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碩士學位論文

Development of Novel Peptide Inhibitors of Cytosolic Phospholipase $A_2\alpha$

朝鮮大學校 大學院

生物新素材學科

姜 成 珍

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새로운 $cPLA_2\alpha$ 억제제들 개발

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Phospholipase A$_2$s (PLA$_2$) 는 인지질의 *sn-2* 위치에서 에스테르기를 가수분해한다. PLA$_2$에 의한 세포막에서의 아라키돈산의 방출은 프로스타글란딘들, 류코트린들, 그리고 다른 에이코사노이드들의 변환들에 의해서 염증을 야기하는데 중요한 역할을 한다. 다른 PLA$_2$들과 달리 85 kDa의 크기의 cytosolic phospholipase A$_2$의 이성질체들 중에서 cPLA$_2$α는 아라키돈산을 포함하는 인지질들 중에서 유일하게 특이성을 가진다. 또한, 이 효소는 대부분의 세포들과 조직들에서 발견되고며 마이크로몰 농도의 칼슘이온들에 의해 조절된다. 또한 cPLA$_2$α는 천식, 관절염과 다른 염증관련 병들과 관련이 있다고 알려져 있다. 따라서 새로운 염증치료 약물들의 개발에 있어 매혹적인 치료적인 목표로써 생각되어진다.

이번 연구에서는, 인간의 cPLA$_2$α유전자를 클로닝하였으며 그 유전자를 HEK293 세포에 과발현시켰다. 안정한 세포 주를 만든 것을 확인하기 위

하여 간단한 측정법인 RT-PCR을 이용하였다. $cPLA_2\alpha$ 단백질은 0.2~0.4 M 농도의 NaCl에서 Q-sepharose를 이용하여 크로마토그래피를 수행하여 부분 정제 하였다. 파아지 디스플레이 방법으로 펩타이드를 선별하였다. 7개짜리 파아지 라이브러리의 패닝 과정을 거쳐서 펩타이드의 시퀀스를 얻어내었다. 새로운 펩타이드들인 PLA4, PLA5 그리고 PLAC는 $cPLA_2\alpha$에 대한 억제제를 확인하였다. 펩타이드들을 합성한 후 $cPLA_2\alpha$에 대하여 활성을 측정하였다. U937 세포를 분화시킨 후 아라키도닉산을 ^3H로 라벨을 붙인 세포막들을 이용 효소와 세포막을 부분적으로 분리하여 $cPLA_2\alpha$에 대한 활성을 측정하였다. 펩타이드들은 인간 적혈구에 처리하였을 때 혈액 용혈성이 나타나지 않았다. 게다가 PLA4, PLA5 그리고 PLAC를 HaCaT 세포에 처리했을 때 낮은 수준의 세포내 독성을 보였다. 세 번의 패닝 결과로 $cPLA_2\alpha$에 결합하는 펩타이드가 보존서열을 포함하고 있다는 것을 확인하였다. 그리고 그 펩타이드들이 세포 안으로 유입되는 것을 확인하였다. 이 펩타이드들 중에서 가장 활성이 좋은 펩타이드는 PLA5였으며 그 활성을 유세포 분석과 형광 현미경분석을 통해서 그 저해 활성을 확인을 하였다. 따라서 PLA5는 염증질환에 있어서 $cPLA_2\alpha$에 대한 억제제로써 국소적인 치료로 사용될 수 있을 것으로 여겨진다.

ABSTRACT

Development of Novel Peptide Inhibitors of Cytosolic Phospholipase $A_2\alpha$

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Phospholipase A_2 (PLA_2) isozymes hydrolyze the ester bond at the *sn-2* position of phospholipids. The release of arachidonic acid from membranes by PLA_2 and its subsequent conversion into prostaglandins, leukotrienes, and other eicosanoids play a key role in the process leading to inflammation. Unlike the other PLA_2s, an 85-kDa group Ⅳ cytosolic phospholipase A_2 isoform ($cPLA_2\alpha$) has a specificity for arachidonate-containing phospholipids. Also, this enzyme is found in most cells and tissues that are regulated by micromolar concentrations of Ca^{2+}. The essential role of $cPLA_2\alpha$ in asthma, arthritis, and other inflammatory disease is now well documented, and $cPLA_2\alpha$ has been identified as an attractive therapeutic target in the development of new inflammatory drugs. In this study, Human $cPLA_2\alpha$ gene was cloned and

overexpressed in HEK293 cells. It was revealed by RT-PCR that cPLA$_2$α gene was stably integrated into chromosomal DNA of the cells. cPLA$_2$α protein was partially purified by 0.2~0.4 M NaCl fractionation using I Q-sepharose column chromatography. Peptides inhibiting cPLA$_2$α were screened by using phage-display system with the partially purified cPLA$_2$α proteins. From the phage display experiments, finally three peptides including PLA4, PLA5 and PLAC were obtained. Inhibition assay of cPLA$_2$α using ^{3}H-labeled membrane as a substrate showed that the peptides could inhibit the enzyme activity. The peptides (PLA4,PLA5 and PLAC) showed low level of cytotoxicity against HaCaT cells. Furthermore, the peptides had no hemolysis activity when they were treated in the human red blood cell. Among three peptides, PLA5 showed the highest inhibiting effect on cPLA$_2$ α, suggesting that it can be a usable therapeutic inhibitor against the enzyme.

I. Introduction

The phospholipase A_2 (PLA_2) superfamily consists of a large number of structurally and functionally distinct proteins that hydrolyze fatty acids from the *sn-2* position of glycerophospholipids. The fatty acid hydrolysis liberates lysophospholipids and free fatty acids, including arachidonic acid (AA). As shown in Fig 1, AA is then converted to prostaglandins by the cyclooxygenase pathway and to leukotrienes by the 5-lipoxygenase pathway and to hydroxyeicosanoic acids by the cytochrome P-450 monooxygenase pathway. These lipid mediators are implicated in the pathophysiology of asthma, arthritis, and other inflammatory diseases (1). Leukotrienes cause airway obstruction in asthmatics through bronchoconstriction, increased mucus secretion, and chemoattraction of inflammatory cells; prostaglandins potentiate pain and edema associated with arthritis (2). In addition, AA and other unsaturated fatty acids are themselves important regulators of specific cellular processes including regulation of protein kinase C and phospholipase Cγ, modulation of ion channels, and cell death (3).

