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Potential Role of Ursodeoxycholic Acid in Suppression of Nuclear Factor Kappa B in Microglial Cell Line (BV-2)

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Expression of the NF-κB-dependent genes responsible for inflammation, such as TNF-α, IL-1β, and nitric oxide synthase (NOS), contributes to chronic inflammation which is a major cause of neurodegenerative diseases (i.e. Alzheimer's disease). Although NF-κB plays a biphasic role in different cells like neurons and microglia, controlling the activation of NF-κB is important for its negative feedback in either activation or inactivation. In this study, we found that ursodeoxycholic acid (UDCA) inhibited IκBα degradation to block expression of the NF-κB-dependent genes in microglia when activated by β-amyloid peptide (Aβ). We also showed that when microglia is activated by Aβ42, the expression of A20 is suppressed. These findings place A20 in the category of "protective" genes, protecting cells from pro-inflammatory repertoires induced in response to inflammatory stimuli in activated microglia *via* NF-κB activation. In light of the gene and proteins for NF-κB-dependent gene and inactivator for NF-κB (IκBα), the observations now reported suggest that UDCA plays a role in supporting the attenuation of the production of pro-inflammatory cytokines and NO *via* inactivation of NF-κB. Moreover, an NF-κB inhibitor such as A20 can collaborate and at least enhance the anti-inflammatory effect in microglia, thus giving a potent benefit for the treatment of neurodegenerative diseases such as AD.

Key words: Nuclear factor kappa B, IκBα, A20, Ursodeoxycholic acid, β-Amyloid peptide, Alzheimer's disease

INTRODUCTION

Nuclear factor-κB (NF-κB) has long been known to play a central role in the immune systems by regulating the expression of genes involved in immune response, apoptosis, inflammation, and the cell cycle. The family of NF-κB transcription factors is a central component of the cellular response to a broad range of extracellular signals, many of which are related to immunological functions and stress. For example, NF-κB controls the expression of a large number of genes including inflammatory cytokines, chemokines, immunological factors, adhesion molecules, cell cycle regulators, and pro- and antiapoptotic factors (Pahl, 1999). A major pathway regulating NF-κB activity involves its nuclear transport. In unstimulated cells, NF-κB is retained in the cytoplasm in an inactive form by IκB proteins. However, when activated by a wide range of extracellular agents, IκB is phosphorylated by a protein kinase (the identity of which remains unknown), resulting in ubiquitination and degradation by the proteosome. This allows NF-κB to translocate to the nucleus where it binds to the κB consensus sequence, generally increasing the expression of the target gene. Several reports have described NF-κB activation in glial and astrocyte cell lines, including murine primary astrocytes, microglia, and Schwann cells (Moynagh *et al.*, 1993; Kaltschmidt *et al.*, 1994; Carter *et al.*, 1996). Activation of NF-κB was found to be regulated in a similar way to that in cells in the periphery, in that NF-κB occurred in a latent form and was activated by pro-inflammatory cytokines such as IL-1 and TNF. In addition, it was shown to be capable of regulating such NF-κB-dependent genes as IL-6, ICAM, VCAM-1, MHC Class I and inducible nitric oxide synthase (iNOS) (Norris *et al.*, 1994; Dhib-Jalbut *et al.*, 1995). This implies that NF-κB could play a role in immune and inflammatory responses in the brain, and further, that the induction of NOS indicated a role for NF-κB in synaptic plasticity. The potential role of NF-κB in neurodegenerative disease in which inflammation in the central nervous system (CNS) is likely to be important, such as multiple sclerosis and

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Alzheimer's disease (AD). Many studies have been reported that the neurotoxic peptide Aβ, which is deposited in the plaques of AD patients, can activate NF-κB (Behl *et al.*, 1994; Kalschmidt *et al.*, 1997). In fact, NF-κB was found to be activated in regions around early stages in AD patients (Kalschmidt *et al.*, 1997). It has also been suggested that inflammatory and immune mechanisms play an important role in the pathophysiology of AD (Aisen and Davis, 1994). Cytokines, such as interleukin-1 (IL-1), IL-6, and TNF, which are an earlier sign of AD due to the mediation of acute phase proteins, and α-1-antichymotrypsin that may become part of the amyloid deposit of senile plaque, have elevated levels both in the serum and brain extracts (Bauer *et al.*, 1991). With respect to this fact, the important thing is that NF-κB controls their expression upon stimulation.

