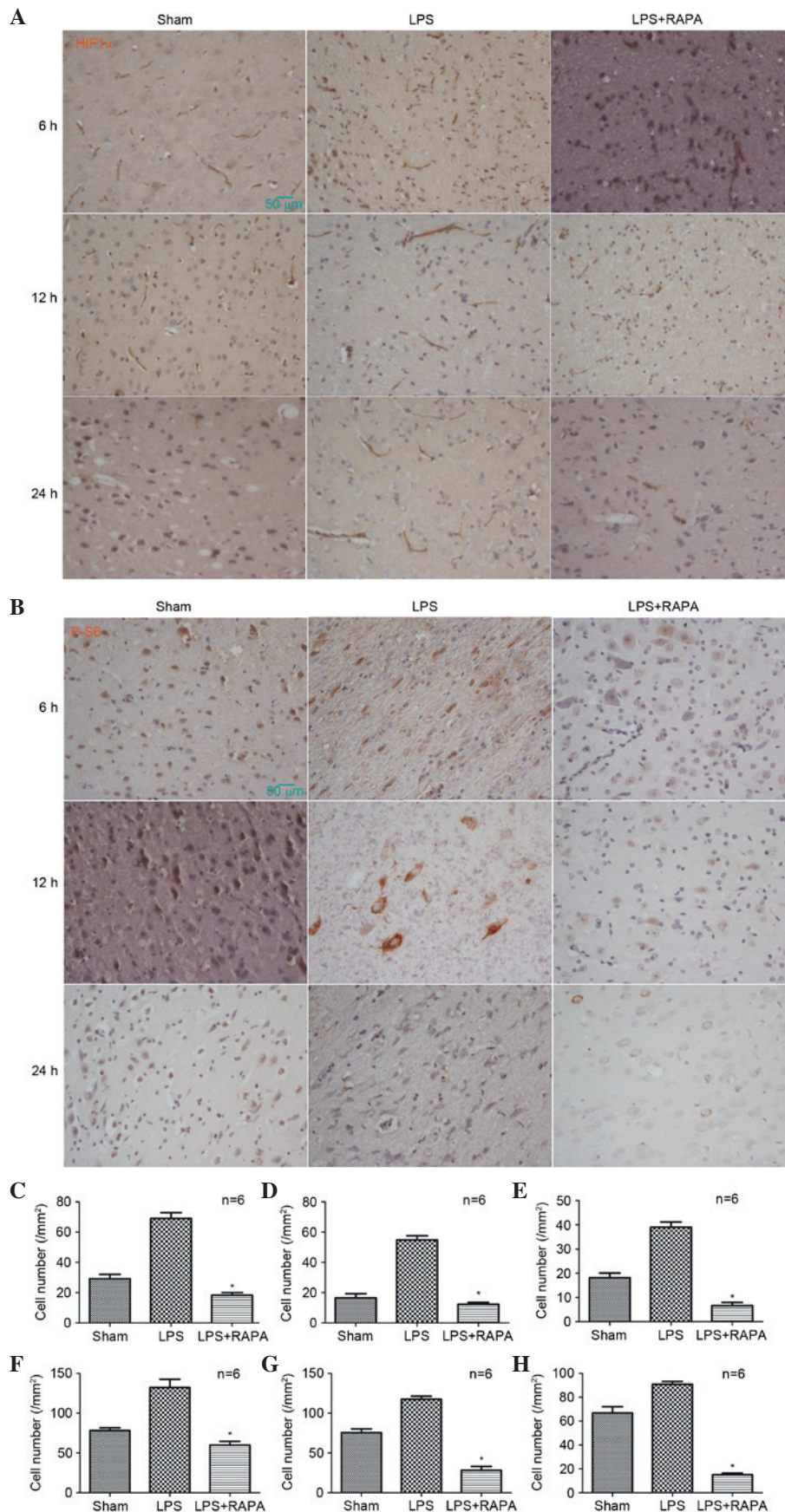


Figure 4. Detection of HIF1 α and p-S6 in rat cerebral cortex by immunohistochemistry, following LPS injection in the absence or presence of RAPA. Cerebral cortex sections from the sham, 100 μ g/kg LPS and 100 μ g/kg LPS + 1 mg/kg RAPA groups were stained with (A) HIF1 α and (B) p-S6 antibodies. Magnification, \times 100. Expression levels of HIF1 α increased following LPS injection; however, this increase was abrogated over time by RAPA treatment at (C) 6, (D) 12 and (E) 24 h. The increased expression levels of p-S6 induced by LPS were attenuated by RAPA treatment at (F) 6, (G) 12 and (H) 24 h. Each section was measured in triplicate and the experiment was repeated twice with similar results. Data are expressed as the mean \pm standard error from independent experiments. * P <0.05 vs. LPS alone. HIF1 α , hypoxia-inducible factor 1 α ; LPS, lipopolysaccharide; RAPA, rapamycin.

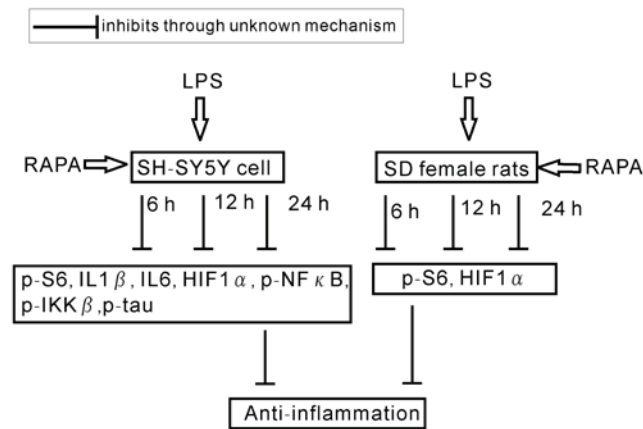


Figure 5. RAPA inhibits LPS-induced neuroinflammation via the mammalian target of rapamycin complex 1 signaling pathway. LPS, lipopolysaccharide; RAPA, rapamycin; SD, Sprague Dawley; p-, phosphorylated; IL, interleukin; HIF1 α , hypoxia-inducible factor 1 α ; NF κ B, nuclear factor κ B; IKK β , inhibitor of NF κ B kinase subunit β .

were significantly suppressed by RAPA treatment. These anti-neuroinflammatory effects may provide neuroprotection.

HIF1 α , which is the functional subunit of HIF1, is typically activated under conditions of hypoxia (25,26). HIF-1 has been demonstrated to be an essential transcription factor that mediates oxygen homeostasis and exhibits an adaptive response to the pathological conditions that result from hypoxia (27), upregulating genes that protect against cerebrovascular diseases and neurodegenerative disorders (28). A previous study suggested that HIF1 α may be a novel therapeutic target for the treatment of cerebral ischemia (29). When inflammation occurs under conditions of hypoxia, an