The group IV A, cytosolic $PLA_2\alpha$ ($cPLA_2\alpha$) is an 85 kDa protein that is activated by a variety of stimuli such as proinflammatory cytokines, growth factors, neurotransmitters, antigens, and endotoxins. This activation is regulated by an increase of intracellular calcium concentrations and phosphorylation (4). $cPLA_2\alpha$ is ubiquitously and constitutively expressed in most cells and tissues. One notable exception is mature T and B lymphocytes (5). Although mammalian cells have structurally diverse forms of phospholipase A_2 enzymes, $cPLA_2\alpha$ has been shown to play a pivotal role in the biosynthesis of inflammatory lipid mediators. Cells and tissues from $cPLA_2\alpha$-deficient mice fail to produce leukotrienes, prostaglandins, or PAF (6) and are more resistant to ischemia-reperfusion injury of the brain, collagen-induced arthritis, inflammatory bone

resorption, intestinal polyposis, bleomycin-induced pulmonary fibrosis, acute respiratory distress syndrome induced by HCl or endotoxin, etc (7). Therefore, cPLA$_2\alpha$ represents a potential useful therapeutic target to control such diseases.

Phage display technique has been used in a number of application (8), including epitope mapping (9), mapping protein-protein contacts (10), and identification of peptide mimics of non-peptide ligands (11), discovery of novel peptide ligands for receptors, developing of antibodies, and discovery of inhibitor for enzymes. The fusion of a small-randomized peptide to the coat proteins of a filamentous phage allows the screening of peptides in binding assays against immobilized target molecules without affecting phage infectivity. Furthermore, the amino acid sequence of the selected peptide can be determined by propagation and sequencing of the phage DNA (12).

In this study, novel cPLA$_2\alpha$ peptide inhibitors was isolated through a selection from a filamentous phage liberating displaying random peptides.

I. Materials and Methods

Mammalian cell cultures

Immature human mast cell line (HMC-1) used in this study has a functional IgE receptor and mutated in the coding region of the C-kit protooncogene and obtained from J. Y. Ro, University of Sungkyunkwan, Suwon, Korea; The cells were cultured in IMDM medium (Gibco., Co) containing L-glutamine, ribonucleosides, deoxyribonucleosides, antibiotic (penicillin-streptomycin) and 10% FBS. HaCaT, HEK293 and NIH3T3 cells were cultured in DMEM containing high glucose in L-glutamine, pyridoxine hydrochloride, 110 mg/L sodium pyruvate. U937 cells were cultured in RPMI 1640 medium. Sodium bicarbonate was added 3.7 g/L with 10% heat inactivated FBS and 4 mM L-glutamine and antibiotics in a humidified 5% CO_2 incubator, anchoring independent cell line.

Preparation of cPLA$_2\alpha$ cDNA

Total RNA was isolated from HMC-1 using TRI regent (Molecular Lab. co., Inc.). 3×10^6 cells (HMC-1 cells, 570 ng/$\mu\ell$) were used for total RNA isolation. First strand cDNA was synthesized and analyzed by RT-PCR. Briefly total RNA 4 $\mu\ell$ (0.464 μg) with Oligo-d(T) 1 $\mu\ell$ (100 pmole/ $\mu\ell$) was incubated at 70℃ for 5 min and quick-chilled at 4℃ for 5 min and hold on ice. The cDNA was synthesized by adding following mixture into sterilized microcentrifuge tube, 5 x reaction buffer

4 ㎕, 25 mM $MgCl_2$ 6 ㎕, 25 mM dNTP 4 ㎕, reverse transcriptase Improm-II™ 1 ㎕, 722 ng/㎕ first strand cDNA was synthesized using Improm- II ™ reverse transcriptase by incubating at 70℃ for 15 min. The $cPLA_2\alpha$ gene was made by PCR using overhanging oligonucleotides: $cPLA_2\alpha$ Forward primer 5'-TGC CGC TCG AGA TGT CAT TTA TAG ATC CTT AC-3' and $cPLA_2\alpha$ Reverse primer 5'-TGC GAA GCT TTT ATG CTT TGG GTT TAC TTA G- 3'; the oligonucleotides encode the 15 residue $cPLA_2\alpha$ gene and incorporate at their ends *Xho*I and *Hind*III restriction sites. PCR was performed using 2 mM $MgCl_2$, pfu DNA polymerase (New England BioLab, Co.) (followed by 30 cycles of denaturation at 94℃ for 40 sec, annealing at 61℃ for 1 min, extention at 72℃ for 4.5 min).

Construction of plasmid DNA

An A tailing procedure for blunt ended PCR fragments were used in pGEM T-easy vector cloning (Because $cPLA_2\alpha$ DNA fragment was amplified with pfu DNA polymerase). Purified PCR fragment was started with 1~7 ㎕, generated by a pfu polymerase with 1 ㎕ pfu DNA polymease 10 x buffer with $MgCl_2$. dATP was added to a final concentration of 0.2 mM. Deionized water was added to a final reaction volume of 10 ㎕. product was incubated at 70℃ for 30 mins, and then next ligated into pGEM T-easy vector (Promega. Co.,). In the ligation of DNA, molar ratio of vector and insert DNA is very important when cloning a fragment into a plasmid vector. The PCR product was purified by electro elution, *Xho*I-*Hind*III digested and ligated with T4 DNA ligase (New England BioLab, Co.), for 12 hr at 4℃, into the pGEM T-easy vector. Competent cell; Inoue method was used for competent cell preparation (Molecular cloning, 3rd edition, volume 1, 1, 112).

Transformation into Top 10 *E. coli* strain; Transferred 10 ㎕ of DNA (insert, vector ligation product) into 200 ㎕ competent cells. Gently flicked the tubes to mix and placed then on ice for 30 mins. Heat shock the cells for 90 secs in a water bath at exactly 42℃. Immediately return the tubes to ice for 2 mins. Added 800 ㎕ LB media to tubes. Incubated for 1 hours at 37℃ with shaking (190 rpm). Centrifuged at 8000 rpm for 10 mins, resuspended in 100 ㎕ LB medium, and plated on LB/ ampicillin/ IPTG/ X-gal plates containing 100 ㎍/ml ampicillin , 80 ㎍/ml X-gal , 0.5 mM IPTG. Incubated the plates overnight (16~24 hours) at 37℃. The constructs were transformed into the *E. coli* Top 10 bacteria cells. Bacteria were plated on LB agar contained of 100 mg/ml ampicillin and incubated overnight at 37℃. The *Xho*I-*Hind*III digested product was purified by electro elution and Ligation of $cPLA_2$ α, *Xho*I-*Hind*III digested pcDNA3.1(-) vector and ligated with T4 DNA ligase (New England BioLab, Co.) at 4℃ for 12 hrs into the pcDNA3.1(-) vector.

Agarose gel electrophoresis

Twenty ㎕ of each aliquot was run onto 1% agarose gel (Sub-Cell GT Agarose gel electrophoresis system, Bio-Rad. Co.) at 80 V/cm for 4 hrs in TAE buffer (89 mM Trisma base, 89 mM Acetic acid, 2 mM EDTA, pH 8.0) according to slightly modified method of Voytas (13). The gels were stained with ethidium bromide (0.5 ㎍/ml) for 30 min and destained in distilled water for 10 mins. The DNA bands were visualized by illumination with short-wave length ultraviolet light and photographed. For quantitative determinations, the integrated intensity of the ethidium bromide fluorescence of the bands (relaxed form) was acquired and

measured by using Gel document system (Bio-Rad Co.,).

