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Ursodeoxycholic Acid Inhibits Pro-Inflammatory Repertoires, IL-1β and Nitric Oxide in Rat Microglia

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Ursodeoxycholic acid (UDCA) is a non-toxic, hydrophilic bile acid in widespread clinical use mainly for acute and chronic liver disease. Recently, treatment with UDCA in hepatic graft-versus-host disease has been given in immunosuppressive therapy for improvement of the biochemical markers of cholestasis. Moreover, it has been reported that UDCA possesses immunomodulatory effects by the suppression of cytokine production. In the present study, we hypothesized that UDCA may inhibit the production of the pro-inflammatory cytokine, IL-1β, and nitric oxide (NO) in microglia. In the study, we found that 100 μg/mL UDCA effectively inhibited these two pro-inflammatory factors at 24 h and 48 h, compared to the Aβ42-pretreated groups. These results were compared with the LPS+UDCA group to confirm the UDCA effect. As microglia can be activated by several stimulants, such as Aβ42, in Alzheimers brain and can release those inflammatory factors, the ability to inhibit or at least decrease the production of IL-1β and NO in Alzheimers disease (AD) is essential. Using RT-PCR, ELISA and the Griess Reagent System, we therefore found that UDCA in Aβ42 pre-treated cultures played a significant role in suppressing the expression or the production of IL-1β and NO. Similarly, lipopolysaccharide (LPS) did not activate microglia in the presence of UDCA. Moreover, we found that UDCA exhibits a prolonged effect on microglial cells (up to 48 h), which suggests that UDCA may play an important role in chronic cell damage due to this long effect. These results further imply that UDCA could be an important cue in suppressing the microglial activation stimulated by massive Aâ peptides in the AD progressing brain.

Key words: Microglia, Ursodeoxycholic acid (UDCA), Alzheimer's disease, IL-1β, Nitric oxide

INTRODUCTION

Ursodeoxycholic acid (UDCA), a naturally-occurring bile acid found in small quantities in normal human bile and in larger quantities in the biles of certain species of bears, is a hydrophilic dihydroxylated bile acid which was first identified in the Chinese black bear (Shoda, 1927). Dried bear bile has been used for centuries as a remedy for liver disease in China, based on a long-standing belief that bear bile has curative properties (Beuers *et al.*, 1998). UDCA's actual mechanism of action is still not clear, but experimental evidence suggests three major mechanisms of action: protection of cholangiocytes against cytotoxicity of hydrophobic bile acids, stimulation of hepatobiliary secretion, and protection of hepatocytes against bile acid-induced apoptosis (Paumgartner and Beuers, 2002). More recently, it has been reported that UDCA can mediate the immunosuppressive effect in the partially hepatectomized rat (Liu *et al.*, 2002). In addition, UDCA suppresses MHC class I and II expression indirectly by reducing the stimulatory influence of hydrophobic bile acids and directly by the activation of glucocorticoid receptors. In this respect UDCA treatment has been shown to significantly reduce HLA class I and II expression (Hirano *et al.*, 1996). In addition to the effects on MHC genes, UDCA inhibits abnormal production of immunoglobulins and cytokines from peripheral blood mononuclear cells (Yoshikawa *et al.*, 1992). Moreover, the anti-inflammatory and chemoprotective effects of UDCA have been studied in an animal model (Invernizzi *et al.*, 1997). According to this study, UDCA inhibits the induction of inducible nitric oxide synthase (*i*NOS) and interleukin-1β (IL-1β) in a dose dependent manner with a substantial inhibition at 500 μM. These results may contribute to the anti-inflammatory actions of

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UDCA.

Although there has been significant development in the elongation of lifespan and the treatment of disease through medical advances, senile diseases have become a striking social affair due to the importance of both the diseases themselves and the associated medical costs. Many publications on senile diseases, especially Alzheimers disease (AD) which has over 11,000 reports in PubMed, reflect the importance and interest. Particularly, AD, a type of senile dementia, has provoked a lot of interest because it is a severe restriction on the healthy life in elderly people in both physical and psychological aspects.

