Apoptosis Inhibition Can Be Threatening in Aβ-Induced Neuroinflammation, Through Promoting Cell Proliferation

Article *in* Neurochemical Research · September 2011

DOI: 10.1007/s11064-010-0259-3 · Source: PubMed

CITATIONS
30

READS
92

5 authors, including:

Azadeh Abdi
Saman daroo 8 biotechnological company
12 PUBLICATIONS **227** CITATIONS
SEE PROFILE

Leila Dargahi
Shahid Beheshti University of Medical Sciences
95 PUBLICATIONS **1,180** CITATIONS
SEE PROFILE

Leila Khalaj
Shahid Beheshti University of Medical Sciences
22 PUBLICATIONS **526** CITATIONS
SEE PROFILE

Abolhassan Ahmadiani
Shahid Beheshti University
196 PUBLICATIONS **3,680** CITATIONS
SEE PROFILE

Neurochem Res (2011) 36:39–48
DOI 10.1007/s11064-010-0259-3

ORIGINAL PAPER

Apoptosis Inhibition Can Be Threatening in Aβ-Induced Neuroinflammation, Through Promoting Cell Proliferation

A. Abdi · H. Sadraie · L. Dargahi · L. Khalaj · A. Ahmadiani

Accepted: 26 August 2010 / Published online: 17 September 2010

Abstract Neuronal apoptosis in neurodegenerative diseases is correlated with inflammatory reactions. The beneficial or detrimental role of apoptosis in neuroinflammation is unclear. In this study, we injected β-amyloid peptide into the rat cortex for induction of neuroinflammation in hippocampus. We observed an increase in TNF-α as an inflammatory cytokine and caspase3 and TUNEL-positive cells as apoptotic marker. As far as ability of TNF-α to induce apoptosis or activate NF-kβ, the question is what will happen if the balance between two pathways is disturbed by inhibition of apoptosis. Using caspase inhibitors, we inhibited apoptosis and assessed NF-kβ, Hsp 70 (a hallmark of cancer), cmyc (proto-oncogene) and p53 (tumor suppressor protein). There was an unexpected decrease in NF-kβ while Hsp70 and cmyc upregulated and p53 decreased. These results imply that inhibition of apoptosis due to increased susceptibility to abnormal mitosis may not provide a reliable strategy for treatment of neuroinflammatory diseases.

Keywords Neuroinflammation · Apoptosis · NF-κB · Caspase-3 · Cell proliferation

A. Abdi · L. Dargahi · L. Khalaj · A. Ahmadiani (✉)
Neuroscience Research Center, Shahid Beheshti University of Medical Sciences, Evin, PO Box 19839-63113, Tehran, Iran
e-mail: aahmadiani@yahoo.com

A. Abdi · L. Dargahi · L. Khalaj · A. Ahmadiani
Department of Pharmacology, Shahid Beheshti University of Medical Sciences, Tehran, Iran

H. Sadraie
Department of Anatomy, School of Medicine, Baqiyatallah University of Medical Sciences, Tehran, Iran

Abbreviations

Aβ	β-Amyloid peptide
NF-κB	Nuclear factor-κB
Hsp70	Heat shock protein 70
TNF-α	Tumor necrosis factor-α
TUNEL	Terminal uridine deoxynucleotidyl transferase dUTP nick end labeling
JNK	c Jun N terminal kinase

Introduction

Inflammation, a physiological response to different types of tissue insults, is a cascade of coordinated chemical and cellular reactions. Typically, the inflammatory cascade involves a local production of cytokines that underlies cell recruitment and cell differentiation by calling into action specific gene expression. Acute neuroin-flammation following stroke or trauma may contribute to the initial extension of the lesion by increasing neuronal loss, but may also promote subsequent functional recovery by enabling neuronal plasticity. On the other hand, chronic neuroinflammatory processes are suspected to sustain neuronal loss in a number of pathological conditions such as auto immune diseases (e.g., multiple sclerosis) and neurodegenerative diseases (e.g., Alzheimer's disease) [1]. Brain inflammation is associated with the synthesis and secretion, particularly from activated glial cells of a number of neuroactive molecules, including cytokines (TNF-α, Il-1β …), reactive oxygen-and nitrogen-free radicals, complement proteins, excitatory amino acids, proteases and etc [2]. Inflammation is generally beneficial to the organism in limiting the survival and proliferation of invading pathogens, promoting tissue survival, repair and recovery, and conserving the energy of the organism. However,

extensive, prolonged or unregulated inflammation is highly detrimental [3].

Beta Amyloid peptide (Aβ), specifically Aβ (1–42) is toxic to neurons and is hence strongly associated with loss of brain function through the course of Alzheimer's disease (AD). This deposition induces migration and activation of astrocytes and microglia that further, mediates neuronal cytotoxic path ways. Extensive studies in recent years have focused on Aβ-mediated microglia activation with regard to secretion of pro inflammatory cytokines such as TNF-α and IL-β, activation of complement factors and release of free radicals [2, 4]. TNF-α, can promote tumors such as prostate cancers and also initiate apoptotic cell death [5].

One of the reasons is that TNF-α elicites its biological effects through the activation of two distinct receptors, the P55 TNF Receptor (TNFRI) and the P75 TNF receptor (TNFRII). TNFRI contain an intra cellular "death domain" and contributes to cell death and TNFRII plays a trophic or protective role in neuronal survival [6, 7]. In another study TNFRI can induce both apoptosis and cell survival. The survival and activation signal induced by TNF-α, involves distinct sets of adapter protein and transcription factors, TRAF-2 (TNF receptor associated factor-2) and RIP (receptor interactive protein). TRAF-2 and RIP stimulate path ways leading to activation of MAPkinase and NF-κB, respectively. Studies in mice and humans have shown that NF-κB is a repressor of apoptosis. How ever, MAPK may inhibit or promote apoptosis [8]. Several pro-inflammatory cytokines and chemokines such as TNF-α, IL-1, IL-6 and IL-8 are associated with NF-κB activation pathway and tumor development and progression [9, 10].

So TNF-α induces two distinct pathways: one of them is apoptosis and the other one is NF-κB activation and eventually cell survival and proliferation.

One of the questions that came to us was whether there is any relation ship between these two path ways and blocking one pathway results in boosting the other one. To probe this issue, we blocked apoptosis and measure NF-κB activation. Another question arose as the role of apoptosis in neuroinflammation. Whether apoptosis is a protective or destructive mechanism? To achieve this goal, we inhibited apoptosis and evaluated some intracellular changes such as: cmyc as a proto-oncogene, Hsp70 as a hallmark of cancer that is upregulated by stress and toxicants and p53 as a tumor suppressor protein.

Experimental Procedure

Animals

Adult male wistar rats (230–280 g) were obtained from Neuroscience Research Center of Shahid Beheshti Medical University. Rats were housed three per box and maintained at a constant temperature on a 12-h light–dark cycle (lights on between 08:00 and 20:00) with food and water. After at least 1 week of habituation in the facilities, animals were admitted to the experimental procedures. All experiments were carried out according to the European Community Council Directive 86/609/EEC and efforts were made to minimize animal suffering and to reduce the number of animals used. In each group four rats were used for western Blotting and three rats were used for TUNEL test.