$cPLA_2\alpha$ sequencing

$cPLA_2\alpha$/pGEM T-easy and $cPLA_2\alpha$/pcDNA3.1(-)/zeo was confirmed. $cPLA_2\alpha$ gene is 2.2 kb, four primer was made for DNA sequencing. T7 promoter primer, $cPLA_2\alpha$-2 primer ; 5'-CTA CGT TGC TGG TCT TTC-3', $cPLA_2\alpha$-3 primer ; 5'-GCT CAA ATT CAT CAG GAT C-3' and $cPLA_2\alpha$ Reverse primer 5'-TGC GAA GCT TTT ATG CTT TGG GTT TAC TTA G-3'.

Transfection and selection

Transferred $cPLA_2\alpha$ gene in a expression vector, pcDNA3.1(-)-$cPLA_2\alpha$ was transfected into HEK293 cells using METAFECTENE^{TM} transfection regent for 4 hrs with pcDNA3.1(-)/zeo as a mock control. In a 6 well tissue culture plate seed 2 $\times 10^5$ cells per dish in 20 ml of fresh suitable complete medium. Incubate the cells at 37℃ in a CO_2 incubator until they are at 30~60% real confluency (growing area should be covered 90~100%). The required time will vary among cell types, but usually this will take 18~24 hrs. The solution of DNA/RNA and METAFECTENE^{TM} transfection regent should have an ambient temperature and should be gently vortexed prior to use. Prepared the following solution using a cell culture grade 96-well plate or other tubes made of glass, polypropylene or polystyrene. Lipid : DNA range of 2~7:1. Solution A is 0.5~1.5 ㎍ of DNA in 100 ㎕ medium free of serum and antibiotics. Solution B is 1.0~6.0 ㎕ (Lipid: DNA

range of 3:1) of METAFECTENE^{TM} transfection reagent in 100 ㎕ medium free of serum and antibiotics. Combined the two solution, mixed gently by carefully pipetting several times (do not vortex or centrifuge), and incubate at room temperature for 15~20 mins. This time is required to form the DNA/RNA-lipid complex. Added the DNA/RNA-lipid complexes to the wells with the cells and mixed gently then incubated at 37℃ in a CO_2 incubator. Depending on cell type and promoter activity, assay cell extracts for gene activity 24~72 hrs following the start of transtection. After transfection, the mix was aspirated, and cells were cultured with DMEM medium with 15% FBS for an additional 24 hrs. Subsequently, For selection cells were incubated with complete medium containing 700 ㎍/ml of zeocin for 2 weeks. Cell clones resistant to zeocin were isolated and analyzed.

Semi-quantative RT-PCR

RNA extraction from the control and cPLA$_2$α expressing cells was conducted using the TRI reagent according to the manufacturer's instructions (Molecular Lab. Co.). Two microgram of total RNA was reverse-transcribed into cDNA by reverse transcriptase and amplification of HEK293 and a control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene sequence. The primers used for the PCR were as follows; cPLA$_2$α forward, 5'-TGC CGC TCG AGA TGT CAT TTA TAG ATC CTT AC-3' and cPLA$_2$α Reverse, 5'-TGC GAA GCT TTT ATG CTT TGG GTT TAC TTA G- 3' (designed to amplify a 2250-bp region); GAPDH forward, 5'-CCA TGG AGA AGG CGG GG-3' and GAPDH reverse, 5'-CAA AGT TGT CAT GGA TGA CC-3' (designed to amplify a 194-bp region). The conditions for PCR reactions of cPLA$_2$α were as follows; denaturation at 95℃ for 40 sec, annealing at 62℃ for 1 min

and extention at 72℃ for 2 min. The PCR exportential phase was determined on 20~30 cycles to allows comparison among cDNAs developed from identical reaction. PCR product were resolved on 1.0% agarose gels, which were then stained with ethidium bromide before being photographed. The optical number of PCR cycles was 28.

Partial purification of cPLA$_2\alpha$ protein

Ammonium sulfate fractionated, the powdered ammonium sulfate was added to the supernatant to leach at 30% saturation concentration and completely dissolved overnight at 4℃. The supernatant was transferred to clean beaker and ammonium sulfate was added to reach at 50%. The solution was centrifuged at 13,000 rpm in the same rotor for 30 mins at 4℃. The resulting pellet was dissolved in buffer A containing 25 mM KPO_4, pH 7.0, 10% (v/v) Glycerol, 1 mM DTT, 0.5 mM PMSF. The suspension was dialysised for 48 hrs in the 25 mM Tris-Cl (pH 7.5). Phosphorylation of cPLA$_2\alpha$; 50 μM ATP treatment, and then 10 min incubation, stimulation. 100ϕ plate media volume; 10 ml. And hen next, 10 mM ATP 50 $\mu\ell$ was treated in 10 ml incubating media (14). Stimulation for 10 mins at 37℃. Adherent cells was washed twice in the dish with ice-cold PBS and drain off PBS. Ice cold modified RIPA was added buffer to cells containing 1 ml per 10^7 cells/ 100 mm dish/ 150 cm^2 flask. Protease inhibitor (Protease inhibitor cocktail, Sigma. Cat# p2714). Adherent cells was scraped off the dish or flask with pipette; cell suspension was transferred on either a rocker or an orbital shaker for 15 mins for lysate cells. Lysate was centrifuged the at 14,000 x g in a precooled centrifuge for 15 mins. Immediately supernatant was transferred the to a fresh tube. Ion-exchange column chromatography on Q-sepharose,

The protein was obtained from the ammonium sulfate fractionation and Q-Sepharose column were loaded onto equilibrated with the buffer A. The bound proteins were eluted with 5 volumes of buffer B containing 25 mM KPO_4, pH 7.0, 10% (v/v) Glycerol, 1 mM DTT, 0.5 mM PMSF, 1 M NaCl under a liner gradient of 0 M to 1 M NaCl. The proteins eluted were collected and assayed for $cPLA_2\alpha$ relaxation activity.

