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A Pharmacological Advantage of Ursodeoxycholic Acid in Cytoprotection in Primary Rat Microglia

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Abstract

Ursodeoxycholic acid (UDCA) has long been used as an adjuvant or first choice of therapy for liver disease. Commonly, UDCA has been reported to play a role in improving hyperbilirubinemia and disorder of bromsulphalein. More commonly, UDCA has been used in reducing the rate of cholesterol level in bile juice that can cause cholesterol stone. The effects on the promotion of bile acid release that leads an excretion of toxic materials and wastes produced in liver cells as well as various arrays of liver disease such as hepatitis. Other than already reported in clinical use, immunosuppressive effect has been studied, especially in transplantation. In the study, we hypothesized that UDCA might have a certain role in anti-inflammation through a preventive effect of pro-inflammatory potentials in the brain macrophages, microglia. We found that the treatment of 200 μg/ml UDCA effectively suppressed the pro-inflammatory mediators (i.e. nitric oxide and interleukin-1β) in rat microglia compared to comparators. Interestingly, RT-PCR analysis suggested that UDCA strongly attenuated the expression of IL-1β that was comparable with cyclosporine A at 48 h incubation. Conclusively, we found that UDCA may play a cytoprotective role in microglial cells through direct or indirect pathways by scavenging a toxic compound or an anti-inflammatory effect, which are known as major causes of neurodegenerative diseases.

Keywords: microglia, Ursodeoxycholic acid (UDCA), Neurodegenerative disease, IL-1b, Nitric Oxide

A trend of aging population is a big social issue in worldwide because this means that a limited number of young people recognized as a labor index will have to care for an increased number of elderly peo-ple within the short-term future. Particularly, Alzheimer's disease (AD), which is a neurodegenerative disease and also known for the most common form of dementia has been an important research area for recent decades due to the irrecoverable pathology in elderly people. In patient with AD, senile plaques (SP) and neurofibrilar tangles (NFT) are a common founding and those founding are reported to play a major role in the disease progression[1-3]. β-amyloid peptide (Aβ) produced from transmembraneous amyloid precursor protein (APP) and microtubule associated protein (tau protein) are known as the major causative factors of AD[4]. The Aβ peptides from APP by dual cleavage action of β & γ secretase stimulate the brain glial cells (i.e. microglia) to release pro-inflammatory mediators such as IL-1β, IL-6, TNF-α, and nitric oxide, which lead to chronic inflammation in the brain by an escalation of reactive oxygen species (ROS), pro-inflammatory cytokines, and neurotoxic factors[5-7]. On the other hand, the Aβ directly induces cytochrome c release from mitochondria through a mechanism that is accompanied by profound effects on mitochondrial membrane redox status, lipid polarity, and protein order. In this relation, Ursodeoxycholic acid (UDCA) and tauroursodeoxycholate (TUDC) can directly suppress Aβ-induced disruption of the mitochondrial membrane structure, suggesting a neuroprotective role for this bile salt[8]. In addition, as the Aβ accumulated intracellularly by A-PP 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[9], it will 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. The "amyloid hypothesis" that has been adopted in tens of AD researches for the last decades and that has been centered in molecular approaches of AD-related studies. In short, "Amyloid (cascade) Hypothesis" is based on the neurotoxic properties of A[10], and posits that increased secretion of A leads to elevated extracellular levels of A as senile plaques, which in turn

