CHAPTER 2

Materials and methods

2.1 Chemicals and materials

The name of the chemicals and materials are presented in Appendix A, and the name of instruments are shown in Appendix B. The particulars in preparation of reagents and buffers utilized for experiments are shown in Appendix C.

2.2 Cell culture

RAW 264.7 macrophage cells line were cultured in DMEM supplement with 10% heat inactivated fetal bovine serum (FBS), 100 units/mL of penicillin and 100 µg/mL of streptomycin. The cell cultures were sustained in a humidified incubator with an ambiance of 5% CO_2 at 37°C. All experiments were started with cells in log phase of growth, as a minimum at 25% confluence, and designed to be completed before 80% confluence.

2.3 Sample preparation

Crebanine was purified from the tuber of *Stephania venosa,* which obtained from Dr. Wilart Pompimon, Department of Chemistry, Faculty of Science, Lampang Rajabhat University. The air-dried powdered of crebanine was percolated with hexane and then extracted with ethyl acetate followed by filtration. The filtrates were evaporated to dryness under reduced pressure to afford hexane and ethyl acetate. Then, crebanine was divided from the ethyl acetate fraction by comprehensive column chromatography. It was achieved as light yellow crystals, mp 117.0-117.8°C, corresponding to $C_{20}H_{21}NO_4$ (13,47).

2.4 Determination of cell viability

Principle

The viability of cell is investigated via MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), This assay based on the capability of a mitochondrial succinate dehydrogenase enzyme thru generate reducing equivalents such as NADH and NADPH from living cells to separate the tetrazolium rings of the yellow MTT and form a purple colored formazan product. Therefore, the color pattern display as a marker of the viable cells. The resulting intracellular purple colored formazan product was dissolved in DMSO and quantified by spectrophotometer.

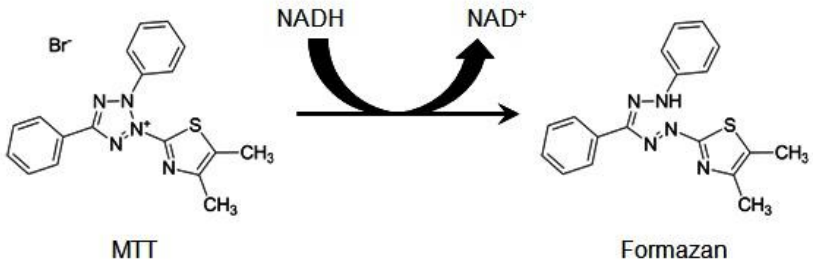

Figure 14. The principle of MTT assay in cells mitochondria (48)

Procedure

MTT assay was used for determination the cytotoxicity of crebanine on macrophages. Briefly, RAW 264.7 macrophages were plated at $7.5x10^3$ cells/well with in DMEM comprising 10% FBS. The cells were given with the concentrations of crebanine at 0-20 µg/mL and incubated for 24 and 48 h. The cytotoxicity of crebanine was purposed by MTT assay. Removal of 100 µL of medium. Then, Adding 15 µL of MTT dye (5 mg/mL) and incubated during 4 h. After incubation, the formazan crystal were dissolved in 200 µL of DMSO, and the absorbance at 570 nm with a reference absorbance of 630 nm was measured by spectrophotometer. In each experiment, the sample were tested in triplicate.

The percent of cell viability was measured by following equation.

$$\text{Cell viability (\%)} = \frac{\text{Mean absorbance in treated wells x 100}}{\text{Mean absorbance in untreated wells}}$$

2.5 The effect of the crebanine on the LPS-stimulated inflammation process in RAW 264.7 macrophages

2.5.1 Determination of pro-inflammatory cytokines IL-6 and TNF-α productions

Principle

Enzyme-linked immunosorbent assay (ELISA) is a simple technique performed on the supernatant to determine the presence of a pro-inflammatory cytokines such as IL-6 and TNF-α. This ELISA is based on a sandwich ELISA assay. Firstly, 96-well plates was coated with IL-6 or TNF-α specific capture antibody for capture soluble proteins including cytokines in the cell supernatant. The bound proteins are then detected with a subsequent detection antibody labeled with biotinylated. And then followed with Avidin-horseradish peroxidase and a colorimetric TMB substrate was then added, which results in a color change based on the amount of pro-inflammatory cytokines captured by using a spectrophotometry (49).

