(S)-Tetrahydroisoquinoline Alkaloid Inhibits LPS-Induced Arachidonic Acid Release through Downregulation of cPLA₂ Expression

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Sepsis, a systemic inflammatory response syndrome, remains a potentially lethal condition. (S)-1- α -Naphthylmethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (CKD712) is noted as a drug candidate for sepsis. Many studies have demonstrated its significant anti-inflammatory effects. Here we first examined whether CKD712 inhibits lipopolysaccharide (LPS)-induced arachidonic acid (AA) release in the RAW 264.7 mouse monocyte cell line, and subsequently, its inhibitory mechanisms. CKD712 reversed LPS-associated morphological changes in the RAW 264.7 cells, and inhibited LPS-induced release of AA in a concentration-dependent manner. The inhibition was apparently due to the diminished expression of a cytosolic form of phospholipase A₂ (cPLA₂) by CKD712, resulting from reduced NF- κ B activation. Furthermore, CKD712 inhibited the activation of ERK1/2 and SAP/JNK, but not of p38 MAPK. CKD712 had no effect on the activity or phosphorylation of cPLA₂ and on calcium influx. Our results collectively suggest that CKD712 inhibits LPS-induced AA release through the inhibition of a MAPKs/NF- κ B pathway leading to reduced cPLA₂ expression in RAW 264.7 cells.

INTRODUCTION

Sepsis (Septicemia, blood poisoning) represents a whole-body inflammatory state that is usually caused by infection with gram-negative bacteria (Levy et al., 2003). Endotoxins from gram-negative bacteria like *Escherichia coli* that are released into the blood stream cause decreased tissue perfusion and oxygen delivery, multiple organ failure (septic shock), and death (Kumar Vinay, 2007). Sepsis is also known as a systemic inflammatory response syndrome (SIRS), and remains a major challenge in medicine (Baron et al., 2006).

CKD712, (S)-1- α -naphthylmethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, is a newly synthesized tetrahydroisoquinoline alkaloid and an (S) enantiomer. It was originally extracted from *Aconitum* (known as Monkshood), and initially used as a

cardio tonic, and has long been considered as a very important drug. It has also been used as a heart stimulant and diuretic in oriental herbal medicine (Zhou and Du, 2003). Kang et al. (1999) reported that the (S)-form, which has shown more potent anti-inflammatory effects than the (R)-form or racemic mixture, significantly inhibited inducible nitric oxide synthase (iNOS) expression, with concomitant decrease in nitric oxide (NO) production, by blocking the activation of nuclear factor- κ B (NF- κ B), leading to increased survival rates in a lipopolysaccharide (LPS)-treated murine model of sepsis (Park et al., 2006; 2011).

A cytosolic form of phospholipase A₂ (cPLA₂) hydrolyzes phospholipids to arachidonic acid (AA) and lysophospholipids; this is the rate-limiting step during pro-inflammatory eicosanoid production (Clark et al., 1995; Leslie, 1997). There are several pathways for AA production through the activation and translocation to cell membrane of cPLA₂; by increases in a) intracellular Ca²⁺ levels (Glover et al., 1995), b) direct activation by ceramide-1-phosphate (Lamour et al., 2009), c) phosphorylation by mitogen-activated protein kinase (MAPK) (Lin et al., 1993), and d) transcriptional expression (Clark et al., 1995). Recent evidence shows that cPLA₂ could be an essential effector in the pathogenesis of the septic shock (Kim et al., 2006; Levy et al., 2000; Roshak et al., 1994) and acute lung injury caused by the sepsis syndrome (Nagase et al., 2000; 2003). Moreover, cPLA₂, and not the secretory form of PLA₂ (sPLA₂), plays an important role in LPS-induced prostaglandin E₂ (PGE₂) formation in human monocytes (Roshak et al., 1994). The disruption of a gene encoding cPLA₂ and subsequent treatment with a potent inhibitor of cPLA₂, such as arachidonyl trifluoromethyl ketone, significantly attenuated LPS-induced acute lung injury in mice (Kim et al., 2006; Nagase et al., 2000; 2003). These studies suggest that the pharmacological inhibition of cPLA₂ could be a novel therapeutic approach to sepsis.

In the present study, we have demonstrated the inhibitory effect of CKD712 on LPS-induced AA release and PGE₂ production. These effects may be due to inhibition of cPLA₂ expression, through the activation of a MAPK/NF- κ B pathway.