increase in the mRNA expression levels of HIF1 α and the activation of mTOR have been observed (30,31). Using RT-qPCR and immunohistochemistry, the present study demonstrated that in the presence of RAPA, the HIF1 α levels were markedly decreased *in vivo* and *in vitro* from 6 to 24 h. This may downregulate the expression of genes responsible for protection against cerebral inflammatory injury, and thus provide neuroprotection. Certain previous studies have demonstrated that myeloid-specific deletion of HIF-1 α impaired inflammatory responses, which was associated with a metabolic defect characterized by lower glycolytic rates and energy generation in the absence of HIF-1 α (32,33).

mTOR integrates signaling pathways that respond to growth factors, energy metabolism, nutrients and in addition, stress (34). Postmortem evidence from the brains of patients with AD indicated that expression levels of p-S6 are increased compared with brains from age-matched controls, thus suggesting that mTORC1 activity is elevated in AD brains (35-41) and that events downstream of mTORC1 are involved in AD (42). The increase in mTOR-dependent signaling has also been positively and significantly correlated with total tau and p-tau expression levels, and the decades-long continuous synthesis of tau in degenerating neurons resulted from upregulated mTOR-dependent signaling (40). Evidence has indicated a direct association

between the neuropathological effects of A β and activation of the NF κ B signaling cascade (43). A β induces the accumulation of inhibitor of κ B, a downstream transcriptional target of the NF κ B signaling pathway. The results of the present study revealed that RAPA inhibited the expression of the mTORC1 downstream target, p-S6, and the NF κ B downstream targets, p-NF κ B and p-IKK β , in addition to suppressing p-tau protein expression levels. This is indicative of anti-neurodegenerative effects mediated by RAPA via mTORC1 and the downstream NF κ B signaling pathway (Fig. 5).