Procedure

For determine the result of crebanine on LPS induce IL-6 and TNF-α production**,** RAW 264.7 macrophages were plated at $6.0x10^5$ cells/well. Treatment condition of crebanine, crebanine was liquefied in DMSO and diluted with DMEM medium, the final concentration of DMSO was less than 0.1% (v/v). The cells were pretreated with the concentrations of crebanine at 0-10 µg/mL for 2 h. Next, 50 µL of LPS (1 µg/mL) were added (except untreated group) and incubated for 24 h. When the incubation was ended, the cell supernatants were collected and measured the level of cytokines production by Mouse IL-6 and TNF-α ELISA kits. In each experiment, the sample were tested in triplicate. The concentration of IL-6 and TNF-α were considered with standard curve of IL-6 and TNF-α respectively.

2.5.2 Measurement of Nitric oxide production

Principle

NO is a biological mediator of various physiological conditions including vasodilation, inflammation and thrombosis. In principled assay, sulfanilic acid was changed to a diazonium salt by response with nitrite in acid mixture. The diazonium salt is then connected with *N*-(1-naphthyl) ethylenediamine to developing an azo dye. This dye can be measured by spectrophotometric method based on the optical density at 540 nm.

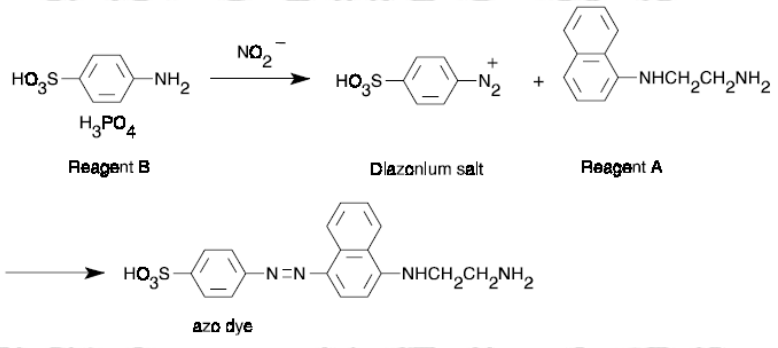

Figure 15. Principle of nitrite measuring via the Griess reaction (50)

Procedure

For examined the influence of crebanine on LPS-stimulated NO production. The nitrite concentration was established via the Griess reagent (0.1% N-1-naphthylenediamine dihydrochloride, 1% sulfanilamide and 2.5% phosphoric acid) as an indication of NO production. Presently, RAW 264.7 macrophages were plated at $3.5x10^5$ cells/well. Pretreatment of the cell with crebanine at concentrations 0-10 µg/mL for 2 h. Then, 50 µL of LPS (1 µg/mL) were added (except untreated group) and incubated for 24 h. When incubation times were ended, the nitrite concentration of the supernatants was determined by adding 150 µL of cell supernatant in 96-well plates. Then, the Griess reagent was added 150 µL. After that, the optical density of was quantified at 540 nm by spectrophotometer. In each experiment, the sample were tested in triplicate. The nitrite concentration was considered with standard curve of sodium nitrite.

2.5.3 Measurement of PGE_2 production

Principle

PGE_2 is other inflammatory mediator, which are generated in several inflammatory conditions, fever, tissue damage and a wide type of cancers. The procedure of assay is ground on the onward competitive attaching method of PGE_2 which present in the supernatants contends with HRP-labeled PGE_2 of binding sites on the specific antibody. PGE_2 present in the supernatant was permitted to fix to the specific antibody in the initially incubation. During the following incubation, HRP-labeled PGE_2 fixes to the outstanding antibody. After, to rinse for remove liberated molecules, a substrate mixture is added. Next, to measure the activity of bound enzyme. The color of mixture is break and the optical density was measured at 450 nm. The concentration of the color is contrariwise proportional to the amount of PGE_2 in the supernatants (51).

Procedure

For determine the influence of crebanine on LPS-stimulated PGE_2 production**,** Briefly, RAW 264.7 macrophages were plated at $3.5x10^5$ cells/well. Pretreatment of the cells with the concentrations 0-10 µg/mL µM for 2 h. After that, the cells were induced with 50 µl of LPS (1 µg/mL) by except untreated group and incubated for 24 h. When incubation times were ended, the supernatants were kept. In each experiment, the sample were tested in triplicate. The PGE_2 level in supernatants was determined by the PGE_2 ELISA kit.