Drug Administration to Rats

β-Amyloid (1–42) fragment (Aβ, sigma, A9810) Was prepared as stock solution (0.1 mg/ml) in steril normal saline and diluted tenfold, then aliquoted (10 µl per vial), frozen on dry ice, and stored at −20°C until use. The peptide was still perfectly soluble after defrosting the aliquots, and 3 µl of the Solution (10 ng/µl) was used for each injection (30 ng Aβ in each site). Sterile normal saline was injected into control animals. The animals were anesthetized with Ketamine (150 mg/kg) and Xylasine (8 mg/kg). They were placed in a stereotaxic frame and Aβ or vehicle was injected in the deep frontal cortex (3.2 mm AP, 2 mm DV relative to bregma, 3 mm depth) [11] injections were bilateral. Each injection (3 µl) lasted 3 min using a 10-µl Hamilton syringe with blunt needle. And the needle was left in place for an additional 1 min before it was slowly retracted.

Caspase Inhibitors

z-VADfmk (V-116, Sigma), a pan-caspase inhibitor and z-DEVDfmk (CO605, sigma), a selective caspase-3 inhibitor, were injected at 2,000 and 400 ng doses respectively. z-VAD fmk was dissolved in 1% DMSO and z-DEVD fmk was dissolved in 0.5% DMSO. 6 µl of z-VAD fmk (333.3 ng/µl) and 3.7 µl of z-DEVDfmk (107.14 ng/µl) were mixed into the 25 µl-Hamilton syringe and were injected intracerebroventricular (icv), −0.4 mm AP, 1.6 mm DV relative to bregma, 4 mm depth. DMSO 1% was injected icv in the control group. Injections were repeated through the cannula (a 23 gauge needle that had been placed into the cerebro vertricle and fixed with Acrylic cement) on 0,1,5,7 and 10 days after the surgery; on the 13th day rats were sacrificed. Hippocampi were removed and stored at −20°C.

Immunostaining

To detect cells undergoing apoptosis, we used the technique of Terminal-Transferase dUTP Nick End labeling (TUNEL Apoptag plus peroxidase in situ Apoptosis detection kit,

S7101, chemicon). After killing, the brains were removed, two hemispheres were separated and rapidly fixed in formalin 10% For 24 h. The tissues were processed and paraffin embedded. The blocks were cronally sectioned by micro tome. Sections (10 μm) were mounted on slides and a proteinase k digestion (20 μg/ml) was carried out for 15 min. Endogenous hydrogen peroxidase activity was quenched in 3% hydrogen peroxide. After a series of rinsing, nucleotides labeled with digoxigenin were enzymatically added to the DNA by terminal deoxy nucleotidyl transferase enzyme (TdT). The incubation was carried out for 60 min the labeled DNA was detected using anti-digoxigenin-peroxidase for 30 min. The chromogen diaminobenzidine tetra hydrochloride (DAB) resulted in a brown reaction product that was evaluated by light microscopy and cells were counted. Positive and negative controls were carried out on slides from the same block. Incubation without TdT served as the negative control.

Western Blots

Western blot analysis was performed on hippocampi homogenates. Briefly, hippocapi were removed from brains and frozed in −20°C.For preparing cytosolic extraction hippocampi of each rat were resuspended in about 500 μl of ice-cold lysis buffer (Tris–HCL 50 mM, Nacl 150 mM, Triton x-100 0.1%, sodium deoxy cholate 0.25%, SDS 0.1%, EDTA 1 mM, protease inhibitor cock tail 1%). The tissues were lysed by tissue homogenizer, and the total protein extract was then obtained by centrifugation for 15 min at 13,000 rpm. Protein concentration was determined by the Brad ford assay, and equivalent amounts (80 μg) of each sample were subjected to SDS–PAGE electrophoresis. The proteins were transferred onto PVDF membranes, according to the manufacturer's instructions (Bio-Rad). The membranes were saturated by incubation with 2% non-fat dry milk (Amersham, Ecl Advance™ blocking agent, cpk 1075), in TBST for 1 h and 15 min. Then incubated with : anti-caspase-3 (cell signaling, 1/1,000 v/v), anti-NF-κB (cell signaling, 1/1,000 v/v), anti-TNF-α (cell signaling, 1/1,000 v/v), anti-cmyc (cell signaling, 1/1,000 v/v), anti-p53 (cell signaling, 1/1,000 v/v), anti-Hsp70 (cell signaling, 1/1,000 v/v), at 4°C overnight. All the antibodies including primary and secondary were diluted by 2% non-fat dry milk in TBST. The membranes were washed three times with TBST, and then incubated with anti-rabbit immunoglobulins coupled to peroxidase (cell signaling, 1/3,000 v/v). The immune complexes were visualized by the Ecl Advanced chemiluminescence method (Amersham, Ecl Advanc™ western Blotting detection kit, RPN2135).

The relative expression of protein bands was quantified by densito metric scanning of the x-ray films with Image J.

Nuclear Extraction

After extracting cytosolic extraction as described above, the supernatants containing cytosolic proteins were removed and stored at −70°C the nuclear pellet was resuspended by cold salin buffer (20 mM Hepes/KOH [pH 7.9], 1.5 mM MgCl2, 0.2 mM EDTA, 650 mM NaCl, 25% v/v glycerol, 1 mM DTT, 0.5 mM PMSF and protease inhibitor) on ice. After centrifugation at 13,000 rpm for 20 min at 4°C, supernatants containing nuclear proteins were removed and stored at −70°C.

Statistical Analysis

Factors including caspase-3, NF-κB, Hsp70, cmyc and p53 were compared among three groups and assessed by one-way ANOVA followed by Tukey post hoc test. TNF-α was compared between two groups and measured by Student's *t*-test. For TUNEL staining, 10 fields were chosen from each groups (3 groups) and the percent of TUNEL-positive cells were calculated according to this relation: %TUNEL-positive neurons = (TUNEL-positive neurons/TUNEL-positive neurons(brown) + normal neurons(green)) × 100. Data were compared by one-way ANOVA and Tukey post hoc; and were expressed as mean ± SE. Statistical significance was determined at $P < 0.05$.

Results

Aβ Increased TNF-α as an Inflammatory Cytokine

It is reported that fibrillar Aβ stimulation of mouse microglia and THP-1 monocytes results in a Syk kinase and NFkB-dependent production of TNFα that is responsible for increased iNOS expression, peroxynitrite production, and subsequent apoptosis in primary mouse neuronal cultures. These data establish a functional linkage between Aβ-stimulated microglial proinflammatory changes and the specific characteristics of neuron loss that occur in AD brains [7]. To confirm that Aβ can cause neuroinflammation, we investigated level of TNF-α as an inflammatory marker following single dose injection of Aβ. Injection of Aβ (1–42), 30 ng in prefrontal cortex in each hemisphere increased TNF-α as an inflammatory cytokine, in hippocampi, significantly, compared with control group (***$P < 0.001$) (Fig. 1).