The above mentioned AD, which is a neurodegenerative disease and is also known as the most common form of dementia, was first reported by Dr. A. Alzheimer in 1907. In patients with AD, senile plaques (SP) and neurofibrillary tangles are a common founding and are reported to play a major role in the disease progression (Lue *et al.*, 2002; Dickson *et al.*, 1993; Selkoe *et al.*, 1991). Aβ peptides produced from transmembraneous amyloid precursor protein (APP) and microtubule associated protein (tau protein) are known as the major causative factors of AD (Bayer *et al.*, 2001). The Aβ peptides from APP stimulate the brain glial cells (i.e. microglia), by dual cleavage action of β & γ secretase, to release pro-inflammatory mediators such as IL-1β, IL-6, and nitric oxide (NO), which leads to chronic inflammation in the brain by an escalation of reactive oxygen species, pro-inflammatory cytokines, and neurotoxic factors (Banati *et al.*, 1996; Giulian *et al.*, 1987, 1986). On the other hand, Aβ directly induces cytochrome c (Cyt c) release from mitochondria through a mechanism that is accompanied by profound effects on mitochondrial membrane redox status, lipid polarity, and protein order. Considering that AD is a chronic neurodegenerative disease, the concept that UDCA plays a certain role in the suppression of pro-inflammatory repertoires, such as NO and IL-1β, may be one prospective way in AD therapy. It has been reported that UDCA and TUDC (tauroursodeoxycholate) directly suppress Aβ-induced disruption of the mitochondrial membrane structure, suggesting a neuroprotective role for this bile salt (Rodrigues *et al.*, 1998). Furthermore, Aβ peptides accumulated intracellularly by APP processing might exert neurotoxicity by interacting with mitochondria and inducing mitochondrial swelling and release of Cyt c, which activates caspase-3 and finally can lead to apoptosis in neuronal cells and to neurodegeneration in AD (Kim *et al.*, 2002). This can also be a meaningful concept in AD therapy if UDCA, as reported previously, plays a role in suppressing the mitochondrial damage by way of oxidative stresses as well as chronic inflammation by cytokines. In this study, we wanted to elucidate the anti-inflammatory effect of UDCA in microglia at the level of the rat brain by screening the NO and cytokine expression (IL-1β) when stimulating microglia with Aβ42, cyclosporine, and the culture media. In addition to this, we expected that UDCA might be a potential element in AD therapy either alone or in combination use within the short-term future.

Based on this interest, we assumed that UDCA, in the presence of the strong stimulator, Aβ42, plays a role in cell protection and regulation of cell stressors such as NO and IL-1β. The purpose of this study was to evaluate the *in vitro* effect of UDCA on the suppression of NO and IL-1β in brain microglial cells, in the presence or absence of Aβ42. Studies were designed to investigate the production of such mediators in the level of gene (PCR) and protein (ELISA).

MATERIALS AND METHODS

Test animal

The study was performed using one-day Sprague Dawley (SD) rats.

Test drug

Ursodeoxycholic acid (UDCA, $C_{24}H_{40}O_4$; MW. 392.58) with 99% purity was provided by Oriental Pharmaceutical Company. Stock UDCA and Aβ42 solutions were prepared by dissolving in microfiltered (0.2 μm) culture media at concentrations of 50 μg, 100 μg, 150 μg, and 500 μg/mL for UDCA, and 20 μg/mL for rat Aβ42 (Bachem). 1 μg/mL of cyclosporin-A (CYS-A) was prepared for a positive control. The solutions were either kept at -20°C for future use or diluted directly in 5% FBS-DMEM culture medium for cell culture experiments.

Reagent and assay kits

FBS (Gibco, U.S.A.), Griess Reagent (Promega, U.S.A.), HEPES (Duchefa Biochem, Netherlands), L-glutamine (Gibco, U.S.A.), Penicillin-Streptomycin (Gibco, U.S.A.), rat IL-1β Enzyme Immunometric Assay Kit (TiterZyme® EIA, U.S.A.), Trypsin (Sigma Chem., U.S.A.), and RT & PCR premix (Bioneer, Korea) were used.

Measurement of NO, Cytokine, and RT-PCR

Study methodologies were based on routine lab methods, NO detection with Griess reagent (Promega), RT-PCR (RT-PCR Premix, Bioneer, Korea) (Table I), and EIA kit (Assay Designs, U.S.A.) under the most optimistic conditions. In brief, the cells at a density of 1.5×10^5/mL were plated in 24-well plate and treated with each sample for the designed culture times. For NO measurement, Griess reagents were used in accordance with the company's instruction. UDCA concentrations were varied from 50 μg/mL to 500 μg/mL and the experiments were performed under various conditions, such as culture media, lipopoly-