2.6 Protein determination

Principle

The protein concentration of condition media, cell lysate, cytosolic extract and nucleus extract were established by the Coomassie plus™ protein assay reagent. The coomassie dye can fixes with protein in acidic condition resulting in instantaneously change in the optical density from 465 nm to 595 nm together with a concomitant color change from brown to blue. The proteins concentrations are approximated by indication to absorbance procured for a sequences of standard protein concentrations.

Procedure

BSA standard solution in various concentrations (2.5, 5, 10, 15 and 20 µg/mL) was prepared from stock 1 mg/mL of BSA. Standard solution or sample for 150 µL was added on appropriate 96 well-plates. After that 150 µL of Coomassie plus™ protein reagent was added and mixed each well. The absorbance was detected at 570 nm by spectrophotometer. The standard curve was prepared and utilized for purpose the protein concentration of each sample.

2.7 Treatment condition of crebanine in preparing of whole cells lysates, cytoplasmic and nuclear fraction for Western blot analysis

Procedure

The whole cells lysates were used to determine the result of crebanine on the expression levels of COX-2 and iNOS in RAW 264.7 cells. In brief, the RAW 264.7 cells were pretreated with crebanine at concentrations at 0-10 µg/mL and induced with or without LPS (1 µg/mL) and incubated for 24 h. The whole cells lysates were used as to determine the result of crebanine on the MAPKs and Akt proteins expression level in macrophages. Curtly, these cells were pretreated with crebanine at concentrations at 0-15 µg/mL and induced with or without LPS (1 µg/mL) for 15 min. When the incubation was ended, the cells were kept with cooling PBS and extracted by lysis solution (50 mM Tris–HCl, 150 mM NaCl, 10 mM EDTA, 1 mM PMSF, 1% Triton X-100, 10 µ g/mL leupeptin and 10 µg/mL aprotinin) on ice during 15 min. The cell extracts were centrifuged at 12,000 rpm at 10 min then whole cells lysates were kept.

The cytoplasmic and nucleus extract were utilized as to investigate the expression of NF-κB and AP-1 transcription factors**.** Briefly, the RAW 264.7 macrophages were pretreated with crebanine at concentrations at 0-15 µg/mL and induced with or without LPS (1 µg/mL) and incubated for 30 min. When the incubation ended, the treated cells were collected with cooling PBS and resuspended with hypotonic solution (10 mM KCL, 0.1 mM EGTA, 0.1 mM EDTA, 10 mM HEPES, pH 7.9, 1 mM NaF, 0.5 mM PMSF, 1 mM DTT and 1 mM Na_3VO_4) then incubated during 15 min on ice. 0.5% Nonidet P-40 was added into the cell lysates and centrifuged at 12,000 rpm at 1 min. The supernatants were kept and act for the cytoplasmic extract. The nucleus pellets were rinsed with cooling PBS and resuspended with extraction solution (20 mM HEPES,pH 7.9, 400 mM NaCl, 1 mM EGTA, 1 mM NaF, 1 mM Na_3VO_4 and 1mM DTT) on ice during 15 min and centrifuged at 12,000 rpm at 10 min. The supernatants were kept and represent the nucleus fraction (52).

2.8 Western blot analysis

Principle

The western blotting is a well-known and widely used an analytical technique for identify and analyze interested proteins in a sample of tissue or cell extract. Using the SDS-PAGE electrophoresis to separate the denatured proteins following by their electrophoretic mobility, which rely on charge, molecule size and structure of proteins. Then, these proteins was transferred onto nitrocellulose membrane. The transferred protein was detected via specific primary antibody and enzyme-labeled secondary antibody.