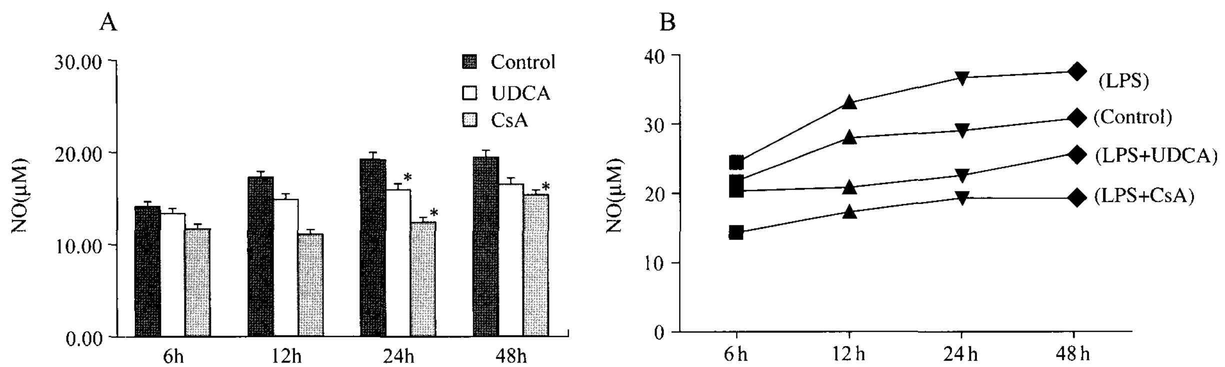

Fig. 1. NO production in primary rat microglia. 200 μg/ml UDCA, 5 μg/ml cyclosporine, and 5 μg/ml LPS were added to cell culture for 48 h and measured the NO concentration under griess reagent system. (A) control, UDCA, and Cyclosporine A (CsA) were compared at four time points, 6, 12, 24, and 48 h. (B) lipopolysaccharide (LPS) was used for microglial activation and the co-culture with UDCA or CsA was analyzed for the attenuation of NO production from cells. Data are mean ± S.D. of a representative experiment repeated three times. $^*p < 0.05$, vs. control.

are toxic to surrounding neurons. Therefore, to prevent such plaques formed from Aβ peptides, fast and effective clearance of Aβ peptides should be done, or if the plaques are already formed, the attenuation of microglial activation should be considered in AD therapy. Virtually, Aβ is a good activator to microglia and is known to play a role to aggregate in the disease states of AD brain. In turn, a massive production of Aβ peptides from APP causes neuritic plaques and activates microglia by fibrillar Aβ (fAβ), which are surrounded the vicinity of plaques. In this state, microglia release proinflammatory cytokine such as IL-1β (5) and nitric oxide[11] and thus lead to chronic inflammation that causes a cell death. In these backgrounds, we expected that UDCA supports the cell survival through an anti-inflammatory effect by attenuating the activation of microglia and further would be a potential candidate for AD therapy.

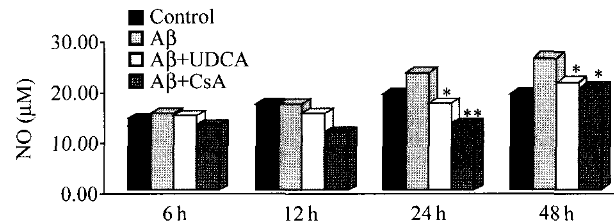

Fig. 2. Production of NO by Aβ42 treatment. 200 μg/ml UDCA and 5 μg/ml cyclosporine were co-cultured with 5 μg/ml Aβ42 for 48 h. The measurement of NO was carried out by Griess reagent described in Materials and Methods. To effectively stimulate microglia, Aβ42 was pre-aggregated for 12 h in 37°C before incubation. Data are mean ± S.D. of a representative experiment repeated three times. $^*p < 0.05$, $^{**}p < 0.01$, vs. Aβ42.

Attenuation of NO Production by UDCA (NO assay)

To investigate the NO production in various time courses, UDCA concentrations from 50 to 300 μg/ml were designed in the study groups, control (culture media), UDCA, LPS, and Aβ42 for 6 to 48 h (Fig. 1, 2). We ignored the NO production from microglia in control group because we did not find the increase of NO during entire incubation time (data not shown). Cyclosporine A (CsA) showed to effectively suppress the NO production at 12 h, whereas it relatively increased after 24 h (Fig. 1A). In comparison, LPS group activated microglia and induced the NO production up to 12 h, but the production of NO was not continued by 48 h. The positive control, CsA, suppressed the NO produciton more effectively than that of UDCA in earlier time phase. The intervals between the pre- & -post time point in UDCA group were less than that of CsA (Fig. 1B). When cells were treated with Aβ42, NO was significantly increased at 24 and 48 h. This reveals that Aβ42 activates microglia in time dependant manner. In addition, when cells were co-treated Aβ42 with UDCA or CsA, NO production was constantly posited under the normal control up to 24 h, although there was no significant upregulation of NO concentration at 48h (Fig. 2). Clearly, UDCA single treatment kept the lower level of NO than that of control group in higher concentration of UDCA, but the dose-dependency of UDCA was not found.