SDS-polyacrylamide gel electrophoresis

10% SDS- polyacrylamide gel electrophoresis was carried out as described by Laemmli (15). Samples were completely denatured in SDS/ sample buffer containing 1.25% Tris-HCl, pH 6.8, 20% Glycerol, 2% β-mercaptoethanol, 0.1% Bromophenol blue, 10% SDS by boiling in a water bath for 2 mins and electrophoresis on 10% to 12% acrylamide gel. After fixed for 2 hrs in a solution A containing 50% Methanol, 10% Acetic acid, 40% H_2O, the gels were stained with coomassie blue solution containing 0.005% coomassie blue R-250 in solution A for 1 hr. The gels were then destained with solution B containing 5% Methanol, 7% Acetic acid, 88% H_2O. The protein bands were visualized by light box and photographed

Western blotting

The cells were lysed in a cell lysis buffer containg 20 mM Hepes, pH 7.4, 2 mM EGTA, 50 mM-glycerol phosphate, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol (DTT), 1 mM phenylmethysulfonyl fluoride (PMSF), 10 $\mu g/ml^{-1}$ and

equal amounts of the protein from each sample was underwent electrophoresis on 10% SDS polyacrylamide gels. For western blot analysis, proteins were separated on polyvinylidene difluoride (PVDF) membranes (Amersham Phamacia. Co, USA). Subsequently, the PVDF blots were blocked in a 5% skim milk for overnight. Primary antibody ($cPLA_2$ antibody, SANTA CRUZ Biotechnology, Inc.) incubations (1 : 1000) were carried out overnight at 4℃, followed by overnight incubations (1 : 20000 dilution in TBS-T) with the appropriate goat anti mouse IgG peroxidase conjugated affinity purified second antibody (CHEMICON International Inc.). Immunoreactive bands were visualized using an ECL detection kit (Amersham Phamacia. Co, USA) according to the manufacturers instructions. Developed films were photographed and captured using the Kodak film and cassette Box.

Phage display

Agarose Top was contained per liter containg 10 g Bacto-Tryptone, 5 g yeast extract, 5 g NaCl, 1 g $MgCl_2 \cdot 6H_2O$, 7g agarose, dispense into 50 ml aliquots. Tetracycline stock was contained 20 mg/ml in Ethanol. LB-Tet plate was contained LB medium, containing 15 g/ l Agar, cooled to below 70℃, add 1 ml tetracycline stock and pour. Blocking buffer was contained 0.1 M $NaHCO_3$ (pH 8.6), 5 mg/ml BSA 0.02% NaN_3-filter sterilize, store at 4℃. TBS was contained 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, PEG/ NaCl: 20% (w/v) polyethylene glycol-8000, 2.5 M NaCl, Iodide Buffer was contained 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 4 M NaI. M13 is not a lytic phage so plaques are due to diminished cell growth rather than cell lysis and are turbid rather than clear (16). The F-factor of ER2738 contains a mini-transposon that confers tetracycline resistance, so plating and propagating on

tetracycline-containing media can select cells harboring the F-factor. Phage tittering, panning of six days and sequencing used to manuscript of Ph.D.-7^{TM} Phage display peptide library kit (16,17) (Rapid screening of peptide ligands with a phage display peptide library, New England Biolabs, Inc.)

Peptide synthesis

Three peptides, PLA4, PLA5 and PLAC were supplied from Anygen (Inc. Gwang-ju, Korea)

U937 cell membrane labeling

Preparation of [^{3}H]arachidonate labeled U937 membranes (18). 1.2 x 10^{6} cells/ml, [^{3}H]arachidonate treat of 0.1 μCi/ml into the cells. After incubation for 24 hrs harvest the cells. Washed the using of DPBS. Lysised using by sonication in PBS buffer. Centrifuged for 10 mins at 3000 rpm using of SS-34 rotor (Sorval Inc.). The supernatant was centrifuged for 30 mins at 2,000 x g. Pellet resuspend in water and autoclaved (inactive any residual phosphatase activity). Centrifuged 50,000 x g for 30 mins. Pellet was resuspend in water and frozen at -80℃.

Quantitative determination of phospholipids

This colorimetric methods was based on the formation of a complex between phospholipids and ammonium ferrothiocyanate (19). Dissolved 27 g of

ferric chloride ($FeCl_3$ $6H_2O$) and 30 g of ammonium thiocyanate (NH_4SCN) in 1 liter water. This solution was stable for months at room temperature. Standard solution of phosphatidylcholine in chloroform (1 mg/ml). Evaporate completely aliquots of phospholipid solutions in glass tubes. Dissolved the phospholipid residue in 2 ml chloroform and added 1 ml thiocyanate reagent. Vortexed 1 min and centrifuged at low speed, removed the red lower layer (chloroform) with a pasteurpipette. Read the absorbance of this solution at 488 nm and compare with known amounts of a standard phospholipid solution (range: 10~100 ㎍ per tube). All phospholipid do not give the same response but this method was useful for measuring rapidly phospholipid in mixtures before further analyses (19).

Measurement of arachidonic acid release from U937 cells

For measurements of the effects of $cPLA_2\alpha$ inhibitors on cellular arachidonate production, U937 cells were differentiated by treating (96 hrs) cells (2.5×10^5/ml) with 1.2% Me_2SO (v/v) (20). Cells were labeled (24 hrs) with [^{3}H]AA (0.1 μCi/ml) and washed in PBS buffer. After two rounds of washing, cells were resuspended in PBS containing 1mM $CaCl_2$. The cell suspension (1.2×10^6 cells/ml) was preincubated with inhibitors for 10 mins at 37℃ and then cultivated with 1.5 μM A23187 for 30 mins at 37℃ (21). After stimulation, the cells were spun down at 4°C and supernatants were collected to measure the released [^{3}H]AA, whereas the cell pellet was resuspended in PBS buffer to measure total cellular [^{3}H]AA incorporation. The quantity of ^{3}H was determined by liquid scintillation spectrometry (PACKARD, Tri-Carb Liquid Scintillation Counter, Co). The percentage of AA release was calculated using the formula $[S/(S + P)] \times 100$, where S and P are the radioactivities measured in equal

portions of the supernatant and cell pellet, respectively (22).

Assay of cPLA$_2\alpha$ activity

cPLA$_2\alpha$ was prepared from cPLA$_2\alpha$ overexpressing HEK293 cells that were disrupted by sonication in 50 mM Tris-HCl buffer, pH 7.5, containing 0.32 M sucrose, 100 μM EDTA, 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Sigma Co.). The homogenated cells were centrifuged at 2,000 × g for 10 min at 4℃, and supernatant was further centrifuged at 100,000 × g for 100 mins at 4℃ to obtain the cytosolic fraction (23). Differentiated U937 membranes were used as a substrate. Twenty-four hrs prior to harvesting the cells, [^{3}H]AA (0.2 μCi/ml) was added to the cell (1.0 × 10^6 cells/ml). After two rounds of washing with PBS buffer, the cells were disrupted by sonication (6 watts, 15 s × 3, Microson Inc.) and the homogenate was centrifuged at 2,000 × g for 10 mins. The supernatant was further centrifuged at 50,000 × g for 60 min after which the pellet was resuspended in water and autoclaved to inactivate any residual phospholipase A_2 activity. This suspension was then centrifuged at 50,000 × g for 60 min, and the pellet was resuspended in distilled water and frozen at -80℃ (23). The estimation of the total amount of phospholipids in these membrane fraction was determined from lipid phosphorous assay (20). To determine the inhibitory activity, 24 ㎍ of protein of cytosolic fraction from HEK293-cPLA$_2\alpha$ was preincubated for 30 min with the synthetic peptides (10 μM) at 37℃ in 50 mM Tris buffer, pH 7.5, which contained 5 mM $CaCl_2$, 40% glycerol. And then, [^{3}H]AA-labeled U937 membrane substrate (22 μM) was added to a final volume of 100 ㎕ of the assay mixture, and incubated for 1 hr at 37℃. The reaction was terminated by mixing with 0.5 ml of isopropyl alcohol/heptane/0.5 M H_2SO_4 (10:5:1). To extract the liberated [^{3}H]AA, heptane (0.7 ml) and water (0.2 ml) were added, and the solution was vigorously mixed

for 15 secs. The heptane phase was mixed with 100 mg of silica gel 60 (70230 mesh; Merck) and centrifuged, and the radioactivity in each supernatant was measured by liquid scintillation counting. (20, 22)