Activated microglia produce cytotoxic molecules, including nitric oxide, oxygen radicals, arachidonic acid derivatives and in addition, cytokines (1,15,44-46). Numerous mechanisms may underlie the contribution of post-traumatic inflammation to neuronal damage (10); the anti-inflammatory effects of early RAPA treatment may reduce microglial activation and enhance recovery from head trauma. RAPA is non-cytotoxic *in vivo* and may be used as an anti-angiogenic agent to inhibit tumor growth (6). The lack of toxicity of RAPA adds weight to the importance of the neuroprotective effects of RAPA revealed in the present study.

In conclusion, the results of the present study demonstrated that RAPA suppresses the neuroinflammation induced by LPS, *in vitro* and *in vivo*. RAPA may therefore be a potential therapeutic agent for the treatment of early AD, or for the treatment of neuroinflammation in non-AD patients to prevent the onset of AD. However, further studies are required to confirm the suitability of RAPA as a treatment strategy.

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References

- Giulian D: Microglia and the immune pathology of Alzheimer disease. *Am J Hum Genet* 65: 13-18, 1999.
- Alzheimer's Association: 2014 Alzheimer's disease facts and figures. *Alzheimers Dement* 10: e47-e92, 2014.
- Golde TE, Petrucelli L and Lewis J: Targeting Abeta and tau in Alzheimer's disease, an early interim report. *Exp Neurol* 223: 252-266, 2010.
- Veerhuis R, Janssen I, De Groot CJ, Van Muiswinkel FL, Hack CE and Eikelenboom P: Cytokines associated with amyloid plaques in Alzheimer's disease brain stimulate human glial and neuronal cell cultures to secrete early complement proteins, but not C1-inhibitor. *Exp Neurol* 160: 289-299, 1999.
- Baker H, Sidorowicz A, Sehgal SN and Vézina C: Rapamycin (AY-22,989), a new antifungal antibiotic. III. In vitro and in vivo evaluation. *J Antibiot (Tokyo)* 31: 539-545, 1978.
- Majumder PK, Febbo PG, Bikoff R, Berger R, Xue Q, McMahon LM, Manola J, Brugarolas J, McDonnell TJ, Golub TR, *et al*: mTOR inhibition reverses Akt-dependent prostate intraepithelial neoplasia through regulation of apoptotic and HIF-1-dependent pathways. *Nat Med* 10: 594-601, 2004.
- Bonow RH, Aid S, Zhang Y, Becker KG and Bosetti F: The brain expression of genes involved in inflammatory response, the ribosome, and learning and memory is altered by centrally injected lipopolysaccharide in mice. *Pharmacogenomics J* 9: 116-126, 2009.
- Hauss-Wegrzyniak B, Lukovic L, Bigaud M and Stoekel ME: Brain inflammatory response induced by intracerebroventricular infusion of lipopolysaccharide: An immunohistochemical study. *Brain Res* 794: 211-224, 1998.
- Burguillos MA, Hajji N, Englund E, Persson A, Cenci AM, Machado A, Cano J, Joseph B and Venero JL: Apoptosis-inducing factor mediates dopaminergic cell death in response to LPS-induced inflammatory stimulus: Evidence in Parkinson's disease patients. *Neurobiol Dis* 41: 177-188, 2011.
- Erlich S, Alexandrovich A, Shohami E and Pinkas-Kramarski R: Rapamycin is a neuroprotective treatment for traumatic brain injury. *Neurobiol Dis* 26: 86-93, 2007.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔC_T} Method. *Methods* 25: 402-408, 2001.
- Lowry OH, Rosebrough NJ, Farr AL and Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265-275, 1951.
- Espinosa-Oliva AM, de Pablos RM and Herrera AJ: Intracranial injection of LPS in rat as animal model of neuroinflammation. *Methods Mol Biol* 1041: 295-305, 2013.
- Vignot S, Faivre S, Aguirre D and Raymond E: mTOR-targeted therapy of cancer with rapamycin derivatives. *Ann Oncol* 16: 525-537, 2005.
- Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, Cole GM, Cooper NR, Eikelenboom P, Emmerling M, Fiebich BL, *et al*: Inflammation and Alzheimer's disease. *Neurobiol aging* 21: 383-421, 2000.
- Griffin WS, Sheng JG, Roberts GW and Mrak RE: Interleukin-1 expression in different plaque types in Alzheimer's disease: Significance in plaque evolution. *J Neuropathol Exp Neurol* 54: 276-281, 1995.
- Griffin WS, Sheng JG, Royston MC, Gentleman SM, McKenzie JE, Graham DI, Roberts GW and Mrak RE: Glial-neuronal interactions in Alzheimer's disease: The potential role of a 'cytokine cycle' in disease progression. *Brain Pathol* 8: 65-72, 1998.
- Heinrich PC, Horn F, Graeve L, Dittrich E, Kerr I, Müller-Newen G, Gröttinger J and Wollmer A: Interleukin-6 and related cytokines: Effect on the acute phase reaction. *Z Ernährungswiss (37 Suppl 1)*: 43-49, 1998.