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MATERIALS AND METHODS

Materials

LPS (from *E. coli*, serotype O128:B12), protease inhibitor cocktail, phenylmethylsulfonyl fluoride (PMSF), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltrazolium bromide], and dithiothreitol (DTT) were purchased from Sigma Chemical Co. (USA). Arachidonyl trifluoromethyl ketone (AACOCF₃), SP600125, PD98059, and SB203580 were obtained from Biomol (Plymouth Meeting, USA). Anti-cPLA₂, anti-COX-2, and anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology (USA). Anti-MAPK antibodies (p42/44, p38 MAPK, and JNK) and anti-phospho-MAPK (p-p42/44, p-p38 MAPK, and p-JNK) antibodies were purchased from Cell Signaling technology® (USA). 1-Stearoyl-2-(1-¹⁴C) arachidonoyl-*sn*-glycero-3-phosphocholine [2-(1-¹⁴C) AA-GPC] (55 µCi/mol) and (5,6,8,9,11,12,14,15-³H) arachidonic acid [³H] AA] were purchased from Amersham Life Science Co. LTD. (UK). The plastic ware for cell culture was purchased from Nunc Co. (Denmark). All other chemicals were of highest purity available from commercial sources. Prostaglandin E2 (PGE2) enzyme immunoassay kit was from Sapphire Bioscience (Australia). The Nuclear Extraction kit and EMSA Gel Shift kit were purchased from Panomics (USA). Fluo-4 NW Calcium Assay Kits was purchased from Invitrogen (Molecular Probes™, USA). (S)-Tetrahydroisoquinoline alkaloid (CKD712) was obtained from the Chong Kun Dang Pharmaceutical Co. (Korea).

Cell culture

RAW 264.7 cells were obtained from ATCC (USA). The growth medium consisted of DMEM (USA) supplemented with 10% fetal bovine serum (Gibco) and 1× antibiotics (penicillin and streptomycin; Gibco). The cultures were maintained in an incubator at 37°C, in an atmosphere containing 5% CO₂.

Assay for arachidonic acid release

Sub-confluent cells in 6-well plates were labeled for 24 h with [³H] arachidonic acid (0.5 µCi/ml) in DMEM containing 10% FBS and antibiotics. After labeling, the medium was removed, and the cells were washed 3 times with serum-free DMEM, followed by 30 min of incubation in this medium. After incubation, the indicated concentrations of CKD712 were added, and the cells were incubated for 1 h. Next, 1 µg/ml LPS was added, and arachidonic acid release was measured at the indicated times. To measure stimulated arachidonic acid release, an aliquot of medium was removed and centrifuged. The radioactivity in 100 µl of the supernatant was measured in a liquid scintillation counter (Tri-Carb 2910TR/Perkin Elmer LAS). The radioactivity of released arachidonic acid was normalized to the total radioactivity of the monolayer extracted with 1% Triton X-100.

Preparation of RAW 264.7-derived cPLA₂

RAW 264.7 cells were grown in 150 × 20-mm petri dishes and washed twice with TBS. The cells were harvested in homogenizing buffer containing 0.12 M NaCl, 1 mM EDTA, and 75 mM Tris-HCl, pH 9.0. The cell suspension was sonicated on ice 6 times for 3 s, with 5 s-intervals between each pulse to maintain ice-cold condition. The sonicated cells were centrifuged at 2,000 × *g* for 10 min at 4°C. The supernatant (lysates) was centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatants (cytosolic fraction) were used as a source of cytosolic PLA₂. For direct binding studies, cPLA₂ was partially purified from the cytosolic fraction of RAW 264.7 cells. Cells were grown in T175

Fig. 1. Structure of CKD712. (S)-1-α-Naphthylmethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline.

flasks, and the cytosolic fractions were prepared by centrifugation at 100,000 × *g* after sonication in 125 mM NaCl, 25 mM Tris, and 1 mM EDTA, pH 9.0 (buffer H). The prepared sample was applied to a HiTrap™ Heparin HP column (1 ml, GE Healthcare) that was pre-equilibrated with buffer H at a flow rate of 0.5 ml/min. Because enzyme activity was recovered in the unbound fraction, unbound path-through (PT) protein was eluted with 5 ml of buffer H and diluted with equal volumes of 1 mM EDTA, and 50 mM Tris-HCl, pH 7.5 (buffer A). The diluted PT fraction was applied to MonoQ™ column (GE Healthcare) pre-equilibrated with buffer A at a flow rate of 1 ml/min. Unbound protein was eluted and rinsed with 25 ml of buffer A. The portion containing active enzyme was eluted at a flow rate of 1 ml/min using a 20-ml linear gradient of buffer A containing 1 M NaCl. An aliquot (20 µl) of each fraction (1 ml) was assayed for cPLA₂ activity. The fraction exhibiting the highest activity was used for testing the direct effects of CKD712 on cPLA₂.