Hemolytic activity

Hemolytic activity for all peptides was determined using human red blood cells (hRBC) from healthy donors, collected on heparin. Fresh hRBC were rinsed three times in PBS (1.5 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM $NaHPO_4$, 135 mM NaCl, pH 7.3) by centrifugation for 10 min at 980 x g and resuspended in PBS. The peptides (final erythrocyte concentration, 4% v/v) samples were incubated with agitation for 60 mins at 37℃. The samples were then centrifuged at 980 x g for 10 mins. The absorbance of the supernatant was measured at 414 nm, and controls for zero hemolysis (blank) and 100% hemolysis consisted of hRBC suspended in PBS and 1% Triton X-100, respectively. Each measurement was made in triplicate.

Cytotoxicity in HaCaT cell

Cells were seeded onto 96-well plates at a density of 2×10^3 cell, respectively. Each concentrations of the peptides were treated. Proliferation and viability assay was determined by MTT [3-94,(5-dimethylthiazole-2-yl)- 2,5-diphrnyl tetrazolium bromide] assay. The plate was then incubated for 24 h before adding to each well 50 ㎕ of MTT reaction solution. The medium containing MTT was removed, and 100 ㎕ of dimethyl sulfoxide (DMSO) was added. Cells were incubated for 10 min at 37℃ with gentle shaking. The absorbance was read on

an ELISA plate reader. The optical density was read at 580 nm in an enzyme-linked immunosorbent assay plate reader after 2 hr of incubation. Cell viability was determined relative to the control. All studies were done in triplicate.

FITC labeling

FITC (Fluorescein isothiocyanate) was amine reactive probe. mine-reactive reagents react with non-protonated aliphatic amine groups, including the amine terminus of proteins and the ε-amino group of lysines. Peptide was dissolved 10 mg in 1 ml of 0.1 M sodium bicarbonate buffer. Amine-reactive compound was dissolved in DMF of (except for sulfonyl chlorides) DMSO at 10 mg/ml. While stirring of vortexing the protein solution, slowly added 50-100 ㎕ of the reactive dye solution. Reaction was incubated for 1 hours at room temperature with continuous stirring. After reaction to stop by adding 0.1 ml of freshly prepared 1.5 M hydroxylamine (pH 8.5). Conjugate was separated the from unreacted labeling reagent by using gel filtration with Sephadex G-25. Each fractions were lyophilized and FITC labeling was confirmed .

FACScan analysis

The cells showing fluorescence were analyzed by the FACScan. U937 Cells were untreated with peptide, treated with PLA5 for 10 and 20 min. Samples were analyzed on an Flow Cytometer (Becton Dickinson, San jose, CA) with FITC excited and collected through FITC filters, respectively. Data from a minimum of

3×10^4. Cells per sample were recorded with triggering on FITC fluorescence.

Confocal analysis

U937 cells were stained for microscopical analysis as described above for flow cytomeric analysis. After PLA5 peptide treatment, Incubation at 10, 20 mins. Intracellular localization was determined by confocal laser scanning microscope (Olympus fluoview FV 300, Japan) capable of laser excitation with green fluorescence filter. For analysis and photography a confocal laser scanning microscope was used.

III. Results and Discussion

As shown in Table 1 and Fig 1, cPLA$_2$α DNA was obtained by PCR using forward and reverse primers. cPLA$_2$α DNA fragment of 2.2 kb in size were eluted from 1% agarose gel and ligated to the *Xho*I and *Hind*III cleaved pGEM T-easy vector with T4 DNA ligase. The ligated DNA was transformed into the *E. coli* Top 10 and the transformants were selected on LB agar plate containing 100 ㎍/ml of ampicillin. The clones that form a white clone on the LB agar plate were selected. As shown in Fig 2, the selected clone harbored a 5.2 kb recombinant plasmid DNA having 2.2 kb insert. From mammalian expression vector pcDNA3.1(-)/zeo, the cloned gene was expressed cPLA$_2$α in the human embryonic kidney (HEK293) cell. This construct was transfected into the HEK293 cell line. Thirty stable transfectant cell lines were established following transfection and selection using zeocin (selection for pcDNA3.1(-)/zeo- cPLA$_2$α) for four weeks. cPLA$_2$α protein were acquired using ammonium sulfate precipitation. cPLA$_2$α were purified partially on Q-sepharose. Protein expression were confirmed by using western blot analysis. Peptides were screened by using phage-display system with purified cPLA$_2$α proteins partially. As shown in Fig 6, the flowchart of the general protocol that was used for phage selection and identification. Four subtraction/selection rounds on cultured cells yielded phage pools that exhibited significantly higher binding efficiency than the nonselected starting pool or control phages. For the selections, the cPLA$_2$α proteins of U937 cells were used. Titration before and after subtraction showed that three consecutive subtractions of an unselected phage library reduced the phage pool (17). Display of peptides may be achieved by fusion to coat proteins of filamentous phage such as M13. The infectivity per particle of the different fusion phage was compared by calculating the number of physical particles per expected, only a fraction (20~30%) of the