Aβ Increased Caspase-3 in Hippocampus

Studies have shown that activation of caspase-2 and -3 is downstream of JNK activation. It is reported that there is activation of both caspase-2 and -3 after Aβ treatment of neuronal cells, but that only caspase-2 activation correlates

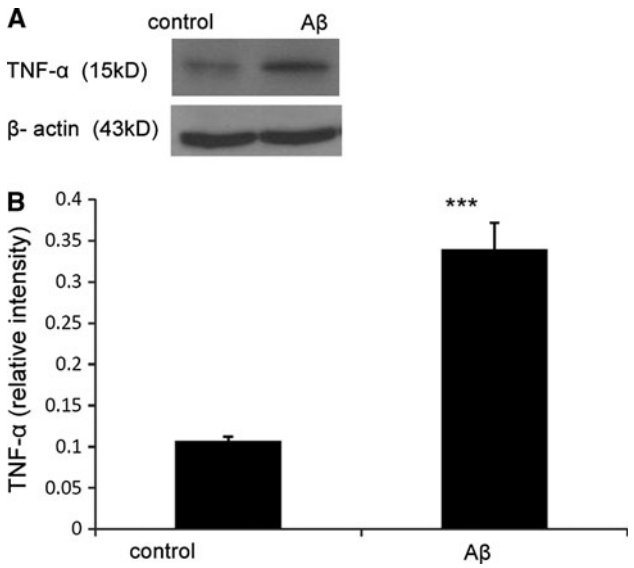

Fig. 1 Aβ injection into the prefrontal cortex increased TNF-α in the hippocampi of rats. **a** Changes in the expression of TNF-α (15KD), were determined by western blot on cytosolic proteins extracted from hippocampus. **b** Data were compared by densitometry scanning of x-ray film. Immunodetection of β-actin is used as an internal standard. Data are shown as relative intensity corresponding to the ratio TNF-α (15KD)/β-actin (43KD). (Mean ± SEM, independent samples *T* Test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

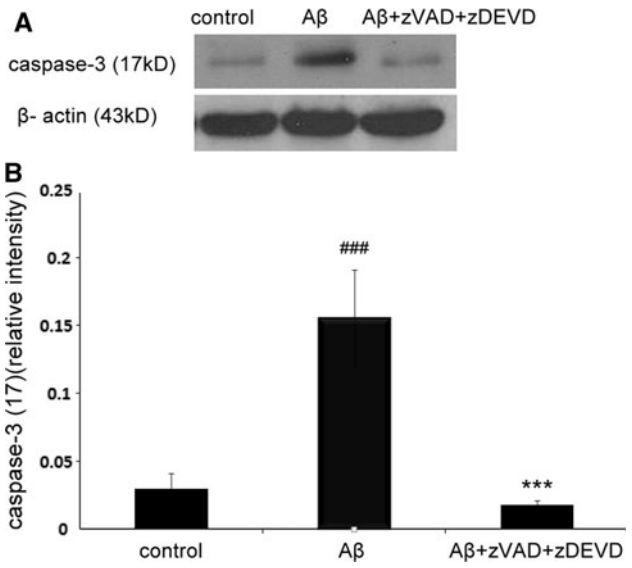

Fig. 2 Hippocapal caspase-3 increased at day 13 after stereotaxic injection of Aβ (1–42) into the rat prefrontal cortex. **a** Changes in the expression of caspase-3 (17 KD), as determined by western blot, carried out with the respective antibodies on the cytosolic Proteins extracted from hippocampus. **b** Data were compared by densitometry scanning of x-ray films. Immunodetection of β-actin is used as an internal standard. Data are shown as relative intensity corresponding to the ratio caspase-3 (17KD)/β-actin (43KD) from the cytosolic fraction (Mean ± SEM, one-way ANOVA, post hoc Tukey, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

causally with death. It is not clear what the function of activated caspase-3 is in this paradigm but it is evident that this activation is also downstream of JNK activation [12]. Huperzine A can protect against Aβ (1–42) insult through a direct inhibition of ROS formation and caspase-3 activation. The potential effectiveness of HupA in the treatment of AD may partially involve its ability to attenuate neuronal apoptosis [13]. According to other different studies,Aβ leads to apoptosis and activation of caspase-3.In this study Single dose Injecting of Aβ (1–42) (30 ng/μl) into the prefrontal cortex bilaterally, increased cleaved caspase-3(17 kD) in hippocampus of rats which were sacrificed on day 13th ($^{###}P < 0.0001$), compared to control group. Other group received a single dose of Aβ but they were sacrificed on day 25th. Cleaved Caspase-3 level in this group, returned to the control level, so this group was not proceeded (data not shown). Icv injection of z-VAD fmk and z-DEVD fmk on the day 0 (5 min before Aβ), 1, 5, 7 after the surgery, caused a significant activated caspase-3 decline compared to the Aβ-injected group ($^{***}P < 0.0001$) (Fig. 2).

Aβ Increased TUNEL-Positive Neurons in Hippocampus

Aβ1–42 exposure significantly decreases neuronal survival as indicated by a decrease in the calcein signal and an increase in the amount of ethidium homodimer signal. It has been shown Following 72 h of Aβ exposure, neurites were thickened and beaded and there was an increase in the number of phase bright cells, indicative of neuronal death compared to vehicle control. Aβ (1–42) exposure also resulted in a significant increase in the number of TUNEL-positive neurons [14]. Numerous studies have proved Aβ-induced apoptosis and TUNEL-positive cells in hippocampal neurons [15]. In the present study we also observed increase of caspase-3 after treatment with Aβ, to verify the apoptosis we used TUNEL staining. Injecting a Single dose of Aβ (1–42) (30 ng/μl) into the prefrontal cortex bilaterally, increased TUNEL-positive neurons significantly ($^{***}P < 0.001$). Inhibiting apoptosis caused a marked decline in the number of TUNEL-positive neurons ($^{###}P < 0.001$) (Fig. 8).

Inhibiting Apoptosis Decreased Nuclear NF-κB

According to different studies, Aβ can increase NF-κB. Jang et al. have performed EMSA using a 32P-labeled oligonucleotide probe containing the Consensus sequence for the NF-κB response element. They have reported Treatment of PC12 cells with Aβ (25–35) led to a transient increase in NF-κB DNA binding activity. They also verified the nuclear translocation of p65 by immunocytochemistry [16]. In the present study after injection of Aβ, nuclear NF-κB increased but it was not significant in comparing to control group. To evaluate the role of