As described above, NF-κB is a transcription factor that regulates the expression in inflammation and survival genes. Importantly, NF-κB is maintained in an inactive state in the cytoplasm bound to the inhibitory protein inhibitor of IκBα, which masks its nuclear location domain (Baldwin, 1996). The phosphorylation process is mediated by the IκB kinase (IKK) complex (Israel, 2000). NF-κB terminates its own activation by inducing the expression of its inhibitor IκBα, which then binds, inactivates, and translocates the transcription factor back to the cytosol (Ghosh and Karin, 2002). NF-κB also induces A20 proteins, which belong to a class of Cys_2/Cys_2 zinc finger proteins and which inhibit IKK-complex activity. This supports the reaccumulation of IκBα in the cytosol (Lee *et al.*, 2000). This implies that severe inflammation and fatal tissue damage can be developed when A20 is not expressed. AD is progressed into more severe disease state due to chronic inflammation caused by senile plaques found in AD brain.

Ursodeoxycholic acid (UDCA) is a hydrophilic bile acid that has been shown to improve clinical and biochemical indices in cholestatic liver disease, hyperbilirubinemia and excretion disorder of bromsulphalein, all of which occur in impaired livers (Cjaza *et al.*, 1999). When administered, UDCA is rapidly conjugated with either glycine or taurine, forming glyco-UDCA (GUDCA) and tauro-UDCA (TUDCA). An entire understanding on the cytoprotective mechanisms is unavailable at the present time, but there are several reports on the effect of cell protection impacted by apoptosis. UDCA and its conjugates can prevent several apoptotic events, including mitochondrial release of cytochrome c, cytosolic caspase activation, and cleavage of the nuclear enzyme PARP (Rodrigues *et al.*, 1999). In previous study, we reported that UDCA suppressed NO and IL-1β in microglial cells (Joo *et al.*, 2003). Based on this interest, we assumed that UDCA may have a potential role in the suppression of chronic inflammation by inactivating NF-κB. In the present study, we assessed the potential role of UDCA in the suppression of NF-κB.

MATERIALS AND METHODS

Stock preparations

Stock UDCA, Aβ42, cyclosporine-A (CsA), SNAP (S-nitroso-*N*-acetylpenicillamine, NO donor), and LPS (lipopolysaccharide) solutions were prepared by dissolving in microfiltered (0.2 μm) culture media at concentrations of 50 μg, 100 μg, 150 μg, and 300 μg/mL for UDCA, 5 μg/mL for rat Aβ42 (Bachem), 5 μg/mL for CsA, 200 μM for SNAP, and 5 μg/mL for LPS. The solutions were either kept at -20°C for future use or diluted directly in 1-5% FBS α-MEM culture medium for cell culture experiments.

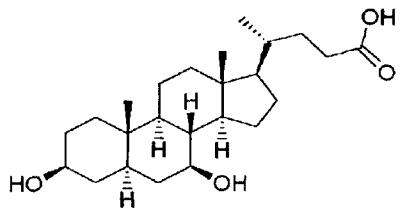

Fig. 1. Chemical structure of Ursodeoxycholic Acid

Cell culture

BV-2 cells were grown in 10% FBS α-MEM (without RNA or DNA) for three days after thawing and refreshed with 5%FBS α-MEM on day four. As <5% FBS did not activate BV-2 cells, all studies were carried out in 1-5% FBS α-MEM media after one-day overnight before use.

Measurement of nitric oxide and iNOS

Study methodologies were based on routine lab methods; NO detection with Griess reagent (Promega), and RT-PCR (Table I) under the most optimistic conditions. In brief, the cells at a density of 1.5×10^5/mL were plated in 6-well plates and treated with each sample for the designed culture times. For NO measurement, Griess reagents were used in accordance with the manufacturers instruction. UDCA concentrations were varied from 50 μg/mL to 300 μg/mL and the experiments were performed under various conditions, such as culture media, LPS, and Aβ42, in the