Assay of PLA₂ activity

PLA₂ activity was assayed using the supernatant and pellet after centrifugation at 100,000 × *g*. Briefly, the standard incubation system (100 µl) for assaying PLA₂ activity contained PLA₂ preparations, 100 mM Tris-HCl, pH 7.4, 5 mM CaCl₂ and 4.5 nmol of substrate 2-[1-¹⁴C] AA-GPC (approximately 55,000 cpm). The reactions were carried out at 37°C for 1 h and stopped by adding 560 µl of modified Dole's reagent (n-heptane/isopropyl alcohol/1 N-H₂SO₄; 400/390/10, v/v) (Kim and Bonventre, 1993), and 110 µl of water. Then, the released [¹⁴C] AA was extracted and quantified.

Immunoblotting

RAW 264.7 cells were plated in 150 × 20-mm petri dishes at a density of ~2 × 10⁶ cells per plate. After 24 h, the media were replaced with serum-free DMEM for 30 min, and the cells were incubated with indicated concentrations of CKD712 for 1 h, and then stimulated with 1 µg/ml LPS for 8 h. Treated cells were washed twice with TBS and collected in homogenizing buffer (75 mM Tris-HCl, 12 mM NaCl, 1 mM EDTA, pH 9.0, 20 µM pepstatin, 10 mM mercaptoethanol, 20 µM leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride). Cells were homogenized in a sonicator and centrifuged at 2,000 × *g* for 10 min to remove debris. Cell lysates were then centrifuged at 100,000 × *g* for 1 h, and the pellet was resuspended in homogenizing buffer. The protein concentrations of the supernatants and pellets were measured using Bradford's reagent (Biorad). Proteins were

Fig. 2. Effect of CKD712 on LPS-induced morphological changes in RAW 264.7 cells. RAW 264.7 cells were incubated for 1 h in serum-free DMEM, and then incubated with the indicated concentrations of CKD712. After 30 min, the cells were incubated with 1 µg/ml LPS for 2 h. Black arrows represent morphologically altered RAW 264.7 cells. The morphology of RAW 264.7 cells was visualized using optical microscopy (200× magnification).

separated by SDS-PAGE using an 8% gel, and then electrophoretically transferred to nitrocellulose membranes (Schleicher and Schuell, USA) in 25 mM Tris-HCl (pH 8.5) containing 190 mM glycine, 4 mM SDS, and 20% methanol. These nitrocellulose membranes were incubated with a blocking buffer containing 5% non-fat dry skim milk for 1 h at room temperature, followed by incubation with mouse anti-cPLA₂ primary antibody (1:2,000 dilution) overnight at 4°C with constant shaking. Unbound antibody was removed with TBST, antibody-binding sites were detected by using alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibody (incubation for 2 h at 4°C), and blots were developed using a chromogenic substrate (Immu-Pure NBT/BCIP Substrate kit, Pierce Co., USA).

PGE2 enzyme immunoassay

The concentration of PGE2 in the medium used to culture RAW264.7 cells was measured using an ELISA kit (Sapphire Bioscience). Assays were performed according to the manufacturer's recommendation. RAW 264.7 cells were incubated with CKD712 at various concentrations. After 1 h, LPS at 1 µg/ml was added, and the cells were incubated for 8 h. The cell culture medium was then sampled for PGE2 detection using anti-PGE2 immunoglobulin.

Calcium influx assay

The effect of CKD712 on calcium influx in RAW 264.7 cells was detected using Fluo-4 NW Calcium Assay Kits (Invitrogen, USA). RAW 264.7 cells were plated in a 96-well black plate at 5×10^4 cells per well. After 24 h, the medium was removed from the well. Then, the procedure was continued according to the manufacturer's recommendation. Calcium influx was detected by fluorescence using the settings appropriate for excitation at 494 nm and emission at 516 nm. Analysis was operated with Synergy™ 2 Multi-Detection Microplate Reader (BioTek®, USA).