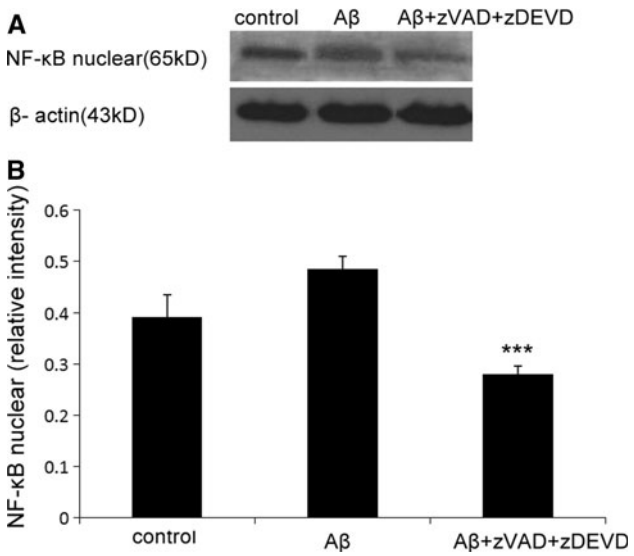

Fig. 3 Hippocampal nuclear NF-κB level decreased after inhibiting apoptosis. Changes in the expression of nuclear NF-κB (65KD). **a** As determined by western blot, carried out with the respective antibodies on the nuclear proteins extracted from hippocampus. **b** Data were compared by densitometry scanning of x-ray film. Immunodetection of β-actin is used as an internal standard. Data are shown as relative intensity corresponding to the ratio NF-κB nucl (65KD)/β-actin (43KD), from the nuclear fraction (Mean $\pm$ SEM, one way ANOVA, post hoc Tukey, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

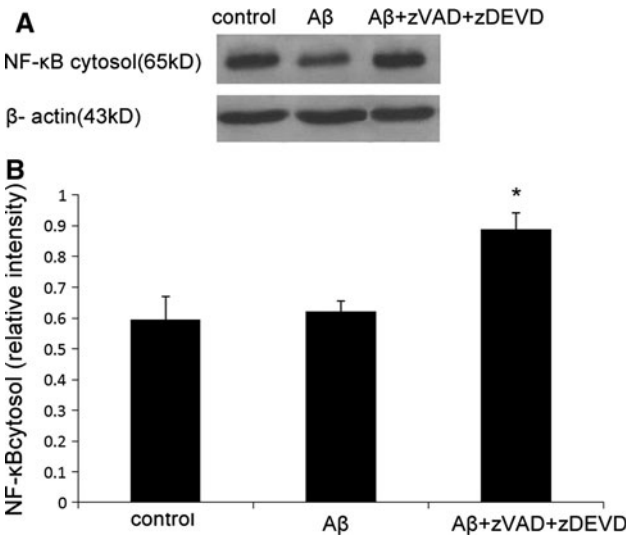

Fig. 4 Hippocampal cytosolic NF-κB level increased after receiving a single dose of Aβ and 5 repeated doses of z-VAD fmk + z-DEVD fmk. **a** Changes in the expression of cytosolic NF-κB were assessed by western blotting. Immunodettection of β-actin is used as an internal standard. **b** Data are shown as relative intensity corresponding to the ratio NF-κB cyto (65KD)/β-actin (43KD). (Mean $\pm$ SEM, one-way ANOVA, post hoc Tukey, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

apoptosis, we inhibited apoptosis and measured the amount of NF-κB into the cytosol and nucleus. After injection of caspase inhibitors (z-VAD fmk + z-DEVD fmk) for five repeated doses, nuclear NF-κB decreased significantly compared to Aβ-injected group (***$P < 0.001$) (Fig. 3).

To confirm our results of NF-κB level into nuclear extraction, we measured total amount of NF-κB in cytosolic extraction. In Aβ-injected group we didn't observe any significant changes in cytosolic NF-κB level; but following apoptosis inhibition, NF-κB amount increased markedly compared to Aβ-injected group (*$P < 0.05$) (Fig. 4).

As β-actin is in both cytosolic and nuclear fraction [17], it was measured as internal control to make sure equal amount of protein were loaded. Even though, the fractionation method has been proved for separation of cytosolic and nuclear proteins, to show the accuracy of fractionation, we used GAPDH and anti histon H4. GAPDH was detected just in cytosolic fraction and histon H4 was detected in nuclear fraction(data are not shown).

Inhibiting Apoptosis Increased Hsp70

One of the important Hsps which increases in neuroinflammation and also Aβ-aggregated neurons is Hsp70 [18]. Expression of certain Hsps such as Hsp70 can be correlated with the carcinogenic process as well as with the degree of differentiation and cell proliferation, and moreover, they have been implicated in the regulation of apoptosis. Therefore, it was reasonable to study the prognostic implications of Hsps, and they emerged as useful in certain cancer types. In addition, the levels of Hsp and anti-Hsp antibodies in the serum of cancer patients are useful in tumor diagnosis [19]. To further explore the probable damages or consequences of inhibiting apoptosis, we assessed Hsp70 as a hall mark of cancer and many dangerous situations that cells may encounter. After injecting a single dose of 30 ng of Aβ into the prefrontal cortex bilaterally (each hemisphere received 30 ng), the amount of Hsp70 increased but not significantly. After inhibiting Apoptosis, we found a noticeable increase in Hsp70 level, Compared to the Aβ-injected group (**$P < 0.01$) (Fig. 5).

Inhibiting Apoptosis Increased c-Myc

In physiological situation, the central role of c-Myc may be its promotion of cell replication in response to extracellular signals, by driving quiescent cells into the cell cycle. According to several observations, c-Myc has been increased in different cancers and can be considered as a marker of cell proliferation [20]. To approach to more consequences of inhibiting apoptosis in neuro inflammatory situation, we focused on a proto-oncogene like c-Myc. Although Aβ injection caused a minor increase in cytosolic c-Myc compared to control group, inhibiting apoptosis markedly increased c-Myc level in comparing with Aβ-injected group (**$P < 0.001$) (Fig. 6).

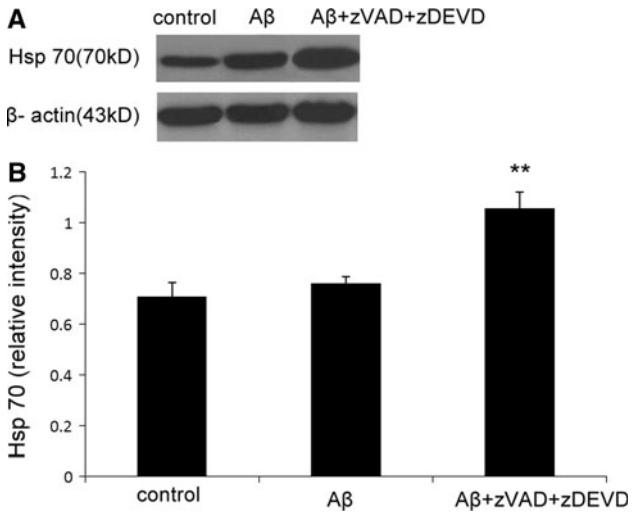

Fig. 5 Cotreatment with Aβ (1–42) and z-VADfmk +z-DEVD fmk increased the level of Hsp70 in the cytoplasmic fraction of hippocampi of rats. **a** By western blot, changes in the expression of Hsp70 were assessed. **b** The relative intensities corresponding to the ratio Hsp70/β-actin from the cytoplasmic fraction from different groups were determined. Immunodetection of β-actin is used as an internal standard. (Mean ± SEM, one-way ANOVA, post hoc Tukey * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