- Hirano T, Nakajima K and Hibi M: Signaling mechanisms through gp130: A model of the cytokine system. *Cytokine Growth Factor Rev* 8: 241-252, 1997.
- Campbell IL: Transgenic mice and cytokine actions in the brain: Bridging the gap between structural and functional neuropathology. *Brain Res Brain Res Rev* 26: 327-336, 1998.
- Gadient RA and Otten UH: Interleukin-6 (IL-6)-a molecule with both beneficial and destructive potentials. *Prog Neurobiol* 52: 379-390, 1997.
- März P, Cheng JG, Gadient RA, Patterson PH, Stoyan T, Otten U and Rose-John S: Sympathetic neurons can produce and respond to interleukin 6. *Proc Natl Acad Sci USA* 95: 3251-3256, 1998.
- Rodriguez M, Pavelko KD, McKinney CW and Leibowitz JL: Recombinant human IL-6 suppresses demyelination in a viral model of multiple sclerosis. *J Immunol* 153: 3811-3821, 1994.
- Yang P, Wen H, Ou S, Cui J and Fan D: IL-6 promotes regeneration and functional recovery after cortical spinal tract injury by reactivating intrinsic growth program of neurons and enhancing synapse formation. *Exp Neurol* 236: 19-27, 2012.
- Jeong YJ, Cho HJ, Magae J, Lee IK, Park KG and Chang YC: Ascofuranone suppresses EGF-induced HIF-1 α protein synthesis by inhibition of the Akt/mTOR/p70S6K pathway in MDA-MB-231 breast cancer cells. *Toxicol Appl Pharmacol* 273: 542-550, 2013.
- Shin JM, Jeong YJ, Cho HJ, Park KK, Chung IK, Lee IK, Kwak JY, Chang HW, Kim CH, Moon SK, *et al*: Melittin suppresses HIF-1 α /VEGF expression through inhibition of ERK and mTOR/p70S6K pathway in human cervical carcinoma cells. *PLoS one* 8: e69380, 2013.
- White NM, Masui O, Newsted D, Scorilas A, Romaschin AD, Bjarnason GA, Siu KW and Yousef GM: Galectin-1 has potential prognostic significance and is implicated in clear cell renal cell carcinoma progression through the HIF/mTOR signaling axis. *Br J Cancer* 110: 1250-1259, 2014.
- Singh N, Sharma G and Mishra V: Hypoxia inducible factor-1: Its potential role in cerebral ischemia. *Cell Mol Neurobiol* 32: 491-507, 2012.
- Umschweif G, Alexandrovich AG, Trembovler V, Horowitz M and Shohami E: Hypoxia-inducible factor 1 is essential for spontaneous recovery from traumatic brain injury and is a key mediator of heat acclimation induced neuroprotection. *J Cereb Blood Flow Metab* 33: 524-531, 2013.
- Argyriou P, Papageorgiou SG, Panteleon V, Psyrris A, Bakou V, Pappa V, Spathis A, Economopoulou P, Papageorgiou E, Economopoulos T and Rontogianni D: Hypoxia-inducible factors in mantle cell lymphoma: Implication for an activated mTORC1-->HIF-1 α pathway. *Ann Hematol* 90: 315-322, 2011.
- Weng Q, Zhang J, Cao J, Xia Q, Wang D, Hu Y, Sheng R, Wu H, Zhu D, Zhu H, *et al*: Q39, a quinoxaline 1,4-Di-N-oxide derivative, inhibits hypoxia-inducible factor-1 α expression and the Akt/mTOR/4E-BP1 signaling pathway in human hepatoma cells. *Invest New Drugs* 29: 1177-1187, 2011.
- Palazon A, Goldrath AW, Nizet V and Johnson RS: HIF transcription factors, inflammation, and immunity. *Immunity* 41: 518-528, 2014.
- Cramer T, Yamanishi Y, Clausen BE, Förster I, Pawlinski R, Mackman N, Haase VH, Jaenisch R, Corr M and Nizet V: HIF-1 α is essential for myeloid cell-mediated inflammation. *Cell* 112: 645-657, 2003.
- Laplante M and Sabatini DM: mTOR signaling at a glance. *J Cell Sci* 122: 3589-3594, 2009.
- An WL, Cowburn RF, Li L, Braak H, Alafuzoff I, Iqbal K, Iqbal IG, Winblad B and Pei JJ: Up-regulation of phosphorylated/activated p70 S6 kinase and its relationship to neurofibrillary pathology in Alzheimer's disease. *Am J Pathol* 163: 591-607, 2003.
- Chang RC, Wong AK, Ng HK and Hugon J: Phosphorylation of eukaryotic initiation factor-2 α (eIF2 α) is associated with neuronal degeneration in Alzheimer's disease. *Neuroreport* 13: 2429-2432, 2002.
- Griffin RJ, Moloney A, Kelliher M, Johnston JA, Ravid R, Dockery P, O'Connor R and O'Neill C: Activation of Akt/PKB, increased phosphorylation of Akt substrates and loss and altered distribution of Akt and PTEN are features of Alzheimer's disease pathology. *Journal of Neurochemistry* 93: 105-117, 2005.
- Onuki R, Bando Y, Suyama E, Katayama T, Kawasaki H, Baba T, Tohyama M and Taira K: An RNA-dependent protein kinase is involved in tunicamycin-induced apoptosis and Alzheimer's disease. *EMBO J* 23: 959-968, 2004.
- Peel AL and Bredesen DE: Activation of the cell stress kinase PKR in Alzheimer's disease and human amyloid precursor protein transgenic mice. *Neurobiol Dis* 14: 52-62, 2003.