Table I. Primer sequences for PCR

iNOS (382bp)	Sense anti-sense	5'- ggc acc gag att gga gtt cg -3' 5'- gcc aga tgt ggg tct tcc ag -3'	30cy
A20 (500bp)	Sense anti-sense	5'- tgc tac gac act cgg aac tg -3' 5'- agg tga gtc gtg ccg tgg tc -3'	
TNF-α (418bp)	Sense anti-sense	5'- gtg aca agc ctg tag ccc ac 3' 5'- tgc ccg gac tcc gca aag tc -3'	
β-Actin (186bp)	Sense anti-sense	5'- tga ccg agc gtg gct aca gc- 3' 5'- acc gct cat tgc cga tag tg - 3'	

presence or absence of UDCA and CsA, for 6, 12, 24, and 48 h. After the culture times, total RNA was obtained from 1×10^7/mL cells using Trizol reagent (Life Technologies). In brief, the cells were lysed using 1 mL Trizol reagent, 200 μL chloroform was added after 1 min incubation at room temperature, and the mixture was centrifuged at 13500 rpm for 15 min. The aqueous layer (about 450 μL) was transferred to another tube and the RNA was precipitated by centrifugation with 450 μL isopropanol at 13500 rpm for 10 min. RNA pellets were washed by 700 μL cold Et-OH (70% in DEPC water) and dried in air. Total reaction volume was 20 μL and the amplified cDNA was separated on a 1.2% agarose gel stained with ethidium bromide. PCR band intensities were expressed as OD values using UVIDocMw program.

Western blot

Protein samples (20 μg) extracted from cytosol or nuclear were heated in SDS loading buffer at 90°C for 5 minutes before separation on 12% SDS-polyacrylamide mini-gels and transferred to PVDF membrane (Appligene). Membranes were blocked using 5% skim milk in TBS-T buffer solution and probed with primary antibodies directed against each protein, IκBα (polyclonal C-21, sc-37; Santa Cruz Biotech), p-IκBα (monoclonal B-9, sc-8404; Santa Cruz Biotech), or NF-κB p65 and p50 (polyclonal C-20; Delta Biolabs). Bound antibodies were detected using species-specific secondary antibodies conjugated to HRP.

Statistical analysis

The data was analyzed as mean±SEM. Statistical significance was determined using Student's *t*-test. Significant differences were found by using SPSS software (ver. 10).

RESULTS

Nitric oxide production and iNOS expression

As a first step to investigate the factor responsible for NF-κB activation, we scrutinized the level of NO in culture media and iNOS gene expression (Fig. 1A,1B), which are known to increase when NF-κB is activated (Akama *et al.*, 1998). To determine the relationship between NO production and iNOS expression in the presence of β-amyloid, which can cause neurodegeneration *via* microglia activation and which is known to stimulate NF-κB, we varied the times from 6 h to 48 h and LPS was used for a stimulative control. In addition, to compare the inhibition of NO, CsA was used as a positive control. As shown in Fig. 1A, we found that NO production was suppressed when treated with UDCA and CsA, and that the amount of NO was minimized at 48 h compared to control (culture media). On the other hand, the expression of iNOS gene showed the same pattern of NO production. Pre-treatment with Aβ42 in UDCA and CsA group was not as stimulative as Aβ42 single treatment at 24 h and 48 h.

Expression of TNF-α

TNF-α, a proinflammatory cytokine, was highly produced in the presence of Aβ42 in both ELISA and RT-PCR observation at 24 h (Fig. 2A, 2B). The level of TNF-α production was compared with that of single treatment and of their co-treatment, such as UDCA+Aβ42 and CsA+Aβ42. The Aβ42-pretreated UDCA group well regulated the production of TNF-α as much as shown in CsA-treated group. No significant difference was found between UDCA+Aβ42 and CsA+Aβ42 groups, but TNF-α production was relatively lower in the CsA+Aβ42 group. TNF-α production did not fluctuate by time variation in culture