physical phage particles infect bacteria; there is no significant difference between the different fusion phage tested. Binding to anti-cPLA$_2\alpha$ does not necessarily implicate binding to the native of cPLA$_2\alpha$ protein. Therefore, binding of phage was tested on U937 cells transfected with expressing high levels of cPLA$_2\alpha$ protein. Binding is specific, because the addition of an excess of cPLA$_2\alpha$ abolishes binding completely. By using phage display was screened peptides that are PLA4, PLA5 and PLAC (Table 2). The peptides were biosynthesized. And then next, In order to study of effect in the human were tested the hemolysis. Hemolysis percentage was measured against the human red blood cells (hRBC) at various peptide concentration and PLA4, PLA5 and PLAC showed no hemolytic activity, while melittin was used like a positive control and to exhibited a strong hemolytic activity. The result is no effect of the human (Table 3). The results of this study contribute to the understanding of our peptide's toxicity required for targeting to the human. As shown in Table 4, cytotoxicity was tested the potential of the PLA4, PLA5 and PLAC peptides to inhibit the growth of the spontaneously immortalized human skin keratinocyte cell lines (HaCaT). These results were founded that the PLA4, PLA5 and PLAC peptides had a little cytotoxicity. The result of cytotoxicity is no effect in a high concentration of our peptides. The results of this study contribute to the understanding of the anti-inflammation response required for targeting to inhibition of cPLA$_2\alpha$. Also, we have found that PLA4 and PLA5 peptide stimulates AA release from U937 cells expressing cPLA$_2\alpha$, but not from cells infected with plasmid DNA containing the vector alone. Beside its role in generation of AA, an intriguing role for cPLA$_2\alpha$ may be in membrane trafficking. Many recent studies have implicated cPLA$_2\alpha$ activity in constitutive membrane trafficking (24). Most recent reports, however, implicate calcium-independent cPLA$_2\alpha$ isoform and because cPLA$_2\alpha$ is only on membrane after an increase in $[Ca^{2+}]_i$, it would not be a good candidate for

regulating constitutive trafficking. On the other hand, cPLA$_2\alpha$ may play a role in trafficking in response to $[Ca^{2+}]_i$ signals, although there have been no studies investigating this possibility. Selective inhibition of cPLA$_2\alpha$ by PLA4 and PLA5, U937 cells treated with [^{3}H]AA incorporate this radiolabeled fatty acid into phospholipid pools. As shown in Table 5, an assay using membranes isolated from these radiolabeled U937 cells as substrate was used to measure the activity of human cell, recombinant cPLA$_2\alpha$. By using semi-quantative RT-PCR, transfection of cPLA$_2\alpha$ was confirmed. cDNAs in HEK293 cells as shown in Fig 3. HEK293-cPLA$_2\alpha$ overexpression was confirmed western blotting as shown in Fig 4. As shown in Fig 5, western blotting of partial purified overexpressed cPLA$_2\alpha$ protein. When assayed in this manner, compound PLA4 and PLA5 was identified as an inhibitor of cPLA$_2\alpha$. As shown in Fig 7, the inhibition gave an IC_{50} value of three peptides. By using Phage-display, we were obtained PLA4 and PLA5. In Fig 6, flowchart was phage display method, briefly. And then next, for the purpose flow cytometry utilizing FITC-conjugates our peptides measure of intracellular targeting was analysis using by FACScan (Fig 8). The result of FACScan analysis is more effect PLA5 than PLA4. And PLAC of conserve region smaller effect then PLA5. These means were that PLA5 was selected inhibitor of cPLA$_2$ α to the last. Therefore, localization of cells confirm confocal analysis using by PLA5 (Fig 9) (25). The Fig 7 shown that PLA5 have intracellular effect of high level. We have shown that our peptides are new inhibitors of cPLA$_2\alpha$. The inhibition of cPLA$_2\alpha$ could control the excessive production of lipid mediators and exert protective effects in inflammatory disorders (26). For example, glucocorticoids is a famous drug that it inhibits a inflammatory response. Glucocorticoids control the inflammatory response through complex mechanisms. These drugs inhibit G protein-dependent activation of cPLA$_2$ activity (27). Glucocorticoids are potent agents for inflammation disorders, nevertheless severe side effects limit the long-term treatment. Recently, two important

papers have been published that demonstrates crucial roles of this enzyme in collagen-induced arthritis and prostaglandin E2-mediated bone resorption associated with inflammation using cPLA$_2\alpha$-null mouse (28). Both reports suggest the potential use of cPLA$_2\alpha$ inhibitors in inflammatory joint and bone diseases. However, this will not be without potential concerns. This upstream component of the metabolic pathways culminating in a large number of PGs, leukotrienes and cytochrome P450 products, will naturally affect the production of much more than PGE2. Because these products have been implicated in many physiological functions, often in counter-regulatory control systems, the effects of systemic inhibition are not entirely predictable (29). Therefore, development of topically usable nonsteroidal agent was needed. To develop the specific inhibitor for cPLA$_2\alpha$, phage display library containing 2×10^{11} random 7-amino acid peptides was applied. The results of third panning, we were able to identified a set of cPLA$_2\alpha$ interacting peptides containing a conserved sequence of ISFGW. Among them, the most efficient peptide PLA5 (YTISFGW) was selected by using activity assay of inhibition of arachidonic acid release from stimulated with calcium ionophore, A23187, [^{3}H]arachidonate labeled U937 cell. Surprisingly, PLA series were confirmed that penetrated cellular membrane without cell damage by FACScan and Confocal analysis (Fig 8, 9). Furthermore, PLA5 has low cytotoxicity and hemolysis activity.

Therefore, PLA5 is a potent therapeutic agent of inflammatory response in the capacity of inhibitor against of cPLA$_2\alpha$. Further study is that research active region of the anti-inflammatory protein and find binding region of cPLA$_2\alpha$ domains with deletion mutant.

Table 1. Primer sequence used in PCR and DNA sequencing

Primer	Sequence	length
cPLA$_2$α F	5'-TGC CGC TCG AGA TGT CAT TTA TAG ATC CTT AC-3'	32 mer
cPLA$_2$α-2	5'-CTA CGT TGC TGG TCT TTC-3'	18 mer
cPLA$_2$α-3	5'-GCT CAA ATT CAT CAG GAT C-3'	18 mer
cPLA$_2$α R	5'-TGC GAA GCT TTT ATG CTT TGG GTT TAC TTA G- 3'	31 mer

Table 2. Sequences of peptides derived from panning of 7-mer phage library. Number of separate times a given DNA and amino acid sequence was obtained from randomly selected plaques.

Peptide Name	Amino acid Sequence	Number
PLA4	His-His-Ile-Ser-Phe -Gly-Trp	3
PLA5	Tyr-Thr-Ile-Ser-Phe-Gly-Trp	7
PLAC	Ile-Ser-Phe-Gly-Trp	-

Table 3. Hemolysis activity of Melittin, PLA4, PLA5 and PLAC. % Hemolysis = (Ab 540 in peptide solution - Ab 540 in PBS + hRBC control) / (Ab 540 in 0.1% Triton X-100 - Ab 540 in PBS + hRBC control) + 100

Peptide	% Hemolysis (μM)									
	100	50	25	12.5	6.25	3.125	1.56	0.78	0.39	0.19
Melittin	100	100	100	100	100	94	60	14.9	0.4	0
PLA4	0	0	0	0	0	0	0	0	0	0
PLA5	1.8	0	0	0	0	0	0	0	0	0
PLAC	0	0	0	0	0	0	0	0	0	0

Table 4. Cytotoxicity of PLA4, PLA5 and PLAC in the HaCaT cell (IC_{50}).