EMSA gel shift assay

RAW 264.7 cells were plated in 6-well plates at a density of $\sim 2 \times 10^5$ cells per well. After 24 h, the medium was replaced with serum-free DMEM for 30 min, the cells were incubated with the indicated CKD712 concentrations for 1 h, and then stimulated

with 1 µg/ml LPS for 4 h. After stimulation, the cell nuclei were extracted using a Nuclear Extraction kit (USA). Cells were washed twice in PBS and lysed on ice with gentle shaking for 10 min in extraction buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 10 mM EDTA, protease inhibitor cocktail – Sigma –, and 4% IGEPAL). Cells were centrifuged at $14,000 \times g$ for 3 min at 4°C. The supernatant was discarded, and the cell pellet was mixed with buffer B (20 mM HEPES, pH 7.9; 400 mM NaCl; 1 mM EDTA; 10% glycerol; protease inhibitor; 10 mM DTT), and vigorously vortexed for 10 s. The mixture was then incubated at 4°C for 2 h with gentle shaking. The mixture was centrifuged for 5 min at $14,000 \times g$, and the supernatant was collected. Protein concentrations were determined by the Bradford assay (Bio-Rad). Nuclear extracts (5 µg) were incubated with 1× binding buffer (20 mM HEPES, pH 7.6, 1 mM EDTA, 1 mM ammonium sulfate, 1 mM DTT, 30 mM KCl, and 0.2% Tween-20) and 1 µg of poly d (I-C) in nuclease-free water for 5 min at room temperature. Biotin-labeled probe was then added; NF-κB: (5' → 3') AGT TGA GGG GAC TTT CCC AGG C. The reaction mixture was incubated at 15°C for 30 min. The samples were electrophoresed on a 6% non-denaturing polyacrylamide gel in 0.5× TBE for 50 min at 120 V, and then transferred in 0.5× TBE onto a nylon membrane at 300 mA for 30 min. After transfer, the sample on the membrane was fixed by UV cross-linking at 120 mJ for 3 min. This membrane was then blocked with 1% blocking reagent (Roche Molecular Biochemicals) at room temperature for 15 min. The biotin-labeled probe was detected with streptavidin-horseradish peroxidase (HRP, 1:20,000 dilution, Pierce), for 15 min at room temperature. After washing 3 times, the membrane was overlaid with lumino/en-hancer and substrate for 5 min at room temperature. The image was acquired using ChemiDoc™ XRS (Bio-Rad).

Statistical analysis

Data are presented as mean values ± standard deviation (S.D.) of the indicated number of experiments. Student's *t*-test was used for statistical analysis. A *p* value < 0.05 was considered statistically significant.

Fig. 3. Effect of CKD712 on LPS-induced [³H] AA release in RAW 264.7 cells. (A) RAW 264.7 cells were incubated for 24 h in DMEM containing 10% FBS with 0.5 μCi/ml [³H] AA, washed 3 times with free DMEM, and then incubated for 8 h with various concentrations of LPS. The supernatants were then collected and tested for radioactivity (Tri-Carb 2910TR/Perkin Elmer LAS). The radioactivity released by untreated cells was considered 100%. (B) [³H] AA release induced by LPS in RAW 264.7 cells. RAW 264.7 cells were incubated for 24 h in complete DMEM with 0.5 μCi/ml [³H] AA, washed 3 times with free DMEM, and then incubated with the indicated concentrations of CKD712. After 1 h, incubated cells were treated with 1 μg/ml LPS and incubated for 8 h. The supernatants were then collected and the radioactivity was counted (Tri-Carb 2910TR/Perkin Elmer LAS). The radioactivity released by untreated cells was considered 100%. After treatment with only CKD712 (C) or CKD712 and LPS (D), the [³H] AA released was detected in the supernatant over an 8-h time course (0, 1, 2, 4, and 8 h). (E) After treatment of AACOCF₃ (0, 5, and 10 μM) with or without LPS treatment, released [³H] AA was detected in the supernatant over an 8-h time course (0, 2, 4, and 8 h). ***p* < 0.01 compared with no treated-control group, ##*p* < 0.01 compared with LPS and 0 μM-treated group.

RESULTS

CKD712 inhibits LPS-induced morphological changes in RAW264.7 cells

LPS is known to induce a morphological change in RAW 264.7 cells (Saxena et al., 2003). Thus, we confirmed the change induced by LPS, and examined the effects of CKD712 on this change using optical microscopy. As shown in Fig. 2, 1 μg/ml LPS significantly induced the morphological change at 2 h, which was subsequently inhibited by CKD712 in a concentration-dependent manner.