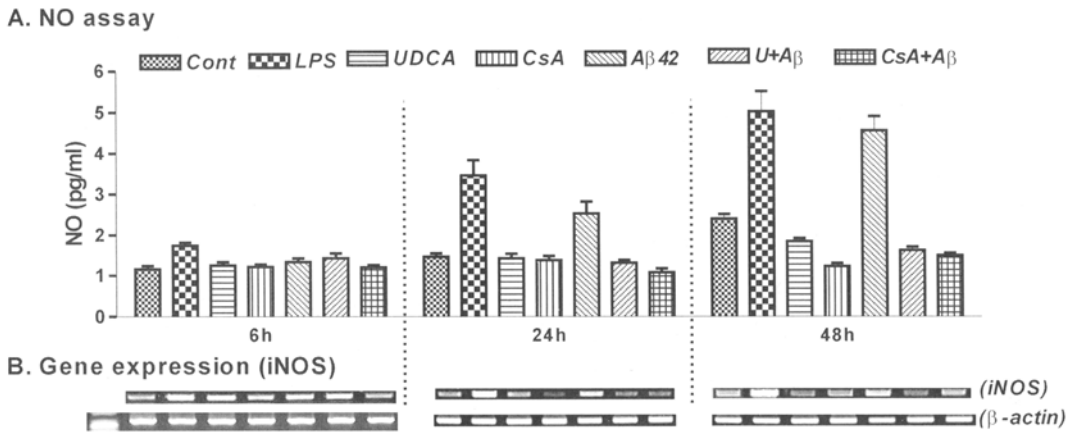

Fig. 1. Comparison of nitric oxide level and gene expression of iNOS. A. Nitric oxide was detected by Griess reagent system from 6 h to 48 h at 540 nm. B. mRNA expression of iNOS was confirmed by using reverse transcription (RT)-PCR. For RT-PCR, total RNA was extracted from the same cell cultures that NO was assayed. Both results demonstrate a similar pattern between iNOS expression and NO production even though they are not exactly identical. Cont; control (culture media), LPS; lipopolysaccharide, CsA; cyclosporine-A, Aβ(42); β-amyloid peptide. All results were repeated three times from independent experiments.

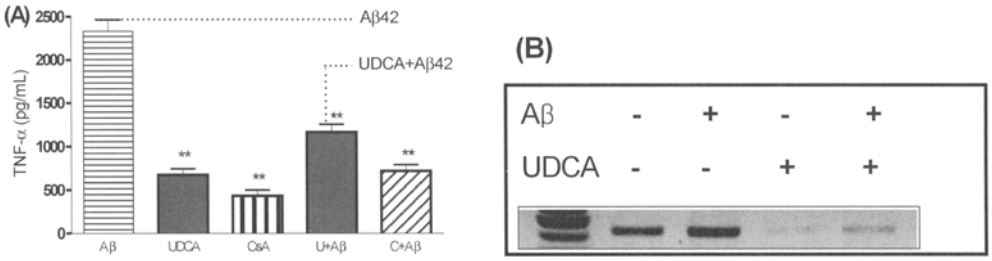

Fig. 2. Production of TNF-α from different treatments in BV-2. TNF-α protein level was determined by ELISA (A), and the expression of TNF-α gene was observed by RT-PCR method to confirm the suppressive effect on TNF-α (B). Final assays were done at 24 h. 100 μg/mL UDCA; 5 μg/mL cyclosporine-A; 5 μg/mL Aβ42. Averages of triplicates ± SD are shown and statistical significances were confirmed by Dunnetts Multiple Comparison Test vs. Aβ group. **, $p < 0.01$.

media, but UDCA and CsA were kept at a lower level (data not shown). However, the Aβ42 groups, including UDCA+Aβ42 and CsA+Aβ42, increased the production of TNF-α in absolute amount (pg/mL) in spite of the fact that UDCA and CsA downregulated the level of TNF-α. Fig. 2B shows that UDCA can suppress the Aβ-induced activation for the expression of TNF-α mRNA.

A20 gene expression

The level of A20 was observed in single groups of UDCA, CsA, and Aβ42, and was compared with Aβ42-treated UDCA and CsA group (Fig. 3) in BV-2 cell line. A20 mRNA levels were quantified by GelDoc system (UVIDocMw) according to their band density with an arbitrary unit. To demonstrate the role of A20 in NF-κB inhibitory function, we examined whether A20 expression is upregulated in the presence of UDCA to scrutinize a possible function of UDCA inhibiting NF-κB activation. Single groups of UDCA, CsA, and Aβ42, and co-treatment groups of UDCA+Aβ42 and CsA+Aβ42, were observed for their impact on A20 mRNA expression. The comparative tendency of the mRNA level is shown in summary graph (Fig. 3). A significant difference was found at 48 h, which indicated that the mRNA was increased in both