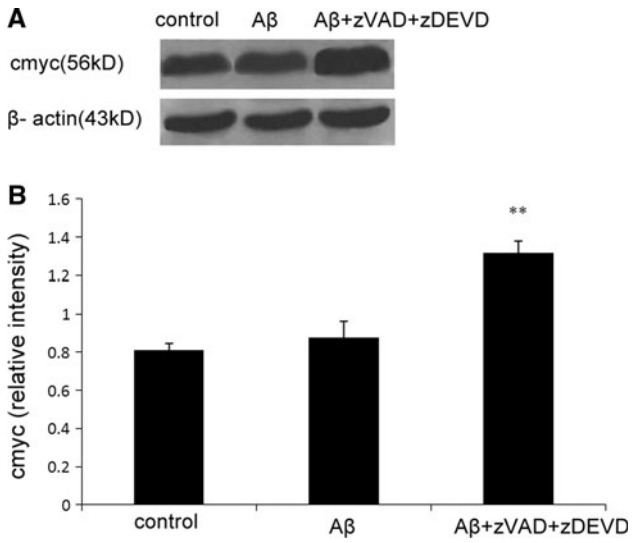

Fig. 6 Cotreatment with Aβ (1–42) and z-VAD fmk + z-DEVD fmk increased the level of cmyc in cytoplasmic fraction of hippocampi of Rats. **a** By western blot, Changes in the expression of cmyc were assessed. **b** The relative intensities corresponding to the ratio cmyc (56 KD)/β-actin(43KD) from the cytoplasmic fraction of different groups were assessed. Immunodetection of β-actin is used as an internal standard. (Mean ± SEM, one-way ANOVA, past hoc Tukey, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

Inhibiting Apoptosis Decreased p53

Overexpression of p53 has been found in many types of human malignancies. There is evidence that supports the existence of a high level of p53 alterations in Hepatocellular carcinoma(HCC). p53 status can be analyzed in combination with several other serological markers for the detection of early HCC. In preneoplastic and neoplastic lesions of rat and human livers, expression of p53, proliferating cell nuclear antigen (PCNA), silverstaining nucleolar organizer regions (AgNOR), and oncogenes has been studied for the use of these molecular parameters as "intermediate" markers in studies of risk assessment and cancer prevention, without having to resort to tumor appearance as an end point [21]. In the present study to probe the role of apoptosis in neuroinflammation, p53 was assessed as a risk factor for cell proliferation after inhibiting apoptosis. In Aβ–injected group the amount of p53 increased significantly compared with control group ($^{\#\#}P < 0.01$),as we observed an increase in the amount of caspase-3 and TUNEL-positive cells which confirms apoptosis. z-VAD fmk + z-DEVD fmk caused a significant decrease in p53 level compared to Aβ-injected group ($^{**}P < 0.01$) (Fig. 7).

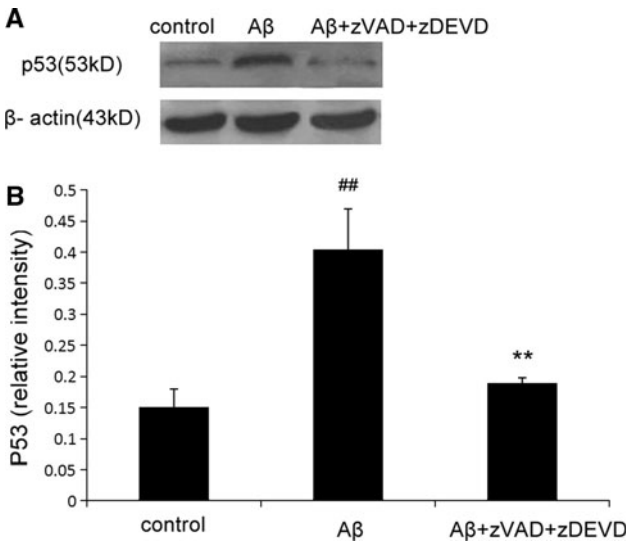

Fig. 7 Cotreatment with Aβ (1–42) and z-VADfmk +z-DEVD fmk declined the level of p53 in the cytoplasmic fraction of hippocampi of rats. **a** By western blot, Changes in the expression of p53 were assessed. **b** The relative intensities corresponding to the ratio p53/β-actin from the cytoplasmic fraction of different groups were determined. Immunodetection of β-actin is used as an internal standard. (Mean ± SEM, one-way ANOVA, post hoc Tukey, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

Discussion

Aβ Induced Inflammation in Hippocampus

Activation of the classical and alternative complement pathways by β-pleated fibrillar Aβ has been proved. All the cytokines and chemokines that have been studied in AD, including IL-Iβ, IL-6, TNF-α, IL-8, Transforming growth

factor-β (TGF-β), and macrophage inflammatory protein-1α (MIP-1α), seem to be unregulated in AD [6, 22, 23]. Inflammatory reaction, induced by Aβ in the CNS involves the release of damaging factors, such as: cytokines(TNF-α), NO and ROS, connecting link between Aβ,TNF-α, iNOS, oxidative stress and the congnitive decline has been shown. TNF-α is one of the earliest mediators in the Aβ-induced Neurodegenerative process [3, 7, 24]. Our results also verified that Aβ initiate inflammation in hippocampal neurons. We measured TNF-α as an inflammatory cytokine, it showed a significant increase in Aβ-injected group (Fig. 1). Inflammation has been suggested as a causal factor in several human tumors, including prostate cancer. In inflammatory process several factors such as: cytokines has been described, and the role of proinflammatory cytokines, such as TNF-α has been well established in prostate cancer. The balance between proliferation and apoptosis in human normal prostate is broken in prostate cancer. Data suggest that several factors which regulate proliferation/apoptosis equilibrium are altered in pathologic conditions [5]. TNF-α produced by tumor cells or inflammatory cells in the tumor microenvironment, can promote tumor cell survival through the induction of genes encoding NF-κB—dependent anti apoptotic molecules such as Bcl-xl, CIAP1 and 2 [25, 26]. In general, chronic inflammation might enhance tumor initiation [9, 10]. NF-κB links inflammation to cancer. Based on many functions of NF-κB target genes, a close relationship between NF-κB and cancer was proposed. The association of NF-κB activation with inflammation—associated tumor promotion, progression and metastasis well documented and was demonstrated in several mouse models [9, 10, 27]. TNF-α can induce apoptosis of oligodendrocytes and in a neuronal cell line in vitro. Apoptosis of oligodendrocytes invivo was induced by the over expression of TNFRI [28]. It has been shown that caspases are essential factors in TNF-α-induced apoptosis in neutrophils [29]. Different studies have shown that TNF-α can provoke two major distinct pathways: one is apoptosis and the other one is cell proliferation through NF-κB activation. It has been shown that inhibition of NF-κB causes a major potentiation of the proapoptotic effect of TNF-α [29]. But it is not known if the activity of NF-κB will increase by inhibiting apoptosis.In the present study we inhibited apoptosis and measured the amount of NF-κB. In contrast to our anticipation, NF-κB decreased after apoptosis inhibition. The probable reason has been explained in this article.