LPS induced AA release, which is inhibited by AACOCF₃, and CKD712 inhibited this AA release

When RAW 264.7 cells pre-incubated with [³H]AA were treated with LPS for 8 h, we observed a concentration-dependent increase in AA release with saturation at 1 μg/ml (Fig. 3A); this release was inhibited in a concentration-dependent manner by CKD712 (Fig. 3B). The inhibitory effect of CKD712 on AA release was observed not only in an LPS-free culture system (Fig. 3C), but also in an LPS-treated system (Fig. 3D). The inhibitory effect of CKD712 was maximal at 100 μM, with almost complete inhibition of AA release. On the other hand, AACOCF₃, a

specific cPLA₂ inhibitor, (Riendeau et al., 1994), was used to identify the forms of PLA₂ that are involved in LPS-induced AA release; AACOCF₃ was added to both LPS-free and LPS-treated systems. As shown in Fig. 3E, AACOCF₃ inhibited AA release in both systems.

Investigation of the mechanism by which CKD712 inhibits LPS-induced [³H] AA release

We examined the direct effects of CKD712 on cPLA₂ activity in a cell-free system. First, to separate cPLA₂ from other forms of PLA₂ such as sPLA₂, or a Ca²⁺-independent form, cPLA₂ was partially purified according to the activity profile from RAW 264.7 cell homogenates by using a Heparin HP hydrophobic column (Fig. 4A) and MonoQ™ anion exchange column (Fig. 4B). To confirm whether the active fractions displayed mostly cPLA₂ activity, fractions were pre-incubated with AACOCF₃ and DTT, a sPLA₂ inhibitor. The PLA₂ activity in the heparin-non-binding fractions was inhibited by AACOCF₃ in a concentration-dependent manner, but not DTT (Fig. 4C), whereas the activity in the heparin-binding fractions was inhibited by DTT, but not AACOCF₃ (Data not shown). The activity of the heparin-non-binding fractions was further purified using a MonoQ™ anion exchange column, and the active fractions incubated with vari

Fig. 4. CKD712 has no direct effect on purified cPLA₂ from RAW 264.7 cells. cPLA₂ was partially purified from RAW 264.7 cells following the procedure outlined in the "Materials and Methods". (A) The prepared sample was applied to HiTrap™ Heparin HP column (1 ml, GE Healthcare) pre-equilibrated with buffer H at a flow rate of 0.5 ml/min. (B) The PT of Hi-Trap™ Heparin HP column, enzyme activity was recovered in unbound path-through (PT), was applied to Mono Q™ column (GE healthcare). (C) Purified cPLA₂ was incubated with AACOCF₃ (cPLA₂ inhibitor) or DTT (sPLA₂ inhibitor) or CKD712 at various concentration for 10 min, and then, PLA₂ activity was assayed with the standard assay system.

Fig. 5. CKD712 downregulates the activity and expression level but not phosphorylation of cPLA₂. (A) RAW 264.7 cells were incubated for 1 h with the indicated concentrations of CKD712. After 1 h, the cells were treated with 1 μg/ml LPS and incubated for 8 h. Cells were then harvested and washed 2 times with TBS, and sonicated in lysis buffer (120 mM NaCl, 7 mM Tris-Cl, and 1 mM EDTA, pH 9.0). The sonicated cells were centrifuged at 2,000 × g for 10 min at 4°C. The supernatants (lysates) were assayed for cPLA₂ activity, and immunoblotted with anti-cPLA₂ antibody. (B) RAW

264.7 cells were incubated with LPS for 30 min after pre-incubation with CKD712 at 0, 1, 10, 50, and 100 μM for 30 min. The lysate was then immunoblotted with anti-cPLA₂ and anti-phospho-cPLA₂ antibodies. **p* < 0.05, ***p* < 0.01 compared with no treated-control group, ##*p* < 0.01 compared with LPS and 0 μM-treated group.

ous concentrations of CKD712. CKD712 showed no significant effect on this cPLA₂ activity (Fig. 4C).