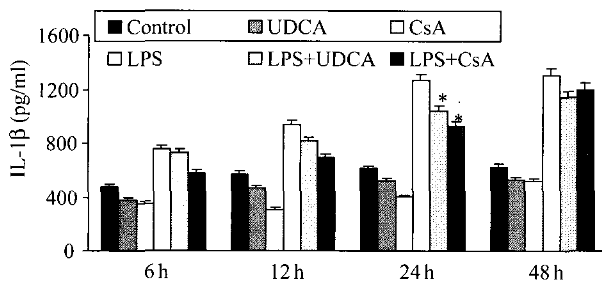

Fig. 3. Measurement of IL-1β production. The anti-inflammatory effect of UDCA was analyzed by examining the concentration of pro-inflammatory cytokine, IL-1β at 6 to 48 h time points using Enzyme Immunometric Assay (EIA). Control, UDCA, and CsA single group were compared with LPS-activated groups for assuring the efficacy of UDCA. CsA was used as a positive comparator. Data are mean± S.D. of a representative experiment repeated three times. $^*p < 0.05$, vs. LPS single group.

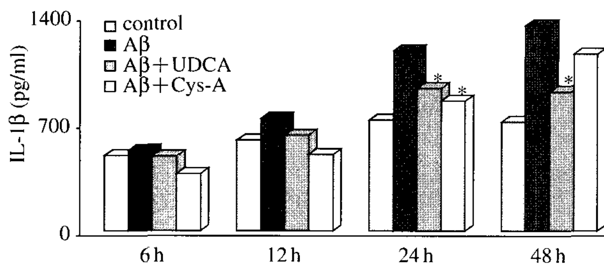

Fig. 4. Suppression of IL-1β production from mocroglia activated by Aβ42. 200 μg/ml UDCA and 5 μg/ml cyclosporine were co-cultured with pre-aggregated Aβ42 (5 μg/ml) for 6 to 48 h in EIA system. Data are mean±S.D. of a representative experiment repeated three times. $^*p < 0.05$, vs. Aβ42 single group.

Suppressive Effect of UDCA for IL-1β (EIA assay)

The production of proinflammatory cytokine, IL-1β, was measured by EIA polyclonal test kit using media from cell culture for each treatment. Four groups, culture media, lipopolysaccharide (LPS), LPS+UDCA, and LPS+CsA were measured for 48 h. As a pre-study, we observed the UDCA and CsA single group in comparison with the control (Fig. 3). We found that the IL-1β was well suppressed when treated with CsA at 12 h. However, the tendency of IL-1β was gradually decreased as time went by, which was not likely UDCA. In Aβ42 group, there was a tiny increase of IL-1β from 6 h to 12 h, whereas it was significantly increased at 24 h and 48 h comparing to the control (Fig. 4). When comparing the group of Aβ42+UDCA and Aβ42+CsA, more decrease of IL-1β was found in Aβ42+CsA group

Fig. 5. Expression of IL-1β mRNA in microglia activated by Aβ42. UDCA and cyclosporine were compared on their suppression effects of cytokine, IL-1β. The gene expression was analyzed by RT-PCR as described in Materials and Methods and β-actin was used as an internal standard. The PCR products were electrophorased in 1.2% agarose gel and the bands were analyzed by UVIDocMw program.

than that in Aβ42+UDCA group. This pattern was reversed at 48 h (Fig. 4).

Suppression of IL-1β Gene Expression (RT-PCR)

Reverse transcription polymerase chain reaction (RT-PCR) assay was carried out under the standard protocol described in methods and materials. The final concentration of UDCA ranged from 50 to 300 μg/ml was evaluated and we finally adopted 200 μg/ml due to more stable result than that found in the higher concentration when repeatedly examined. We found that the suppression of the release of pro-inflammatory cytokine, IL-1β, was detected in 200 μg/ml UDCA treatment. To compare the cell activation and suppression, 5 μg/ml Aβ42 was pre-incubated to form a slight aggregation for at least 12 h in 37°C with gentle mix by tapping smoothly two or three times during incubation at low pH (<6.0). Additionally, 5 μg/ml CsA was used as a positive control. The results are shown in Fig. 5 using UVIDocMw program. β-actin was used for an internal standard. The treatment of microglial cells with UDCA, CsA, and Aβ42 resulted in different PCR products according to their stimulating activities. As shown in Fig. 5, Aβ42 was a strong stimulant for IL-1β, whereas UDCA and CsA in co-culture with Aβ42 suppressed the production of IL-1β for given chasing times (6, 24, and 48 h). The most significant result of suppression was found in CsA treatment in 6 h and 12 h. However, the trends were reversed in UDCA and CsA group at the end of incubation phase (Fig. 5). Moreover, the continuity of the suppressive effect was found in UDCA