Procedure

To determine the expression of COX-2, iNOS, p-p38, p38, p-ERK1/2, ERK1/2, p-JNK, JNK, p-Akt, Akt, p-NF-κB p65, NF-κB p65, p-c-Jun, c-Jun and β-actin in the whole cell lysate. The expression of p-NF-κB p65, NF-κB p65, p-c-Jun, c-Jun and PARP-1 in the cytoplasmic and nucleus fraction, Western blot analysis was used to determine the protein levels. Briefly, equal amounts of whole cell lysate, cytoplasmic and nucleus fraction were isolated by 10% SDS-PAGE and transferred onto a nitrocellulose membranes**.** Then, the membrane was instantaneously blocked with 5% (w/v) skim milk in PBST buffer during 1 h for diminish the non-specific binding sites. After blocking, these nitrocellulose membranes was washed with PBST buffer. Then, the membrane was probed with a specific primary antibody at 4^{o}C overnight. The dilution of primary antibodies were used as monoclonal anti-inducible NO synthase (1:1000), polyclonal anti-COX-2 (1:1000), monoclonal anti-phospho p38 MAPK (1:1000), polyclonal anti-p38 MAPK (1:1000), monoclonal anti-phospho ERK1/2 (1:1000), polyclonal anti-ERK1/2 (1:1000), monoclonal anti-phospho JNK (1:2000), polyclonal anti-JNK (1:1000), polyclonal anti-phospho NF-κB p65 (1:1000), monoclonal anti- NF-κB p65 (1:200), monoclonal anti-phospho c-Jun (1:1000), monoclonal anti-c-Jun (1:1000), polyclonal anti-phospho Akt (1:1000), polyclonal anti-Akt (1:1000), monoclonal anti-β-actin (1:5000) and polyclonal anti-PARP (1:200). The membrane was washed for 5 times with PBST buffer, the membrane was incubated with the second antibodies at normal condition during 2 h in 5% (w/v) skim milk in PBST buffer. The second antibodies were anti-rabbit IgG, HRP-

conjugated (1:10,000) or anti-mouse IgG, HRP-linked (1:10,000). After that, the membrane was washed for 5 times. Finally, the labeled-antibody was considered with a Supersignal West Pico Chemiluminescent Substrate and exposed to high performance chemiluminescence X-ray film and quantities by scanning densitometry.

2.9 Effect of MAPKs and PI3-K/Akt inhibitors on LPS-induced RAW 264.7 macrophages

2.9.1 Effect of MAPKs and PI3-K/Akt inhibitors on LPS-induced AP-1 and NF-κB phosphorylation.

Procedure

In order to study the association of MAPKs and PI3-K/Akt signaling pathways in the activation of AP-1 and NF-κB, RAW 264.7 macrophages were treated with MAPKs or PI3-K/Akt inhibitors and the phosphorylation NF-κB and AP-1 were analyzed. Briefly, RAW 264.7 cells were plated at $3.0x10^6$ cells/well in DMEM containing 10% FBS with a specific inhibitors, 30 μM of p38 MAPK inhibitor (SB202190), 50 μM of JNK inhibitor (SP600125), 50 μM of ERK1/2 inhibitor (PD98059) and 20 μM of PI3-K/Akt inhibitor (LY294002) for 2 h. And then, 50 μL of LPS (1 μg/mL) were added to each well except control well and incubated for 30 min. When the incubation was ended, the whole cells lysates were used to determine the expression of phospho NF-κB p65 and phospho c-Jun by Western blot analysis.

2.9.2 Effect of MAPKs and PI3-K/Akt inhibitors on LPS-induced inflammatory mediators productions.

Procedure

To investigate the regulatory influences of MAPKs and PI3-K/Akt signaling pathways in LPS-induced inflammatory gene expression, the effect MAPKs or PI3-K/Akt inhibitors on the expression of IL-6 and NO were determined. Shortly, RAW 264.7 macrophages were plated at $3.0x10^6$ cells/well with a specific inhibitors, 30 μM of p38 MAPK inhibitor (SB202190), 50 μM of JNK inhibitor (SP600125), 50 μM of ERK1/2 inhibitor (PD98059) and 20 μM of PI3-K/Akt inhibitor (LY294002) for 2 h before being with incubated 1 μg/mL of LPS for 24 h. When the incubation was ended, the cell supernatant were collected to determine the production of IL-6 by ELISA and NO by Griess reagent reaction.

2.10 Statistical analysis

All experiments were presented in triplicate. Quantifications were described as mean ± standard deviation of triplicate-independent experiments. Statistical significances of difference during the present study were estimated by one-way ANOVA, proposed by Dunnett's test. A difference between the investigational groups was significantly considered whenever the *p* value is < 0.01 or < 0.05.