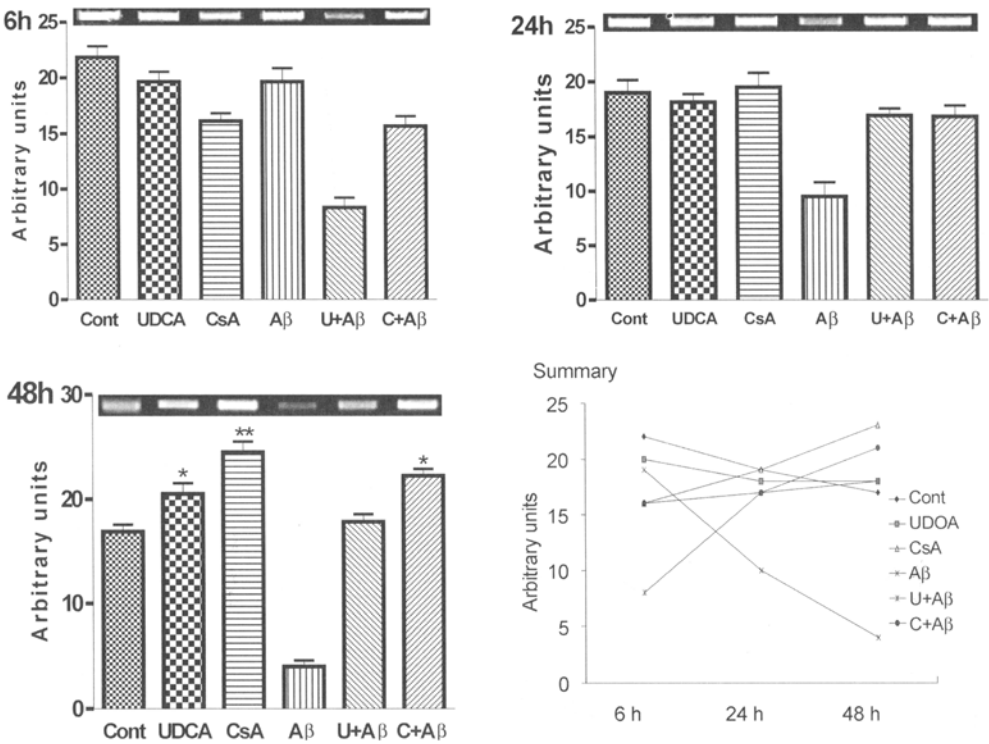

Fig. 3. Expression of A20 mRNA after treatment with different samples, UDCA, cyclosporine-A, Aβ42, and co-treatment with Aβ42 in the presence of UDCA or cyclosporine-A. A significant increase of A20 mRNA was detected from 48 h compared to control, whereas Aβ42-treated group was dramatically decreased in a time dependent manner. Patterns of mRNA expression are shown in the summary figure. A20 mRNA was detected by using reverse transcription (RT)-PCR. The data represent means ± SD of three determinations. *, $p<0.05$; **, $p<0.001$.

UDCA and CsA single treatments and that the Aβ42-pretreated UDCA and CsA groups were not affected by Aβ42 treatment.

Regulation of NF-κB and IκBα protein levels

NF-κB is transcriptionally active when it is not bound to its inhibitor IκBα. Degradation of IκBα is initiated *via* phosphorylation mediated by the IKK (IκB kinase) complex. The IKK complex is activated by a variety of stimuli, including oxyradicals, TNF-α, and amyloid β-peptide (i.e. Aβ42). As part of the feed-back regulation after NF-κB activation, IκBα mRNA expression is induced leading to reaccumulation of IκBα protein and to inactivation of NF-κB. A20 exerts its NF-κB inhibitory function by supporting the reaccumulation of IκBα protein in the cytosol (Lee *et al.*, 2000). We examined whether NF-κB activation can be inhibited by both A20 and reaccumulation of IκBα, and whether phosphorylated IκBα is reduced due to the collaboration of A20 and IκBα. BV-2 cells were incubated either with LPS, SNAP, UDCA, CsA, Aβ42, UDCA+Aβ42, or CsA+Aβ42 for 24 h and cytosolic and nuclear protein extracts were subjected to Western blotting with an IκBα, phosphorylated IκBα, and NF-κB (p65)/(p50) specific antibody (Fig. 4A, 4B). LPS and SNAP (NO donor) were used for NF-κB stimuli. The phosphorylated IκBα that loses its inhibitory function and is soon to be degraded by the proteasome (Baeuerle, 1996) was greatly reduced when treated by UDCA and CsA, and co-treatment with Aβ42 also led to downregulation compared to control groups and Aβ42 single treatment (Fig. 4A). In addition, IκBα was increased in the presence of UDCA. As shown in Fig. 4B, p50 (also called NF-κB1), which is known to bind specifically to the κB-site of the gene, was reduced in both UDCA and CsA treatments and the protein was downregulated even in the presence of Aβ42 compared to Aβ42 single treatment. p65 in cytosol was not comparable with other groups, but it was found to reduce in nuclear extracts when treated with UDCA and CsA (Fig. 4A). Moreover, p50 showed a meaningful change suggesting that UDCA regulates the translocation of p50 when comparing to positive controls and Aβ single treatment (Fig. 4B).