saccharide (LPS), and Aβ42, in the presence or absence of UDCA and CYS-A, for 6, 12, 24, and 48 h. After the culture times, EIA kit for IL-1 was prepared and secreted cytokines in cell culture supernatant fluid were determined at 450 nm using the plate reader. Total RNA was obtained from 2.5×10^5/mL cells using Trizol reagent (Life Technologies). In brief, the cells were lysed using 300 μL Trizol reagent and 30 μL chloroform was added after 1 min incubation at room temperature, and centrifuged at 15000 rpm for 5 min. The aqueous layer was transferred to another tube and the RNA was precipitated by centrifugation with 200 μL isopropanol at 15000 rpm for 10 min. RNA pellets were washed by 700 μL cold EtOH (70% in DEPC water) and dried in air. Total reaction volume was 20 μL and the amplified cDNA was separated on a 1.8% agarose gel stained with ethidium bromide (Sigma). PCR band intensities were expressed as OD normalized for β-actin expression using UVIDocMw program.

Cell culture

Microglial cells for primary culture were isolated from mixed glia prepared from newborn Sprague-Dawley rats (1-day-old, SD rats) in accordance with the lab protocol (Joo *et al.*, 2002), which has been modified from that of previous reports (Paresce *et al.*, 1997, Webster *et al.*, 2000). To prepare primary cultures of mixed glia, we obtained neocortical tissues of newborn SD rats, removed the meninges, minced well with micro tips and incubated the tissues in 0.05% trypsin (Gibco) for 15 minutes. After incubation, the tissue suspension was centrifuged for 7 minutes at 1450 rpm and resuspended in Dulbeccos modified Eagles medium (DMEM) (Life Technologies, Inc) with no serum for the titration of primarily minced tissues with finely fire-polished, Pasteur pipettes by diminishing the tip holes to 1/4 in diameter. The supernatant was then centrifuged once more and the pellets resuspended with DMEM supplemented with 10% heat-activated fetal bovine serum (FBS), 100 units/mL penicillin and 100 mg/mL streptomycin (10% FBS-DMEM) (Gibco). Cells were then grown in 75 cm^2 culture flask (1.5 cortices per flask) containing 10 mL of 10% FBS-DMEM at 37°C in a 5% CO_2 humidified air atmosphere for 2 weeks. After the primary culture (1 week later), the microglia were detached from the flask by tapping softly the side edges several times with mild fluctuation of the media and filtered by passing though a 33 μm nylon mesh to remove astrocytes and clumped unnecessary cells. The supernatant was centrifuged at 1450 rpm and then microglia were resuspended and replaced in a 25-cm^2 culture flask containing 5% FBS-DMEM at a density of about 2.0×10^7. The cultures were shaken to release microglia twice a week for 2 to 3 weeks in accordance with the study designs. In principal, we kept the cell density at $>1.0\times10^5$ per experimental well. Cell purity was confirmed by DiI-LDL uptake of microglia under fluorescence microscopy (>98%).

RESULTS

Prior to entering into the main study, we carried out a series of pre-studies on the culture media to ascertain whether microglia were stable in 5% FBS culture media for a short or long term period because this can affect the final assessment when analyzing data. However, no significant fluctuation in NO production at various time intervals was found except for a small increase of NO from 6 h to 24 h at a constant rate (data not shown). A positive control, CYS-A, effectively suppressed NO production in all time frames (Fig. 1). Contrarily, the LPS group activated microglia and induced NO production up to 24 h, but no more NO increase was found at 48 h. As expected, the

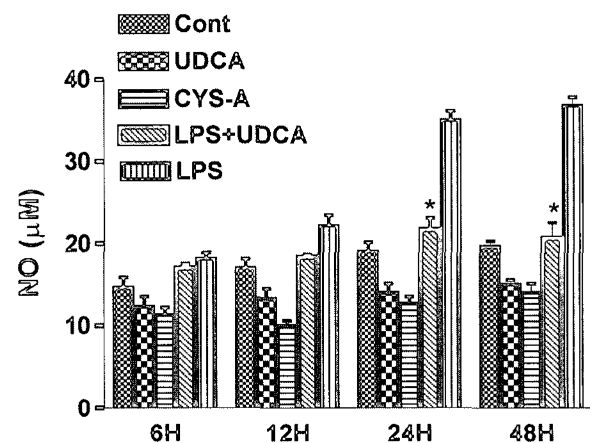

Fig. 1. Measurement of nitric oxide (NO): effect of UDCA in combination with lipopolysaccharide (LPS) on NO inhibition in rat microglial cells. The cells were treated with 100 μg/mL UDCA, 1 μg/mL cyclosporine, and 1 μg/mL LPS over the time period from 6 h to 48 h. NO production (μM/mg) was plotted. Culture media (Cont), Ursodeoxycholic acid (UDCA), CYS-A (cyclosporine-A), Lipopolysaccharide (LPS). Each column represents mean ± SD by experiments in triplicate. *$p<0.05$, UDCA vs. LPS+UDCA, *t*-test.