Concentration (μM)	PLA4	PLA5	PLAC
100	3	45	11
50	1	26	0
10	0	0	0
5	0	0	0

Table 5. For measurements of the effects of $cPLA_2$ inhibitors on cellular arachidonate production. activity test with AA labeling peptides. U937 cells were labeled with 0.5 μCi/ml [^{3}H]AA and incubated at 37°C for 16 hrs. The cell suspension ($2X10^6$/ml) was pre-incubated with inhibitor for 10 mins at 37℃ and then incubated with 10 μM A23187 for 30 mins at 37℃. Radioactivity of released [^{3}H]AA was measured by liquid scintillation counting.

Peptide	IC_{50}
PLA4 (HHISFGW)	80 μM
PLA5 (YTISFGW)	50 μM
PLAC (ISFGW)	>200 μM
$AACOCF_3$	80 μM

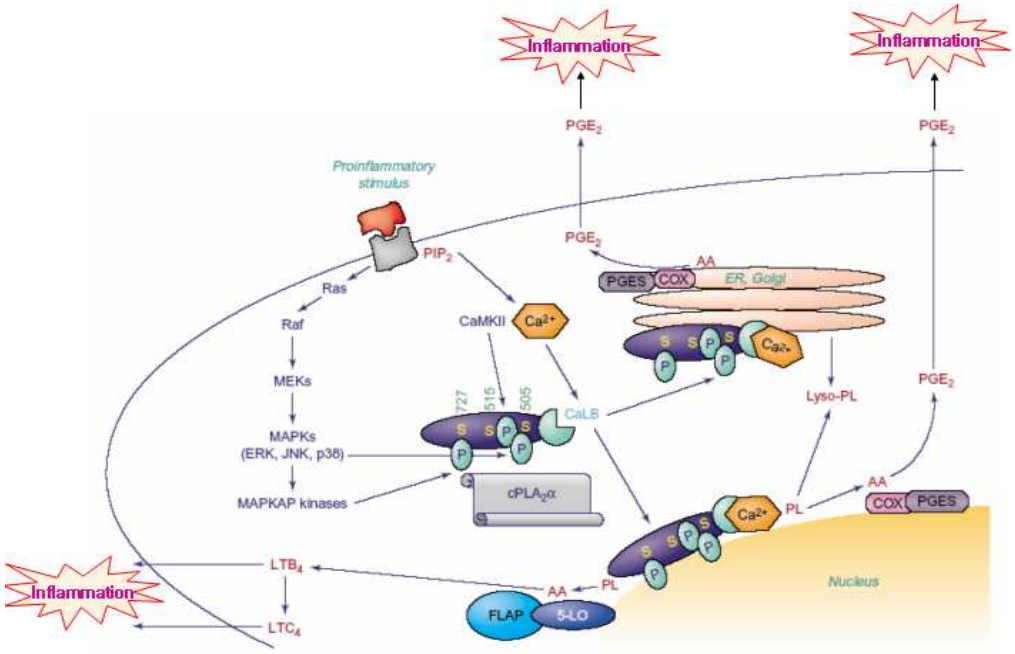

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Figure 1. A schematic model for cPLA$_2$α activation in cells.

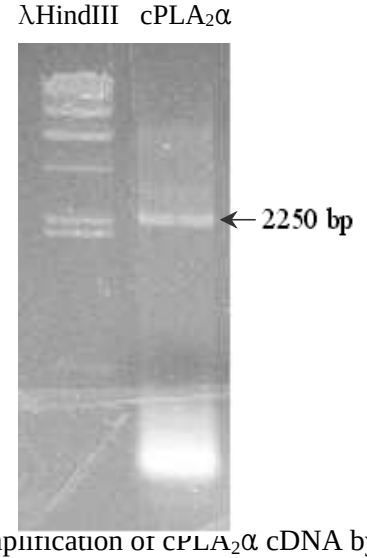

Figure 2. Amplification of $cPLA_2\alpha$ cDNA by PCR

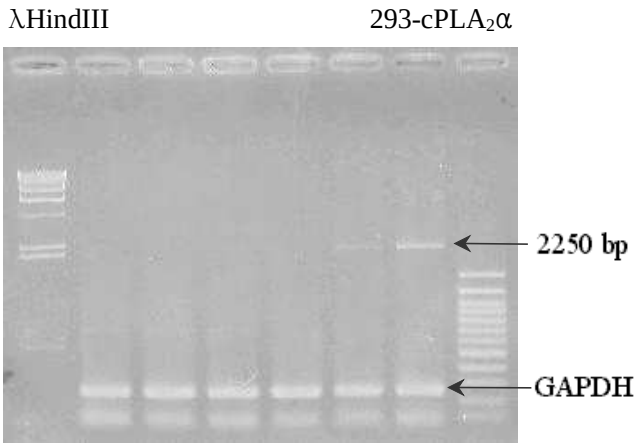

Figure 3. Localization of cPLA$_2$α cDNAs in HEK293 cells.

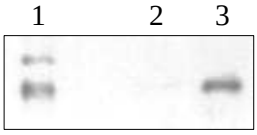

Figure 4. Western blotting of HEK293-cPLA$_2$α overexpression. HEK293-cPLA$_2$α overexpressed cell lysate, 1; Size marker, 2; Normal cell, 3; cPLA$_2$α overexpressed cell

Figure 5. Western blotting of partial purified cPLA$_2$α protein. The proteins (10 μg) were electrophoresis on an 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and blotted with anti-cPLA$_2$α antibody (Santa Cruz Inc.). 1, 293 cell only; 2, 293 cells transfected with cPLA$_2$α gene; 3, 293 cells transfected with cPLA$_2$α gene following with ATP activation (50 μM); 4, Ammonium Sulfate precipitation (50% saturation); 5, 0 ~ 0.2 M NaCl fraction (Q-sepharose fast flow); 6, 0.2 ~ 0.4 M NaCl fraction (Q-sepharose fast flow); 7, 0.4 ~ 0.6 M NaCl fraction (Q-sepharose fast flow)