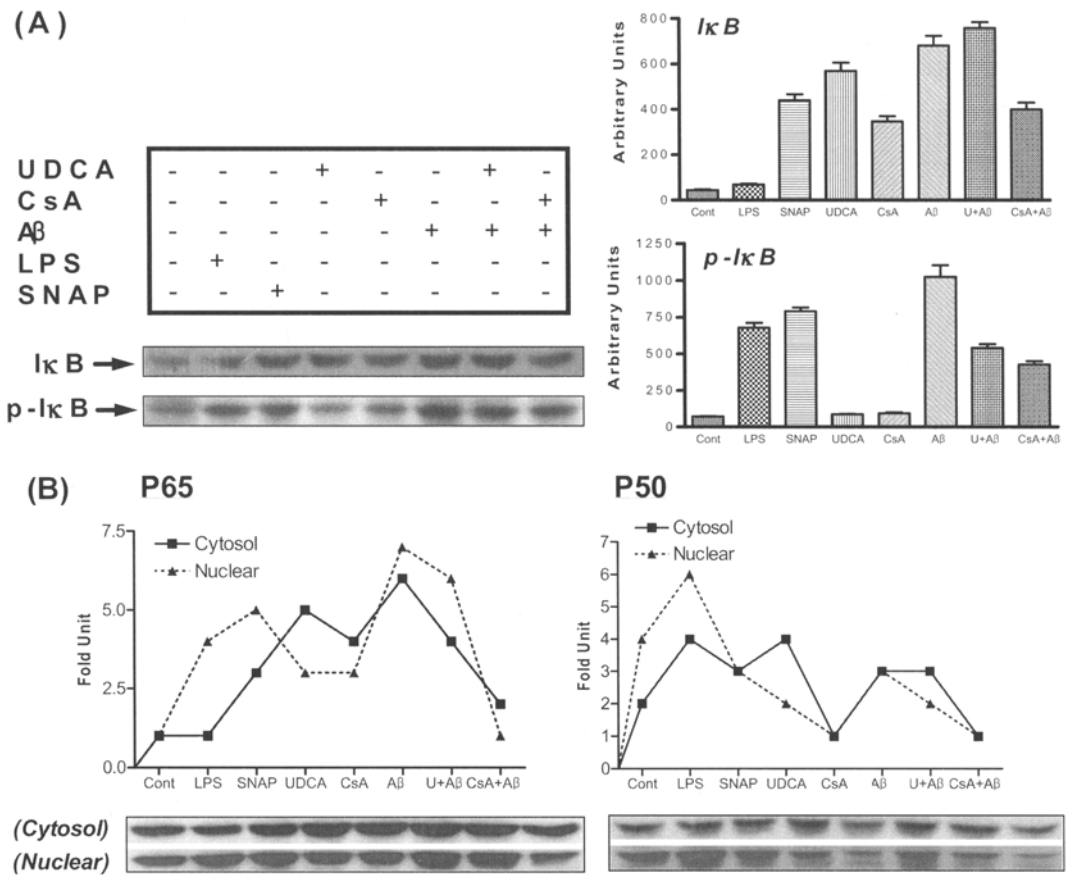

Fig. 4. Protein expression of NF-κB and IκBα after treatment of each sample for 24 h. Western blots of cytosolic and nuclear protein extracts probed with antibodies directed against IκBα, p-IκBα, p65, or p50 are shown. Cells were harvested at 24 h. (A) Degradation of IκBα was confirmed by detection of the phosphorylated IβBα. (B) NF-κB proteins, p65 and p50 in cytosol, were separately probed with each primary antibody and compared with nuclear extracts. 200 μM *S*-nitroso-*N*-acetylpenicillamine (SNAP) was used as NO donor for NF-κB activation.