Aβ Caused Apoptosis in Hippocampus

Neurons treated with Aβ in culture, have shown an increase in JNK(c Jun N terminal Kinase), caspase-2 and -3 activation. This pathway has been implicated in several neuronal death [12]. Neurons exposed to Aβ have demonstrated a typical apoptosis, including condensation of cell bodies, blebbing,DNA fragmentation as shown by morphological observation [13], and up regulation of p53, Bax, Mitochondrial membrane permeability transition and release of cytochrom c and activation of the apoptosome, resulting in caspase-3 activation [30]. Also Aβ treatment has resulted in the appearance of only few TUNEL—positive cells and caspase-3 activity in dentate gyrus [31–33]. In our experiment, 13 days after injection of Aβ (1–42), activated caspase-3(17kD) was increased significantly compared to control group (nearly about fourfold), which can consider as apoptosis, (Fig. 2). For morphological study we used TUNEL technique to confirm apoptosis. In different slices of hippocampus of this group there were several TUNEL—positive cells vs. Control slides (Fig. 8).

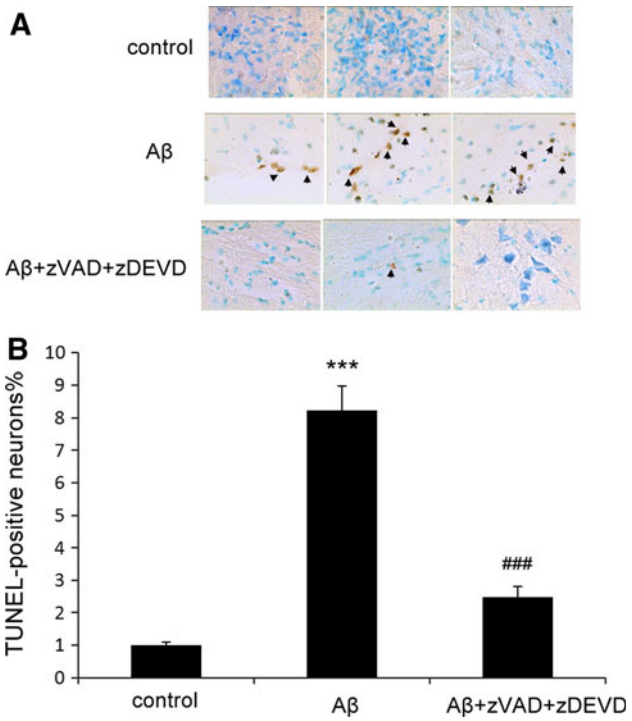

Fig. 8 Hippocampal TUNEL-positive neurons 13 days after stereotaxic injection of Aβ (1–42) into the rat prefrontal cortex. **a** *Top panel* shows staining in control group. There were no TUNEL-positive neurons in this group. *Middle panel* shows Aβ (1–42) injected group. Several TUNEL-positive neurons (*arrows*) were observed. *Bottom panel* shows the group co-treated with Aβ and z-VAD fmk + z-DEVD fmk. In this group number of TUNEL-positive neurons have been reduced significantly. Normal cells (*green*), TUNEL-positive cells (*brown*). Light microscope (×1,000) was utilized for taking pictures. **b** Represents the quantitative analysis of the data. For interpretation of the references to color in this figure legend, the reader is referred to the online version of this article

Inhibiting Apoptosis Decreased Nuclear NF-κB

As the aim of this study is to detect the probable relationship between apoptosis and NF-κB activation and also

determine the role of apoptosis in neuro inflammation, so we inhibited apoptosis, then measured NF-κB activation. Further more we pursued some cellular and molecular changes to explore whether apoptosis is beneficial or detrimental in neuroinflammation. Caspase-3 as a key protease in executing apoptosis is an appropriate target for in habiting apoptosis. z-VAD fmk(a pan-caspase inhibitor) and z-DEVD fmk (a selective caspase-3 inhibitor), were administered to inhibit apoptosis completely.

Dual roles of NF-κB activation in the cell fate have been demonstrated. However, NF-κB activation is increased in the neurons associated with Aβ deposits in the early stages of AD. NF-κB activation may act as a pro-apoptotic factor [34, 35]. There is evidence suggesting that NF-κB has a protective effect in cells of the nervous system [35, 36]. MAPK-NF-κB pathway activation plays an important role in the activation of microglial cells during inflammation, a pathological hall mark of AD [16, 37]. In the present study, Aβ caused an increase in nuclear NF-κB (P65), but it was not significant in comparing with control group. After inhibiting caspase-3, nuclear NF-κB level decreased significantly about 2–2.5 fold (Fig. 3).

In a study (by youn sook song et al. 2004), NF-κB had been activated during induction of cell death by Aβ in differentiated PC12 cells,and NF-κB activation and Aβ-induced cell death were reduced in over expressed Bcl2 cells. One of the possible explanations for this issue is that, over expression of Bcl2 may inhibit NF-κB activation by down regulation of caspase-3 which degrades IκB to activate NF-κB [34]. According to this study, caspase-3 is one of the most important elements in NF-κB activation. Inhibiting this enzyme could decline degrading IκB. IκB is NF-κB inhibitor that prevents NF-κB activation by binding to it into the cytosol. Caspase-3 can break this connection and results in NF-κB activation. In the present study, caspase-3 inhibitors (z-VAD fmk and z-DEVD fmk) prevented this degradation by caspase-3 and kept NF-κB inactivated in cytosol and resulted in NF-κB reduction in nucleus.

Inhibiting Apoptosis Increased Cytosolic NF-κB

In most cases, NF-κB resides in the cytoplasm in inactive form in the absence of stimulation and is retained by an inhibitory protein, IκB, which binds to P65. Loss of IκB exposes nuclear localization signals on P65, which facilitate transport of NF-κB dimer to the nucleus. Measurements of NF-κB protein levels in nuclear extracts reflect levels of activated NF-κB [38].

By this explanation we expected if NF-κB (P65) decreases in nuclear extraction (reduced NF-κB activation), it should increase in cytosolic extraction. For confirming our data about NF-κB activation, we measured cytosolic NF-κB. Data showed that inhibiting caspase-3, decreased NF-κB into the nucleus but increased its level into the cytosol (Fig. 4).These results imply that inhibiting caspase-3 leads to declining NF-κB activation.

Inhibiting Apoptosis Increased Cmyc

Cmyc is a proto-oncogene that regulates cell proliferation and apoptosis. Reduction of cmyc expression and its inappropriate expression can be associated with cellular apoptosis [39].Promotion of cell cycle progression by c-Myc can also be achieved by suppression of transcription of growth inhibitory genes, examples of these genes include gadd45, cdk (cyclin-dependent kinase) inhibitors p21cip1, p19ARF and probably also p27kip1. There is evidence that under certain conditions the role of c-Myc in cell cycle progression may require only its activity of transactivation, for instance, c-MycS, which lacks the transactivation activity but retains the trans-suppression activity, can still promote proliferation of several types of cells in culture. Further, it has been suggested that the trans-suppression may actually be more important for cell proliferation than the transactivation [20].