group compared to CsA group, which was correspondent to the EIA and NO assay. Microglia participate in an inflammatory response, signaling other glial and neuronal cells via cytokines by secreting a variety of immune-related substances such as complement, generate free radicals, as well as act as a clean-up crew in charge of clearing amyloid deposits. Reactive microglial products mediate activation of astrocytes as well as neuronal injury. Substances that lead to the activation of microglia (e.g. LPS) are correlated with neuronal toxicity[13], and differences in the density of microglia may explain why some part of the brain are more prone to inflammation or found to develop a higher density of senile plaques[14]. Microglial cytotoxicity has bolstered the concept that AD may be a form of chronic inflammation, and evidence that supports this hypothesis is the appearance of inflammatory markers in plaque-lesioned areas. In addition, because microglia are the main inflammatory response cells of the brain and activated microglia are enriched around amyloid plaques, microglia could play an important role in causing the chronic inflammation, which may lead neurocytotoxicity in the areas around the plaques[6,15]. It is also certain that when microglia initiate to secrete inflammatory mediators followed by multiple immune reaction as well as inflammation[16], they may take a part in damaging neuron cells, directly or indirectly[17]. Inflammatory mediators, such as NO, IL-1β, and TNF-α, are secreted by microglia and astroglia in the AD brain[18]. IL-1β, secreted predominantly by reactive microglia, occurs at elevated levels early in the development of a plaque and activates the production and processing of APP (amyloid precursor protein) in the tissue, leading to a possible increase in Aβ production[19,20]. In this way, IL-1β contributes to the formation of new sources of amyloid in a kind of positive feedback that can accelerate formation of plaques and destruction of neurons. IL-1β also activates astrocytes, promoting their secretion of IL-6, TNF, and S100-beta[21]. IL-1β has also shown to be directly toxic to neurons (in vitro) at high concentrations. Evidence thus points to IL-1β as a major factor driving the disease[21]. 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[22]. As a neurodegenerative disorder, AD is linked to NO, and the Aβ-associated free radical oxidative stress plays a pathological role in neurotoxicity[23]. In our experiments, we found that IL-1β and NO was suppressed by UDCA as well as cyclosporine A, a positive control, in cultures pre-treated with Aβ42 or LPS. As it has been known that cyclosporine A is the strongest suppressor of NO and IL-1β, it was a meaningful result that UDCA also suppressed the NO and IL-1β with the similar pattern with cyclosporine A. When pre-activated with Aβ42 or LPS before co-culturing with UDCA, microglia produced high NO and IL-1β. However, the patterns were reversed when added with UDCA (Fig. 1-4). The results imply that UDCA plays a role in suppressing NO production and IL-1β expression and the effect can be prolonged for longer time (48 h) comparing with Cyclosporine A (Fig. 2, 5). As NO and IL-1β are generally known as pro-inflammatory mediators and oxidative stressors in cells, their expression in nuclear or the production out to extracellularly have something great to do with cell damage and neurodegenerative disease which are the major cause of the AD. Therefore, the results found in the study may be certain cues in the prevention or treatment of AD in terms of clinical used of UDCA. The mechanisms of UDCA in AD treatment are not certain, but proposedly it plays a role in suppressing AD causatives from microglia, provokzing cell damage or cell death when stimulated by massive Aβ peptides. According to our previous study[24], the results from co-cultures of UDCA and Aβ42 seem to correlate with the signals of transcription factor NF-κB, which is activated by oxidative stressors including NO and IL-1β[25]. Furthermore, UDCA might be involved in inhibition of mitochondrial activation[8] that protects cells from apoptosis, which is a major cause of neurodegenerative diseases.

Methods

Cell Culture

The study was performed by using 1-day old Sprague Dawley (SD) rat. Microglia were isolated from mixed glial cultures prepared from newborn Sprague-Dawley rat brains, as previously described[12]. The mixed glial cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 mg/ml streptomycin (Life Technologies, USA) for 10-14 days at 37°C in a 5% CO_2 humidified air atmosphere before harvesting microglia. Microglia were harvested by orbital shaking for 15-20 min at 200 r.p.m. and centrifuged for 3 min at 1350 r.p.m. and plated on six-well tissue culture plates at 1.5×10^5 cells/well or onto four-well chamber slides at a density of 1×10^4 cells/well.

