**INTRODUCTION**

Sepsis, a systemic inflammatory response syndrome, remains a potentially lethal condition. (S)-1-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-κB 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-κB pathway leading to reduced cPLA2 expression in RAW 264.7 cells.

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**Keywords:** CKD712, cPLA2, MAPKs, NF-κB, sepsis
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-diphenyltetrazolium bromide], and dithiothreitol (DTT) were purchased from Sigma Chemical Co. (USA). Archichondylin 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-glyero-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 Amer sham 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 collected in the 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.

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).

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 a 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 nM 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 (Blidorad). Proteins were