Next, we examined in an indirect manner, the regulatory mechanism by which CKD712 inhibits cPLA₂. The activity of cPLA₂ enzyme could be stably increased by its phosphorylation, Ca²⁺ binding to its C2 domain (Leslie, 1997), or upregulation of enzyme expression. We examined the effect of CKD712 on the expression levels of cPLA₂ and its activating mechanism. As shown in Fig. 5A, the protein level of cPLA₂ in cell homoge-

nates increased by 1 μg/ml LPS and was attenuated by CKD712 in a concentration-dependent manner. On the other hand, the inhibitory effect of CKD712 on cPLA₂ expression was paralleled with that of its activity. CKD712 decreased cPLA₂ activity in both the control and LPS-treated cells (Fig. 5A). To examine whether CKD712 inhibits cPLA₂ phosphorylation, the cells were pre-treated with CKD712 at various concentrations, and stimulated with LPS for 0.5 h. As shown in Fig. 5B, whereas CKD712 decreased LPS-induced expression of cPLA₂, the level of

Fig. 6. Effect of CKD712 on cPLA₂ activation by calcium influx. RAW 264.7 cells were plated in 96-well black plates at 5×10^4 cells per well. After 24 h, calcium influx was detected with Fluo-4 NW Calcium Assay Kits (Invitrogen, USA) at 516 nm wavelength. Calcium influx into the cytosol was detected in cells treated with LPS (A) and A23187 (B) for 10 s (arrows).

Fig. 7. Inhibition of LPS induced NF-κB activation by CKD712. After CKD712 was treated at indicated concentrations for 1 h in free DMEM, RAW 264.7 cells were treated with 1 μg/ml LPS and incubated for 4 h. Lysates and nuclear extracts of cells were prepared using a Nuclear Extraction kit (Panomics, USA). (A) Five micrograms of nuclear extract was incubated with a biotin-labeled probe; NF-κB: (5' → 3') AGT TGA GGG GAC TTT CCC AGG C, at 15°C for 30 min. Images of probes bound with nuclear extract were acquired using ChemiDoc™ XRS (Bio-Rad), and the lysates were immunoblotted with anti-cPLA₂ and anti-COX-2 antibodies (B) After incubation with CKD712 and LPS for 8 h, released

PGE₂ was detected, in the medium used to grow the cells, by using anti-PGE₂ antibody (PGE₂ ELISA kit; Sapphire Bioscience). ** $p < 0.01$ compared with no treated-control group, ## $p < 0.01$ compared with LPS and 0 μM-treated group.

phosphorylation of cPLA₂ was not affected even at 100 μM CKD712.

Next, to examine whether CKD712 regulates cPLA₂ activity through the influx of Ca²⁺, the cells were pre-treated with CKD712 at various concentrations and assayed for Ca²⁺ influx by detecting the intensity at 516 nm using Fluo-4 NW Calcium Assay Kits. A23187 immediately evoked Ca²⁺ influx, which gradually decreased (Fig. 6B), whereas LPS did not induce Ca²⁺ influx (Fig. 6A). In addition, pre-treatment with CKD712 even up to a high dose (100 μM) failed to decrease Ca²⁺ influx (Fig. 6B). These results collectively suggest that CKD712 may inhibit cPLA₂ activation by inhibiting the expression of cPLA₂, and not via its phosphorylation or increased Ca²⁺ influx.

Effect of CKD712 on LPS-induced NF-κB activation

To investigate how CKD712 inhibits the expression of cPLA₂, we focused on the activation of the NF-κB transcription factor and MAPK pathway that the effects of LPS are known to be highly dependent upon (Dieter et al., 2002; Gilroy et al., 2004). First, to elucidate whether CKD712 blocked NF-κB activation by LPS, the cells were pretreated with CKD712 at various concentrations, and then stimulated by LPS. After 4 h, the interac-

tion of nuclear proteins with a 65-kDa NF-κB probe was detected using an EMSA gel shift assay. Figure 7A shows that CKD712 significantly blocked LPS-induced activation and nuclear translocation of NF-κB. On the other hand, our data show that CKD712 inhibited LPS-induced expression of cPLA₂ and COX-2 (Fig. 7B). Accordingly, Fig. 7C shows that LPS significantly increased PGE₂ levels, and CKD712 blocked PGE₂ release from RAW264.7 cells.