40. Pei JJ, Björkdahl C, Zhang H, Zhou X and Winblad B: p70 S6 kinase and tau in Alzheimer's disease. *J Alzheimers Dis* 14: 385-392, 2008.
41. Pei JJ and Hugon J: mTOR-dependent signalling in Alzheimer's disease. *J Cell Mol Med* 12: 2525-2532, 2008.
42. Lipton JO and Sahin M: The neurology of mTOR. *Neuron* 84: 275-291, 2014.
43. Tan L, Schedl P, Song HJ, Garza D and Konsolaki M: The Toll->NFkappaB signaling pathway mediates the neuropathological effects of the human Alzheimer's Abeta42 polypeptide in *Drosophila*. *PLoS one* 3: e3966, 2008.
44. Garwood CJ, Cooper JD, Hanger DP and Noble W: Anti-inflammatory impact of minocycline in a mouse model of tauopathy. *Front Psychiatry* 1: 136, 2010.
45. Walker D and Lue LF: Anti-inflammatory and immune therapy for Alzheimer's disease: Current status and future directions. *Curr Neuropharmacol* 5: 232-243, 2007.
46. Liu B, Gao HM and Hong JS: Parkinson's disease and exposure to infectious agents and pesticides and the occurrence of brain injuries: Role of neuroinflammation. *Environ Health Perspect* 111: 1065-1073, 2003.