Stock preparation

Ursodeoxycholic acid (UDCA, $C_{24}H_{40}O_4$; MW. 392.58) with 99% purity was a gift from a research laboratory of Oriental Pharmaceutical Company, Korea. The stock UDCA, Aβ42, cyclosporine-A (CsA), and LPS (lipopolysaccharide) solutions were prepared by dissolving in microfiltered (0.2 μm) culture media at concentrations of 50 μg to 300 μg/ml for UDCA, 5 μg/ml for rat Aβ42 (Bachem, UK), 5 μg/ml for CsA, and 5 μg/ml for LPS. The solutions were either kept at −20° C for future use or diluted directly in Dulbecco's modified Eagle's medium (D-MEM) with 1-5% heat-inactivated fetal bovine serum (FBS).

Reagent

Cyclosporine A (Sigma Chem., USA), methanol (Merck, Germany), FBS (Gibco, USA), Griess Reagent (Promega, USA), HEPES (Duchefa Biochem, Netherlands), L-glutamine (Sigma, USA.), Lipopolysaccharide (E. coli, serotype 0111:B4) (Sigma Chem., USA), Penicillin-Streptomycin (Gibco, USA), Rat IL-1β & TNF-α EIA Kits (Assay Designs, USA), Trypsin (Gibco, USA), Aβ42 (Tocris, USA) were used. In addition, other buffers and reagents used in the study were formulated in lab with the first or super graded reagents.

Nitric Oxide Assay and Reversed Transcription PCR (RT-PCR)

The amount of NO produced by microglia under the different sample conditions was determined by examining the culture supernatants for the stable end product, nitrite, using an automated procedure based on the Griess reaction. Briefly, an aliquot (100 μl) of the culture medium was incubated with 50 μl of 0.1% sulfanilamide in 5% phosphoric acid and 50 μl of 0.1% *N*-1-anphthyl-ethylenediamine dihydrocholate (Sigma, USA). After a 10 min incubation period at room temperature, the absorption was measured at 540 nm. For RT-PCR, the cells at a density of 1.5×10^5/ml were plated in 6-well plates and treated with each sample for the designated culture times (6 to 48 h). After the designated culture time, the total RNA was obtained from 1×10^7/ml cells using Trizol reagent (Life Technologies, USA). Briefly, 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. The RNA pellets were washed in 700 μl cold Et-OH (70% in DEPC water) and dried in air. The total RNA (0.5 μg) from each group was examined by RT-PCR (MJ Research, USA) using the specific primers for IL-1β (520 bp; sense, 5-GAA GCT GTG GCA GCT ACC TAT GTC T-3 and anti-sense, 5-CTC TGC TTG AGA GGT GCT GAT GTA C-3) and for β-actin (526 bp; sense, 5-GTG GGG CGC CCC AGG CAC CA-3 and anti-sense, 5-GTC CTT AAT GTC ACG CAC GAT TTC-3). The total reaction volume was 20 μl, and the amplified cDNA (annealing temperature 57°C for 30 s) was separated on a 1.2% agarose gel stained with ethidium bromide. The PCR band intensities are expressed as OD values using the UVIDocMw program.

Cytokine EIAs (Enzyme Immunometric Assay)

EIA kits (TiterZyme EIA, Assay Designs, U.S.A.) were used for the quantitative determination of level of the cytokines (IL-1β) produced from microglia in response to the treatment of UDCA or positive/negative controls. The kit is an assay tool designed to use polyclonal antibody to rat cytokines immobilized on a microtiter plate to bind the rat cytokines in the sample. This was carried out in accordance with the manufacturer's protocol and read the optical density of the plate at 450 nm by the plate reader (Bio-RAD, Japan). In brief, cultured plates were taken into clean bench and 100 μl of samples were pipetted into the wells of provided microplate, tapped the plate gently to mix the contents, and sealed with the plate sealer. We then incubated it at 37°C for 1 hour. After incubation, the plate was washed three times by adding 200 μl of wash solution with multi-channel pipette, and took 100 μl of the labeled antibody into each well, and sealed the plate and incubated at 4°C for 30 min. The plate was washed entirely by adding 200 μl with wash solution and 100 μl of the substrate was added to each well. After the incubation, the reaction was completed by adding 100 μl of stop solution. The concentration of cytokines was represented in pg/ml through the calculation from the average OD and standard curve.

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