Table I. Primer sequences for PCR

β-actin (526 bp)	Sense anti-sense	5'- GTG GGG CGC CCC AGG CAC CA -3' 5'- GTC CTT AAT GTC ACG CAC GAT TTC -3'	30cy
IL-1β (520 bp)	Sense anti-sense	5'- GAA GCT GTG GCA GCT ACC TAT GTC T -3' 5'- CTC TGC TTG AGA GGT GCT GAT GTA C -3'	30cy

positive control, CYS-A, suppressed more effectively than UDCA in the earlier time phase. Comparing these results with LPS, a strong activator of NO production from microglia, UDCA was also considered to be comparable in the level of the suppression. Additionally, we found that UDCA maintained its effectiveness during the entire incubation time even though the level was slightly increased. When treating with Aβ42, NO was significantly increased at 24 h and 48 h. This indicates that Aβ42 activates microglia in a time dependant manner, and that the UDCA in combination with Aβ42 group was constantly posited under the non-treated group (culture media) up to 12 h, although there was non-significant increase of NO at 24 h and 48 h compared to culture media (Fig. 2). However, this NO increase was not as large as that which occurred in the Aβ42 alone group, indicating that UDCA played a meaningful role in suppressing NO production. The single treatment of UDCA showed a lower production of NO than the control group in microglia at 200 μg and 100 μg/mL. However, the highest concentration, 500 μg/mL, did not show a dramatic result comparable to 100 μg/mL UDCA, which suggested that the effect was not dose-dependent. The NO levels in the Aβ42 group were upregulated compared to the non-treated group (culture media), but the combination with UDCA was reduced at 50 μg/mL and significantly at 100 μg/mL. However, no more reduction of NO was observed at the higher concentrations of 200 μg/mL and 500 μg/mL (data not shown).

The protein levels of IL-1β in culture media were measured by ELISA Kit (Figs. 3 and 4). Seven groups, culture media, UDCA, CYS-A, LPS, Aβ42, LPS + UDCA, and Aβ42 + UDCA were cultured for 24 h to 48 h. Prior to that,

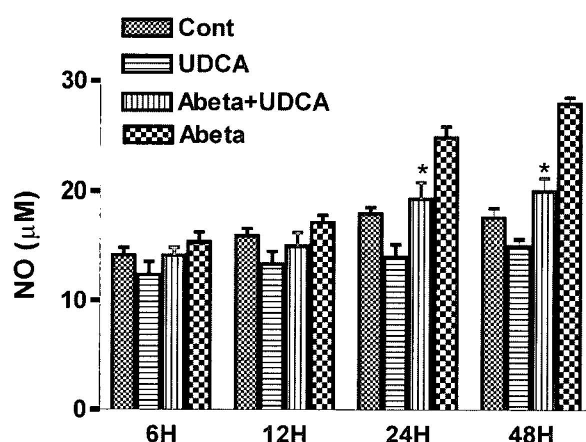

Fig. 2. Measurement of nitric oxide (NO): effect of UDCA in combination with Aâ peptides on NO inhibition in rat microglial cells. The cells were treated with 100 μg/mL UDCA, and 5 μg/mL Aβ42 over the time period from 6 h to 48 h. NO production (μM/mg) was plotted. Culture media (Cont), Ursodeoxycholic acid (UDCA), Abeta (Aβ42). Each column represents mean ± SD by experiments in triplicate. *p<0.05, UDCA vs. LPS+UDCA, *t*-test.