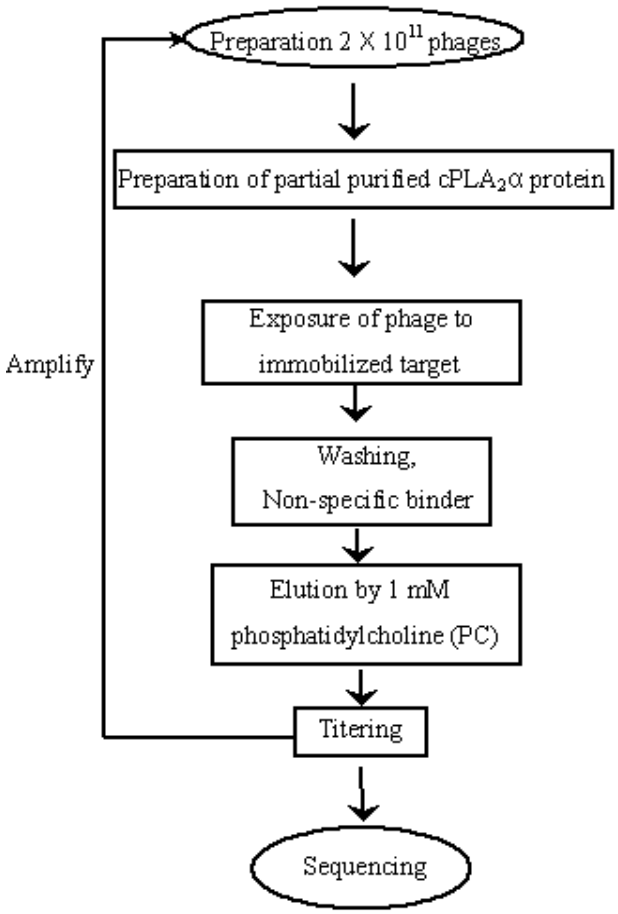

Figure 6. Flowchart of the phage selection protocol. The entire cycle was repeated four times.

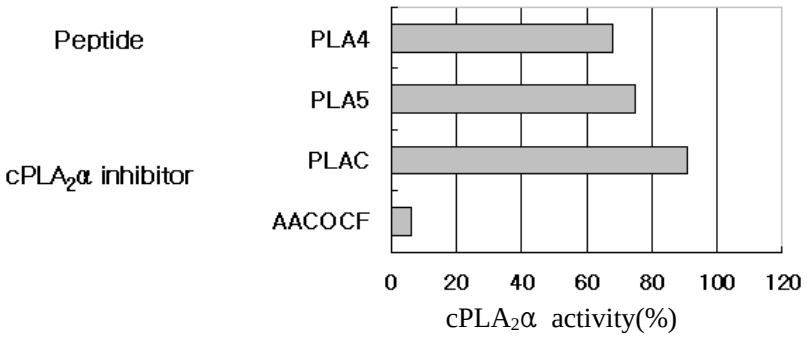

Figure 7. Effects of peptides on $cPLA_2\alpha$ activity. Assay of $cPLA_2\alpha$ activity using the [^{3}H]arachidonate-labeled U937 membranes as substrate used partially purified enzyme and membrane substrate (20 μM) in 25 mM Tris-Cl buffer, pH 7.5, containing 5 mM $CaCl_2$, 5 mM DTT.

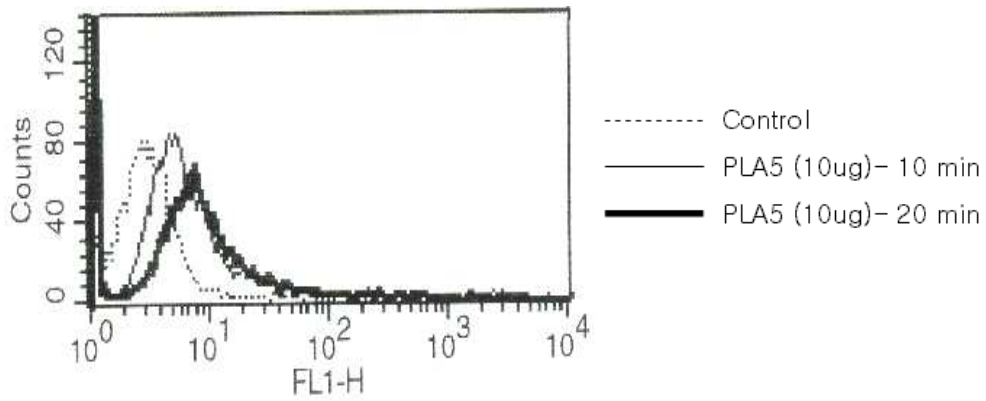

Figure 8. The cells showing fluorescence were analyzed by the FACScan flow cytometer. (A) Cells were untreated with peptide; (B) treated with PLA5 for 10 mins; (C) treated with PLA5 for 20 mins. FITC labeled peptides and U937 cells (2×10^4) were incubated for 10, 20 min.

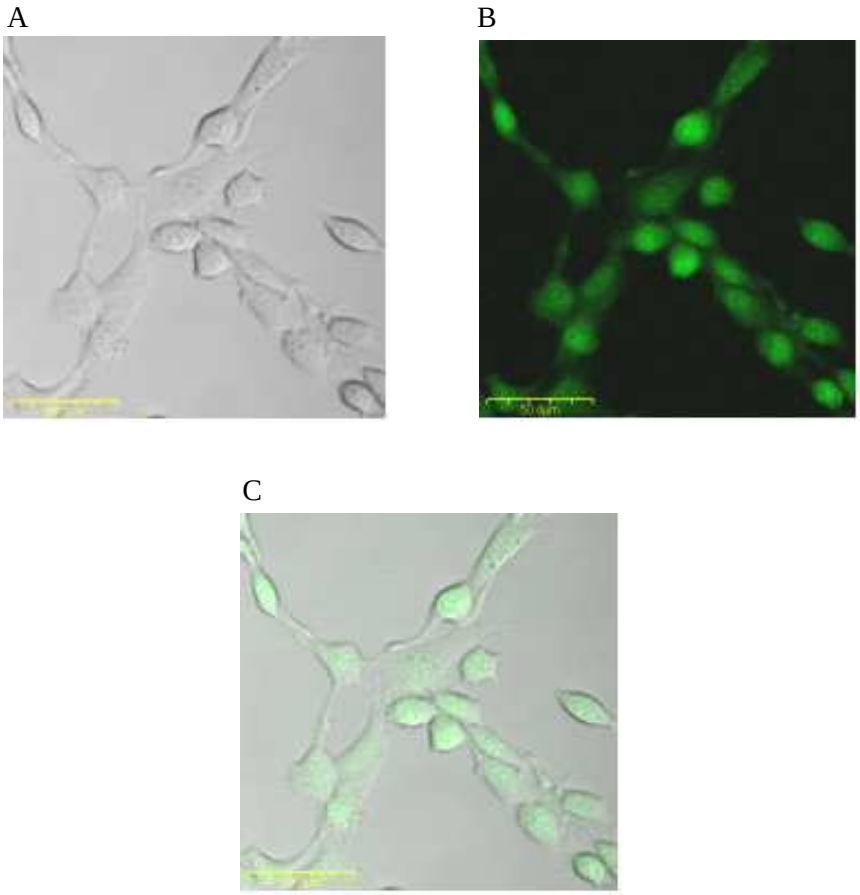

Figure 9. Confocal fluorescence microscopy of NIH3T3 cells. Treated with FITC-labeled PLA5. Cells were incubated for 30 mins at 37℃ with FITC-labeled PLA5. A; cell, B; FITC-labeled PLA5, C; Merge

VI. References

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