In the present study, inhibiting apoptosis caused an increase in cmyc cytosolic level about 1.5–2 fold, compared to control group (Fig. 6). Increasing cmyc as a proto-oncogene and one of the cancer hall marks can be a sign of cell proliferation following inhibiting apoptosis.

Inhibiting Apoptosis Increased Hsp70

Heat shock proteins (Hsps) are overexpressed in a wide range of human cancers and are implicated in tumor cell proliferation, differentiation, invasion, metastasis, death, and recognition by the immune system. Although Hsp levels are not informative at the diagnostic level, they are useful biomarkers for carcinogenesis in some tissues and signal the degree of differentiation and the aggressiveness of some cancers. In addition, the circulating levels of Hsp and anti-Hsp antibodies in cancer patients may be useful in tumor diagnosis. Furthermore, several Hsp are implicated with the prognosis of specific cancers, most notably Hsp27, whose expression is associated with poor prognosis in gastric, liver, and prostate carcinoma, and osteosarcomas, and Hsp70, which is correlated with poor prognosis in breast, endometrial, uterine cervical, and bladder carcinomas. Increased Hsp expression may also predict the response to some anticancer treatments. For example, Hsp27 and Hsp70 are implicated in resistance to chemotherapy in breast cancer. Hsp27 predicts a poor response to chemotherapy in leukemia patients, whereas Hsp70 expression predicts a better response to chemotherapy in osteosarcomas. Implication of Hsp in tumor progression and response to therapy has led to its successful targeting in

therapy by 2 main strategies, including: (1) pharmacological modification of Hsp expression or molecular chaperone activity and (2) use of Hsps in anticancer vaccines, exploiting their ability to act as immunological adjuvants [19, 40]. Most Hsps are constitutively expressed inside cells and serve routine function in assisting protein folding and transport, which occur in all living cells. Proteins active in this way are also referred to molecular chaperons [18]. The over expression of Hsp70 is associated with metastasis, whereas the repression of Hsp70 results in the inhibition of tumor cell proliferation [19, 41]. In our experiment, after injection of Aβ, Hsp70 increased but not significantly. Inhibiting apoptosis increased Hsp70 nearly twofold compared to control and Aβ-injected group (Fig. 5). Elevated expression of members of the Hsp70 family has also been reported in high-grade malignant tumors. In breast tumors, elevated expression of Hsp70 is associated with short term disease-free survival, metastasis, and poor prognosis among patients treated with combined chemotherapy, radiation therapy and hyper thermia [42]. So elevated level of Hsp70 in the present experiment, implied that inhibiting apoptosis can have vicious consequence in the hippocampus received Aβ.

Inhibiting Apoptosis Declined Cytosolic p53

p53 is the most commonly mutated gene in human cancers and more than 50% of human cancers contain p53 mutations. In the past decade, the roles of p53 in human cancers have been investigated extensively in many aspects and intervention to restore wild-type p53 activities is an attractive approach for cancer therapy. Also, cancer predisposition, onset and therapeutic response can be critically determined by the integrity of the tumor suppressor p53 [21].

The p53 tumor suppressor gene encodes a nuclear phosphoprotein that functions as a key regulator of DNA repair, cell cycle progression, and apoptosis. The p53 protein is upregulated in response to a diverse array of cellular stresses, including DNA damage, hypoxia, oxidative stress and oncogene activation. p53 promotes apoptosis by modulation the expression of some proapoptotic genes such as: Bax and fas [42–45]. Hyper thermia was shown some years ago to markedly inhibit cell proliferation and tumor growth. Both p53-dependent and independent mechanisms have been evoked in the induction of apoptosis by hyper thermia [46], so decreased p53 level can be a menace of cell proliferation to the cell.

According to the present data, Aβ caused a significant increase in cytosolic p53. But inhibiting apoptosis reduced p53 1.5–2 fold compared to Aβ-injected group (Fig. 7). This shows apoptosis blocking digresses the cell fate to the cell proliferation.

Conclusion

In this study we induced neuroinflammation in hippocampus by injecting Aβ (1–42). TNF-α was increased as a marker of inflammation. Aβ also induced apoptosis. Caspase-3 increased and we observed TUNE1-positive cells. After inhibiting apoptosis, nuclear NF-κB level didn't increase significantly. So we guess there is not any considerable relationship between apoptosis and NF-κB pathways which are initiated by increasing TNF-α level. If there were any relationship, by blocking apoptosis pathway, TNF-α activation would shift from apoptosis to NF-κB activation and disturb the balance between apoptosis and cell survival. Although we couldn't show any increase in NF-κB activation, we have evidences that shows cell survival.

After inhibiting apoptosis, p53 as a proapoptotic factor decreased cmyc, a proto-oncogene increased. Hsp70 acts as a chaperon and increases when cells encounter dangerous situations and has been mentioned as a hall marke of cancer. Inhibiting apoptosis resulted in a significant increase of Hsp70. All these changes lead to cell proliferation and maybe increase the danger of carcinogenesis.

It may be that inhibiting apoptosis doesn't convey to carcinogenesis, but it has shown some high risk cellular and molecular changes. So, it implies inhibiting apoptosis is not an appropriate treatment strategy in neuroinflammatory diseases like AD.

Acknowledgments The authors would like to thank Neuroscience Research Center for financial support of this study.

References

1. Chauveau F, Boutin H, Van Camp N et al (2008) Nuclear imaging of neuroinflammation: a comprehensive review of [11C] PK11195 challengers. Eur J Nucl Mol Imaging 35:2304–2319
2. Gahtan E, Overmier BC (1999) Inflammatory pathogenesis in Alzheimer's disease: biological mechanisms and cognitive sequel. Neurosci Biohav Rev 23:615–633
3. Allan SM, Rothwell NJ (2003) Inflammation in central nervous system injury. Phil Trans R Soc Lond B 358:1669–1677
4. Monsonego A, Imitola J, Zota V et al (2003) Microglia-mediated nitric oxide cytotoxicity of T cells following amyloid β-peptide presentation to Th1 cells. J Immunol 171:2216–2224
5. Royuela M, Rodriguete-Berriguete G, Fraile B et al (2008) TNF-α/IL-1/NF-κB transduction pathway in human cancer prostate. Histol Histopathol 23:1279–1290
6. Akiyama H, Barger S, Barnum S et al (2000) Inflammation and Alzheimer's disease. Neurobiol Aging 21:383–421
7. Combs CK, Karlo JC, Kao SC, Landreth GE et al (2001) β-amyloid stimulation of microglia and monocytes results in TNF-α-dependent expression of inducible nitric oxide synthase and neuronal apoptosis. J Neurosci 21(4):1179–1188
8. Gupta S (2002) A decision between life and death during TNF-α-induced signaling. J Clin Immunol 22(4):185–194
9. Karin M, Greten FR (2005) NF-κB: linking inflammation and immunity to cancer development and progression. Nature 5:749–759