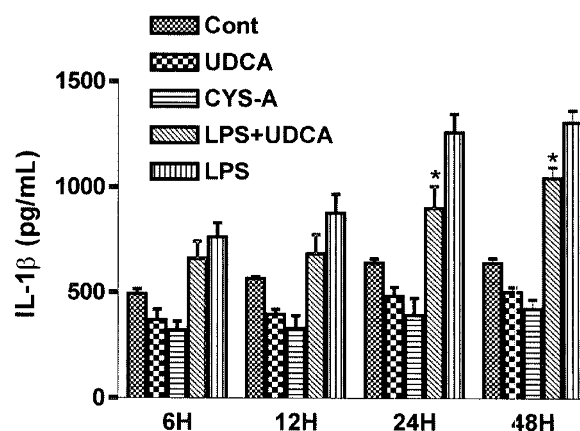

Fig. 3. Suppression effect of UDCA on lipopolysaccharide (LPS) in rat microglial cells. IL-1β was measured over 6 h to 48 h n the same pattern as for NO measurement after cells were stabilizec in 5% FBS culture media. The volume of IL-1β, in pg/mL, was detected by using colorimetric ELISA Kit according to the manufacturers protocol. Culture media (Cont), Ursodeoxycholic acid (UDCA), CYS-A (cyclosporine-A), Lipopolysaccharide (LPS). Each column represents mean ± SD by experiments in triplicate. *p<0.05, UDCA vs. LPS+UDCA, *t*-test.

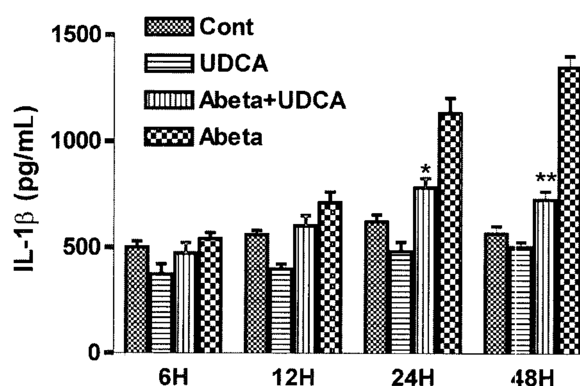

Fig. 4. Suppression effect of UDCA on Aβ peptides in rat microglial cells. IL-1β was measured over 6 h to 48 h in the same pattern as for NO measurement after cells were stabilized in 5% FBS culture media. The volume of IL-1β, in pg/mL, was detected by using colorimetric ELISA Kit according to the manufacturers protocol. The cells were treated with 100 μg/mL UDCA, and 5 μg/mL Aβ42. Culture media (Cont), Ursodeoxycholic acid (UDCA), Abeta (Aβ42). Each column represents mean ± SD by experiments in triplicate. *p<0.05, UDCA vs. LPS+UDCA, *t*-test.

UDCA and CYS-A alone groups were compared with the non-treated group (culture media). Results showed that the lowest IL-1β concentration was detected in single treatments with UDCA and CYS-A (Fig. 3). In the Aβ42 group, the IL-1β level was increased as much as that of LPS treatment. On the other hand, UDCA when co-treated with Aβ42 decreased IL-1β levels compared with Aβ42 alone treatment (Fig. 4). The patterns of IL-1β inhibition by UDCA and CYS-A treatment in microglia showed a similar manner. However, CYS-A more effectively

inhibited IL-1β than UDCA did. Moreover, significant differences between single treatment and co-treatment with UDCA were found.

In a parallel study, we varied the time from 24 h to 48 h and the concentration from 50 μg/mL to 500 μg/mL to investigate differences between the time courses at various concentrations for IL-1β. We found that 100 μg/mL was the most effective concentration around the 24 h and 48 h time points (data not shown). The IL-1β pattern between the UDCA group treated with Aβ42 and the Aβ42 alone group was remarkably differentiated after 24 h and it was almost doubled at 48 h (Fig. 4) compared with that at 6 h and 12 h. This indicates that UDCA may downregulate the effect of Aβ42 in IL-1β production.

For RT-PCR, we uniformly set all the experimental conditions required for RT or PCR. The final dose of UDCA ranged from 50 μg/mL to 500 μg/mL. From this available range, we adopted 100 μg of UDCA because this level showed more stable data when the experiment was repeatedly conducted, and because we wanted to determine the most suitable dose for suppressing the release of proinflammatory cytokine from microglia. Cells were adjusted to a density of 1×10^5/mL in each experimental well by counting them under a glass cell counter. After isolating and placing the cells in culture wells (6-, 12-, or 24-well plates), we stabilized them for at least 24 h under the same conditions as the primary culture. RT-PCR schedules were designed to be conducted on the same day in order to maintain constant values. Firstly, 20 μg/mL Aβ42 was prepared by incubating the peptides for at least 24 h at 37°C with gentle mixing by tapping two or three times during incubation at pH <6.0. Additionally, 1 μg/mL cyclosporine was used as a positive control. The results were prepared using the UVIDocMw program and are shown in Fig. 5. β-actin was used as an indicator for study validation. Treatment of rat microglia with UDCA, CYS-A, and Aβ42 resulted in different gel bands according to their stimulating activities. As shown in Fig. 5, Aβ42 was a strong stimulant for IL-1β, but UDCA and CYS-A in combination with Aβ42 suppressed IL-1β production when compared to the non-treated group (culture media) and Aβ42 alone treatment for a given incubation time.