DISCUSSION

Studies from AD patients have revealed increased NF-κB activity in cells involved in the neurodegenerative process. Several studies have shown that Aβ-peptide can activate NF-κB in cultured neurons (Barger *et al.*, 1995), suggesting a molecular mechanism by which amyloid may act during AD pathogenesis. This activation of NF-κB may be neuroprotective, since TNF-α can protect neurons against Aβ-induced death *via* NF-κB-mediated mechanism. In other words, activation of NF-κB in neurons induces production of antiapoptotic gene productions involved in modulating synaptic plasticity, whereas activation of NF-κB in glial cells results in production of proinflammatory cytokines, and potentially neurotoxic reactive oxygen species and excitotoxins. Therefore, inhibitors of the NF-κB signaling pathways may downregulate the inflammatory properties of activated microglia. In our study, LPS and Aβ42 were used as inducers of proinflammatory properties in BV-2 cell line, for which Aβ42 was regarded as a causal factor for AD. The NF-κB-regulated A20, which is involved in the feedback termination of NF-κB activity, can function as a potent inhibitor of NF-κB-dependent gene expression. Evidence for this function has been observed in a study that found overexpression of A20 blocked the activation of NF-κB by TNF, IL-1, LPS, and hydrogen peroxide in different cell types (Ferran, 1998). IκBα has been known to have a similar effect, which directly binds NF-κB and retains it as the cytoplasm in a dormant form. This protein itself has several κB sequences in its promoter region, so that NF-κB induces its synthesis. IκBα can then enter the nucleus to bind to the activated NF-κB and cause the return of NF-κB to the cytoplasm, thus terminating activation (Arenzana-Seisdedos *et al.*, 1995).

In our study, we have demonstrated that UDCA can play a role in inhibiting NF-κB activation by several signal factors, such as A20, IκBα, and NF-κB dimer (p65/p50). We have shown that key proinflammatory cytokine and NO were suppressed to release in a microglial cell line, and several signal transductions were considered as its trigger. As mentioned above, in microglia NF-κB can act as an inducer of proinflammatory process, which is strongly correlated with AD pathogenesis. In the present study, we found that UDCA effectively controlled the production of NO in both NO (Griess system) and iNOS gene expression (RT-PCR) (Fig. 1B). Particularly, UDCA pulled the level of NO production, even in co-treatment with Aβ42, to a similar level to that of the CsA result. In addition, TNF-α, in ELISA, was well controlled in the UDCA group (Fig. 2). However, TNF-α suppression in Aβ-co-treated UDCA was not as high as that in UDCA single treatment. It was therefore considered that the activation by Aβ42 was stronger than that of UDCAs suppressive role. Although study results longer than 48 h were not shown in the figure, we found that the tendency of TNF-α production was to decrease.

The expression of A20 was compared between UDCA and other controls such as LPS, Aβ42, and CsA (Fig. 3). Interestingly, we found that A20 mRNA was significantly suppressed to express from 6 h to 48 h when treated with Aβ42, whereas the UDCA and CsA treated group did not affect A20 expression up to 24 h. However, A20 gene expression started to increase from 48 h. This suggests that UDCA may play a role in enhancing the expression of A20 gene in BV-2, and thus it can inhibit the activation of NF-κB by a negative feedback in its signal mechanism. This result was supported by the signaling proteins,IκBα, phosphorylated IκBα, and NF-κB heterodimers (p65/p50). In turns, we were able to show that the expression of A20 in BV-2 can be correlated with IκBα and NF-κB heterodimers (p65/p50) in western blot studies of those proteins. As shown in Fig. 4A and 4B, UDCA reduced the level of phosphorylated IκBα and NF-κB (p50) in cytosol/nuclear and this phenomena was shown in the Aβ42-pretreated UDCA group, even though IκBα and NF-κB (p65) were not significantly affected. These results imply that IκBα degradation by phosphorylation can be inhibited when treated with UDCA, directly or indirectly by increasing the production of IκB (Fig.4A). The decreased p50 may reduce the frequency of being dimerized with p65, which is known to translocate to the nucleus and induce gene expression (Hou *et al.*, 2003).

In conclusion, UDCA appears to be a promising drug candidate for the anti-inflammatory process by suppressing proinflammatory repertoires such as TNF-α, IL-1β, and NO. Several pieces of evidence found in our study may introduce a new potential scientific access to AD treatment as well as prevention, since UDCA may have the ability to control the activation of NF-κB via a negative feedback mechanism and thereby to inactivate NF-κB, even though some factors stimulate, and IκB kinase activates, NF-κB complex. It will be important to determine how UDCA clearly contributes to treat neurodegenerative diseases such as early- or late-onset AD.

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