10. Aggarwal BB (2004) Nuclear factor-κB: the enemy within. Cancer Cell 6:203–208
11. Stelt MV, Mazzola C, Esposito G et al (2006) Endocannabinoids and β-amyloid-induced neurotoxicity in vivo: effect of pharmacological elevation of endocannabinoid levels. Cell Mol Life Sci 63:1410–1424
12. Troy CM, Rabacchi SA, Xu Z et al (2001) β-Amyloid-induced neuronal apoptosis requires c-Jun N-terminal kinase activation. J Neurochem 77:157–164
13. Xiao XQ, Zhang HY, Tang XC (2002) Huperzine A attenuates amyloid β-peptide fragment 25–35-induced apoptosis in rat cortical neurons via inhibiting reactive oxygen species formation and caspase-3 activation. J Neurosci Res 67:30–36
14. Nilsen J, Chen S, Irwin RW et al (2006) Estrogen protects neuronal cells from amyloid beta-induced apoptosis via regulation of mitochondrial proteins and function. BMC Neurosci 7(47):1–14
15. Watson K, Fan GH (2004) Macrophage inflammatory protein 2 inhibits β-amyloid peptide (1–42)-mediated hippocampal neuronal apoptosis through activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase signaling pathways. Mol Pharmacol 67:757–765
16. Jang JH, Surh YJ (2005) β-Amyloid-induced apoptosis is associated with cyclooxygenase-2 up-regulation via the mitogen-activated protein kinase-NF-κB signaling pathway. Free Radical Biol Med 38:1604–1613
17. Darin MD, Gustavo C, Christi A et al (2006) Nucleoplasmic β-actin exists in a dynamic equilibrium between low-mobility polymericspecies and rapidly diffusing populations. JCB 172(4): 541–552
18. Noort JM (2008) Stress proteins in CNS inflammation. J Pathol 214:267–275
19. Ciocca DR, Calderwood SK (2005) Heat shock proteins in cancer: diagnostic, prognostic, predictive, and treatment implications. Cell Stress Chaperones 10(2):86–103
20. Lio DJ, Dickson RB (2000) c-Myc in breast cancer. Endocr Relat Cancer 7:143–164
21. Guan YS, He Q, La Z (2006) Roles of p53 in carcinogenesis, diagnosis and treatment of hepatocellular carcinoma. J Can Mol 2(5):191–197
22. Halliday G, Robinson SR, Shepherd C et al (2000) Alzheimer's disease and inflammation: a review of cellular and therapeutic mechanisms. Clin Exp Pharmacol Physiol 27:1–8
23. Padmanabhan J, Levy M, Dickson DW et al (2006) Alpha 1-antichymotrypsin, an inflammatory protein overexpressed in Alzheimer's disease brain, induces tau phosphorylation in neurons. Brain 129:3020–3034
24. Medeiros R, Prediger RDS, Passos GF et al (2007) Connecting TNF-α signaling pathways to iNOS expression in a mouse model of Alzheimer's disease: relevance for the behavioral and synaptic deficits induced by amyloid β protein. J Neurosci 27(20):5394–5404
25. Lin WW, Karin M (2007) A cytokine-mediated link between innate immunity, inflammation, and cancer. J Clin Invest 117: 1175–1183
26. Luo JL, Maeda S, Hsu LC et al (2004) Inhibition of NF-κB in cancer cells converts inflammation-induced tumor growth mediated by TNF-α to TRAIL-mediated tumor regression. Cell 6: 297–305
27. Luo JL, Kamata H, Karin M (2005) IKK/NF-κB signaling: balancing life and death—a new approach to cancer therapy. J Clin Invest 115(10):2625–2632
28. Lee YB, Yune TY, Baik SY et al (2000) Role of tumor necrosis factor-α in neuronal and glial apoptosis after spinal cord injury. Exp Neurol 166:190–195
29. Cowburn AS, White JF, Deighton J et al (2005) z-VAD-fmk augmentation of TNF-α-stimulated neutrophil apoptosis is compound specific and does not involve the generation of reactive oxygen species. Blood 105:2970–2972
30. Mattson MP (2004) Pathways towards and away from Alzheimer's disease. Nature 430:631–639
31. Stepanichev MY, Zdobnova IM, Yakovlev AA et al (2003) Effects of tumor necrosis factor-alpha central administration on hippocampal damage in rat induced by amyloid beta-peptide (25–35). J Neurosci Res 71:110–120
32. Muller T, Meyer HE, Egensperger R et al (2008) The amyloid precursor protein intracellular domain (AICD) as modulator of gene expression, apoptosis, and cytoskeletal dynamics-relevance for Alzheimer's disease. Prog Neurobiol 85:393–406
33. Behl C (2000) Apoptosis and Alzheimer's disease. J Neural Transm 107:1325–1344
34. Song YS, Park HJ, Kim SY et al (2004) Protective role of Bcl-2 on β-amyloid-induced cell death of differentiated PC12 cells: reduction of NF-κB and p38 MAP Kinase activation. Neurosci Res 49:69–80
35. Kinsky TK, Papaconstantinou J, Perez-Polo JR (1997) Age-associated alterations in hippocampal and basal forebrain nuclear factor kappa B activity. J Neurosci Res 48:580–587
36. Alexanian AR, Bamburg JR (1999) Neuronal survival activity of S100β is enhanced by calcineurin inhibitors and requires activation of NF-κB. FASEB J 13:1611–1620
37. Liu Q, Zhang J, Zhu H et al (2007) Dissecting the signaling pathway of nicotine-mediated neuroprotection in a mouse Alzheimer's disease model. FASEB J 21:61–73
38. Tripathy D, Grammas P (2009) Acetaminophen inhibits neuronal inflammation and protects neurons from oxidative stress. J Neuroinflammation 6:1–9
39. Kim HS, Jeong SY, Lee JH et al (2000) Induction of apoptosis in human leukemia cells by 3-deazaadenosine is mediated by caspase-3-like activity. Exp Mol Med 32:197–203
40. Helmbrecht K, Zeise E, Rensing L (2000) Chaperones in cell cycle regulation and mitogenic signal transduction: a review. Cell Prolif 33:341–365
41. Wu G, Osada M, Guo Z, Fomenkov A et al (2005) ΔNp63α up-regulates the HSP70 gene in human cancer. Cancer Res 65(3): 758–766
42. Jolly C, Morimoto RI (2000) Role of the heat shock response and molecular chaperones in oncogenesis and cell death. J Natl Cancer Inst 92:1564–1572
43. Morrison RS, Kinoshita Y, Johnson MD et al (2003) P53-dependent cell death signaling in neurons. Neurochem Res 28:15–27
44. Miller FD, Pozniak CD, Walsh GS (2000) Neuronal life and death: an essential role for the P53 family. Cell Death Differ 7:880–888
45. Culmsee C, Zhu X, Yu Q et al (2001) A synthetic inhibitor of p53 protects neurons against death induced by ischemic and excitotoxic insults, and amyloid β-peptide. J Neurochem 77:220–228
46. Basile A, Biziato D, Sherbet GV et al (2008) Hyperthermia inhibits cell proliferation and induces apoptosis: relative signaling status of P53, S100A4, and Notch in heat sensitive and resistant cell lines. J Cell Bio Chem 103:212–220