Effect of CKD712 on LPS-induced MAPK activation

LPS-induced NF-κB activation is known to be associated with the activation of the MAPK pathway (Luo et al., 2006). Therefore, to examine whether LPS mediates the activation of MAPK pathway, the effects of LPS on the phosphorylation of MAPKs was detected using Western blot analysis, after the cells were treated with PD98059, SP600125, and SB203580, the inhibitors for ERK1/2, SAP/JNK, and p38 MAPK, respectively. As shown in Fig. 8A, LPS markedly phosphorylated MAPKs. Our data show that SAP/JNK and p38 MAPK phosphorylation was blocked by the respective inhibitors; however, PD98059 did not inhibit LPS-induced activation of ERK1/2 under the conditions used in this experiment (Fig. 8A). We then examined the effects

Fig. 8. Inhibition of phosphorylation of ERK1/2 and JNK/SAP by CKD712. After the cells were treated with MAPK inhibitors at 5 μ M (A) or CKD712 at 0, 1, 10, 50, and 100 μ M (B) for 0.5 h in free DMEM, RAW 264.7 cells were treated with 1 μ g/ml LPS and incubated for 0.5 h. Next, the cells were harvested and washed 2 times in TBS, and then sonicated in lysis buffer (120 mM NaCl, 75 mM Tris-Cl, and 1 mM EDTA, pH 9.0). The sonicated cells were centrifuged at $2,000 \times g$ for 10 min at 4°C. Supernatants (lysates) were immunoblotted with anti-MAPK, anti-phospho-MAPK, anti-cPLA₂, and anti-phospho-cPLA₂ antibodies. GAPDH was used as a protein-loading control; its phospho-protein form (A) was detected by anti-GAPDH, not by anti-phospho-GAPDH. For immunoblotting of phospho-proteins, BSA was used instead of skim milk, and NaN₃ was excluded from all processing.

of CKD712 on the activation of MAPKs. Figure 8B shows that CKD712 inhibited LPS-induced phosphorylation of ERK1/2 and SAP/JNK in a concentration-dependent manner, but failed to inhibit LPS-induced phosphorylation of p38 MAPK.

To examine the effects of CKD712 and MAPK inhibitors on cPLA₂ and AA release, the activity and expression of cPLA₂ was analyzed. Although MAPK inhibitors decreased LPS-induced release of AA, CKD712 inhibited LPS-induced release of AA to lower than the control level as a stronger inhibitor compared to any of the MAPK inhibitors (Fig. 9A). Concomitantly, LPS increased the expression and activity of cPLA₂ by 1.4 folds and ~1.8 folds, respectively (Figs. 9B and 9C). CKD712, PD98059, and SP600125 blocked both the expression and activity of cPLA₂ up to the activity of the control or lower, whereas SB203580 showed mild inhibition of the LPS-induced effects (Fig. 9C). Our results show that CKD712 inhibited LPS-induced expression of cPLA₂, which is mediated by NF- κ B activation through ERK1/2 and/or SAP/JNK pathway rather than p38 MAPK.

DISCUSSION

The cPLA₂-induced release of AA is known to play a crucial role in the inflammatory response (Magrioti and Kokotos, 2009) and sepsis (Uozumi et al., 2008). In the present study, we showed that CKD712 inhibited LPS-induced release of AA through inhibition of cPLA₂ expression. CKD712 inhibits the LPS-induced inflammatory response through various pathways: inhibition of HMGB1 secretion by inhibiting PI3K and classical PKC (Oh et al., 2011), selective inhibition of VCAM-1 (Tsoyi et al., 2009), and inhibition of HO-1 and JAK-2/STAT-1 (Tsoyi et al., 2008). Although the inhibitory effect of CKD712 on TXA₂

(Pyo et al., 2007), iNOS, and COX-2 (Tsoyi et al., 2008) was also reported, whether it regulates cPLA₂ as a key enzyme in the inflammatory response remains unknown. In fact, the precise mechanism regarding LPS/TLR signal-induced activation and cPLA₂ expression remains unclear. A recent study showed that MAPKs and NF- κ B pathways were involved in LPS-induced up-regulation of cPLA₂ (Luo et al., 2006). Moreover, MAPKs are reported to mediate both cPLA₂ expression, and phosphorylation (Zhi et al., 2007). Several lines of evidence demonstrate that various stimulators beside LPS induce the expression of cPLA₂ through MAPK activation. Bradykinin-induced activation of PKC activates ERK, and then induces the expression of cPLA₂ (Yang et al., 2005). Induction of cPLA₂ and COX-2 by oncogenic Ras is mediated through the JNK and ERK pathway, rather than p38 MAPK (Van Putten et al., 2001).