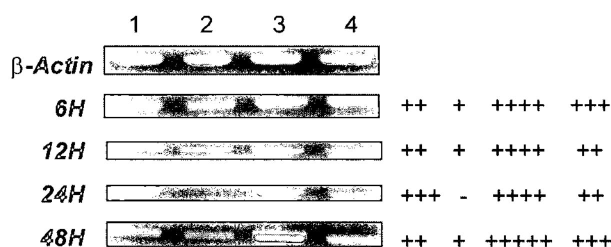

Fig. 5. Comparison of mRNA expression of IL-1β by RT-PCR. Inhibitory effects of UDCA on Aβ42 peptides were evaluated over the time period from 6 h to 48 h. The expression of IL-1β mRNA was primarily compared with that of β-actin mRNA and secondly with IL-1β mRNA. Culture media (1), UDCA (2), Aβ42 (3), UDCA+Aβ42 (4). (+) Intensity of PCR products (band).

CONCLUSION

The results demonstrated that microglia participate in an inflammatory response, signaling other glial and neuronal cells via cytokines by secreting a variety of immune-related substances such as complements, generate free radicals, and act as a clean-up crew in charge of clearing amyloid deposits. Reactive microglial products mediate the activation of astrocytes as well as neuronal injury. Substances that lead to the activation of microglia (e.g. LPS) are correlated with neuronal toxicity (Possel *et al.*, 2000), and differences in the density of microglia may explain why some parts of the brain are more prone to inflammation or are found to develop a higher SP density (Kim *et al.*, 2000). When initiated, inflammatory cytokines activate microglia, sustain inflammatory and immune responses (Benveniste *et al.*, 1992), and may interact directly or indirectly with neurons (Strijbos *et al.*, 1995). Therefore, the inflammatory responses of microglia may elicit a deleterious effect on neurons. A broad inflammatory repertoire, including NO, IL-1β, and TNF-α levels, even though IL-6 and macrophage colony stimulating factor (M-CSF) may be involved, is secreted by microglia and astroglia in the AD brain (Lue *et al.*, 2001). Of this inflammatory repertoire, IL-1β is predominantly secreted by reactive microglia, occurs at elevated levels early in the development of plaque and activates the production and processing of APP in the tissue, leading to a possible increase in Ab production (Forloni *et al.*, 1992). IL-1β has also been shown to be directly toxic to neurons (*in vitro*) at high concentrations (Mrak *et al.*, 1995). On the other hand, NO, the product of a five-electron oxidation of amino acid L-arginine mediated by NO synthesis, is a major mammalian secretory product that initiates host defense and homeostatic and developmental functions by direct effect of intracellular signaling. In addition, NO is a key molecule that stimulates host defenses in the immune system, maintains blood pressure in the cardiovascular system and modulates neural transmission in the brain. As a direct effector, NO is thought to activate regulatory proteins, kinases and proteases that are directed by reactive oxygen intermediates. In particular, NO acts as a neurotransmitter in the central and peripheral nervous systems and, therefore, is critical in the pathogenesis of stroke and other neurodegenerative disorders (Jaffrey *et al.*, 1995). In such neurodegenerative disorders, AD is linked to NO, and the Ab-associated free radical oxidative stress plays a pathological role in its neurotoxicity (Varadarajan *et al.*, 2000).

As shown in the results, we found that UDCA atten-

uated the production of NO and IL-1β in Aβ42-pretreated microglia (Figs. 2 & 4). This result was confirmed by RT-PCR assessments (Fig. 5) that showed a lower expression of IL-1β mRNA than in the control and the Aβ42 alone group. In conclusion, the significant anti-inflammatory effect of UDCA *in vitro* suggests that it may regulate the expression or production of inflammatory mediators. Such an effect would be a cue to protect neurons from cell death or damage in the chronic stage of AD caused by activated microglia in the presence of massive Aβ peptides. Therefore, if AD is caused by damaged neurons from chronic inflammation, UDCA may be applicable to people with a chronic neurodegenerative disease, such as AD, as an adjuvant therapy in combination with a routine prescription. Clearly such a possible treatment modality warrants more in-depth *in vitro* and *in vivo* studies for confirmation of its therapeutic effect.

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