It is known that cPLA₂ can promote the release of pro-inflammatory cytokines (McGuirk et al., 2006; Wang et al., 2008). However, it is unlikely that the expression levels of cPLA₂ change through an NF- κ B-mediated autocrine production of a pro-inflammatory cytokine such as TNF- α (Lee et al., 2011). LPS-induced release of AA seems to be a long-term effect in our system, because AA was significantly increased after 4 h. Moreover, the expression of cPLA₂ and the activation of NF- κ B were significantly increased after 8 h and 4 h, respectively (Figs. 7A and 7B). On the other hand, LPS induced the phosphorylation of MAPKs after 30 min (Fig. 8). Additionally, in this study, we determined that CKD 712 could inhibit perfectly LPS-induced AA release from RAW 264.7 cell even at 10 μ M (Fig. 3D) but, protein level of cPLA₂ was significantly reduced at > 50 μ M of CKD712 (Fig. 5A). Perhaps, in low concentration of CKD712, an additional pathway may regulate AA release not

Fig. 9. LPS-induced AA release and cPLA₂ expression is related to the MAPK-activating pathway. (A) [³H] AA release was induced by LPS in RAW 264.7 cells, which were incubated for 24 h in complete DMEM with 0.5 μCi/ml [³H] AA, washed 3 times with free DMEM, and then incubated with MAPK inhibitors or CKD712. MAPK inhibitors were added at IC₅₀ concentrations that downregulate cytokine levels in monocytes: SP600125 (JNK/SAP inhibitor), IC₅₀: 5-10 μM, SB203580 (p38 MAPK inhibitor), IC₅₀: 50-100 nM, and PD98059 (ERK1/2 inhibitor), IC₅₀: 2 μM. After 0.5 h, the incubated cells were treated with 1 μg/ml LPS and incubated for 8 h. [³H] AA was detected in the supernatant over an 8-h time course (0, 2, 4, 40, and 8 h), using Tri-Carb 2910TR/Perkin Elmer LAS. (B) RAW 264.7 cells were incubated for 0.5 h with CKD712 or MAPK inhibitors. After 0.5 h, cells were treated with 1 μg/ml LPS and incubated for 8 h. Cells were then harvested and washed 2 times with TBS, followed by sonication in lysis buffer (12 mM NaCl, 75 mM Tris-Cl, and 1 mM EDTA, pH 9.0). The sonicated cells were centrifuged at 2,000 × g for 10 min at 4°C. The RAW 264.7 cell lysate was immunoblotted with anti-cPLA₂ antibody, and assayed for cPLA₂ activity. ***p* < 0.01 compared with no treated-control group, #*p* < 0.05 ##*p* < 0.01 compared with LPS only-treated group.

only regulation of cPLA₂ expression pathway. In order to verify this hypothesis, we plan some additional experiments recently.

Several studies showed that MAPKs, including p38 MAPK (Leoncini et al., 2006), ERK (Su et al., 2004), and JNK (Casas et al., 2009), also induce cPLA₂ phosphorylation. However, our results indicate that phosphorylation of cPLA₂ was not observed after 2 h of stimulation with LPS (data not shown). Instead, our study showed that MAPK inhibitors diminished not only LPS-induced release of AA, but also cPLA₂ expression, suggesting that MAPKs may mediate the expression of cPLA₂.

Despite our use of MAPK inhibitors at different concentrations that can inhibit the expression of cytokines such as IL-2 and TNF-α as applied in studies investigating cPLA₂ expression by using NF-κB (Barancik et al., 2001; Bennett et al., 2001; Xin et al., 1999), each of the MAPK inhibitors showed a different effect on LPS-induced AA release. PD98059 at 2 μM, SP 600125 at 110 nM, and SB203580 at 70 nM, could inhibit the phosphorylation of MAPKs (data not shown). That is, SB203580

slightly inhibited LPS-induced release of AA at 1 μM, whereas SP600125 and PD98059 completely inhibited the release at 5 μM and 1 μM after 8 h, respectively (data not shown). As shown in Fig. 9, MAPKs were involved in LPS-induced AA release. Furthermore, CKD712 inhibited the phosphorylation of ERK1/2 and JNK/SAP, but not p38 MAPK, suggesting that CKD712 could completely inhibit the release of AA by inhibiting the activation of ERK1/2 and SAP/JNK.

The results from this study collectively show that CKD712 inhibited LPS-induced activation of NF-κB by inhibiting ERK1/2 and SAP/JNK, rather than p38 MAPK, thus inhibiting the release of AA through the inhibition of cPLA₂ expression. Our findings may provide a rationale for using CKD712 as a potent therapeutic agent for sepsis through its reduction of AA, a precursor to inflammatory mediators such as eicosanoids.

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