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Article

Cycloartenyl Ferulate downregulates lipopolysaccharide stimulated iNOS mRNA via NF-κB suppression in RAW 264.7 macrophages

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Abstract: Cycloartenyl ferulate (CAF) is a major bioactive phytosteryl ferulate purified from rice bran γ-oryzanol. Previously we reported that CAF ameliorates DSS-induced colitis in mice. The present study was undertaken to investigate the effects of CAF on LPS (lipopolysaccharide) stimulated murine RAW 264.7 macrophages. Immunohistochemistry analysis demonstrated that LPS (10ng/mL) treatment exhibited nuclear translocation of NF-κB-p65 in RAW macrophages, which was markedly inhibited CAF (30μM). LPS (10ng/mL) stimulation for 1-4 hours significantly upregulated iNOS and COX-2 mRNA in RAW 264.7 macrophages, but COX-2 mRNA was faster than that of iNOS mRNA. Macrophages pretreated with CAF greatly inhibited the LPS stimulated iNOS mRNA in a dose dependent manner (1-30μM), but CAF weakly inhibited COX-2 mRNA. Interestingly, CAY 10404 (COX-2 inhibitor) inhibited LPS stimulated iNOS mRNA, but not COX-2. In addition, PGE2 (1μM) upregulated iNOS mRNA but did not show any remarkable effects on NF-κB-p65 nuclear translocation in RAW macrophages. PD98059 (p44/42 MAP kinase inhibitor) inhibited iNOS mRNA, but not COX-2. On the other hand, PD169316 (p38 MAP kinase inhibitor) neither inhibited iNOS mRNA nor COX-2. Our results suggest that iNOS mRNA expression by LPS is mediated via p44/42 MAP kinase pathway in RAW 264.7 macrophages, which depends on the preceding expression of COX-2 expression. CAF downregulates iNOS mRNA via an inhibition of nuclear translocation of NF-κB with different mode of action on COX-2 gene expression.

Keywords: cycloartenyl ferulate; iNOS; COX-2; NF-κB; RAW 264.7 macrophage

1. Introduction

Natural products have received great attention for disease prevention owing to their various health benefits, noticeable lack of toxicity and side effects, and the limitations of chemotherapeutic agents. Cycloartenyl ferulate (CAF) derived from rice bran oil is such a natural compound possess promising health-related benefits in the prevention of different diseases, including cancer, hyperlipidemia, fatty liver, hypercalciuria, kidney stones, and heart disease (Jariwalla, 2001). Previously we have reported that phytosteryl ferulates mixture, γ-oryzanol and its component CAF inhibit NF-κB activity in dextran sulfate induced colitis in mice (Islam *et al.*, 2008).

iNOS and COX-2 are transcriptional regulatory molecules in macrophages (Jung *et al.*, 2008), which play an important in body pathophysiology. iNOS is induced in inflamed tissues and generates large amounts of NO that can promote mutagenic changes in DNA through DNA oxidization and protein nitrosylation (Kitasato *et al.*, 2007). COX-2 is upregulated by inflammatory stimuli leading to increased prostaglandin (PG) production (Posadas *et al.*, 2000) which promote tumor growth, angiogenesis, anti-apoptosis and chronic activation of immune system (Kim *et al.*, 2007). The MAPKs are a group of signaling molecules that appear to play

important roles in inflammatory process. LPS induces phosphorylation of p38 MAPK, extra cellular signal-regulated kinase-1/2 (ERK-1/2) and c-Jun NH_2-terminal kinase (JNK), leading to the activation of NF-κB in macrophages. The immune cells, macrophages/monocytes, play a crucial role in eliciting the inflammatory response cascades of NF-κB in the acute phase of inflammation and produce a number of cytokines, chemokines and enzymes such as iNOS and COX-2 for the primary protection of the host. However, uncontrolled accumulation of these defense molecules leads to a more severe and acute level of inflammation (Rajapakse *et al.*, 2008). Activation of NF-κB and the induction of iNOS by lipopolysaccharide involve the transcriptional and translocation of NF-κB to the nucleus (Griscavage *et al.*, 1996).

In this study, we aimed to investigate the effect of CAF on LPS-stimulated NF-κB activity and mRNA expression of iNOS in relation to COX-2 mRNA regulation in RAW 264.7 murine macrophage cell line.

2. Materials and Methods

2.1. Cell culture and treatment

The mouse macrophage cell line RAW 264.7 (ATCC, TIB-71) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100U/ml penicillin and 100µg/ml streptomycin. Cells were seeded at 1×10^6 cells/ml and grown at 37 °C in 5% CO_2 for 2-4 days in DMEM supplemented with 10% fetal bovine serum. Treatment with test compounds and/or LPS (10ng/mL) and PGE2 (1µM) were carried out under 1% FBS.

2.2. Viability and cytotoxicity

The mouse macrophage RAW 264.7 cells were cultured in a 6-well tissue culture plates pretreated with 30µM CAF overnight or LPS for 4 hours. Before harvesting the cells, morphological analysis was done under phase-contrast microscope. The cells were harvested and then exposed to 0.04% trypan blue for 5-10 minutes. Viability was determined by calculating the percentage of macrophages alive after dyeing the cells with trypan blue solution under microscopy examination.

2.3. RNA extraction and quantification of iNOS and COX-2 mRNA

Total RNA from RAW 264.7 macrophages was extracted using Trizol reagent (Invitrogen, Tokyo, Japan).Concentration of RNA was adjusted to 1µg/µl with RNase free distilled water. First strand cDNA was synthesized using a random 9-mer primer and avian myeloblastosis virus (AMV) Reverse Transcriptase XL at 30^0 C for 10 min, 42^oC for 45 min, 99^oC for 5 min and 4^oC for 5 min. PCR amplification was performed using AmpliTaq Gold polymerase. The oligonucleotide primers for iNOS were GCCTCGCTCTGGAAAGA (forward) & TCCATGCAGACAACCTT (reverse) and for COX-2 (NM_011198.2) were AAGCCGAGCACCTTTGGAG (forward) & ATTGATGGTGGCTGTTTTGGTAG (reverse) and the suitable size of synthesized cDNA were 500bp and 147bp, respectively. The oligonucleotide primers for mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as housekeeping gene, designed from mouse were TGT TCC TAC CCC CAA TGT GT (forward) and CCC TGT TGC TGT AGC CGT AT (reverse), and the suitable size of the synthesized cDNA was 269bp. After initial denaturation at 94º C, amplification at 98º C for 10s, 58ºC for 1.0 min and 72º C for 1.0 min were performed using a thermal cycler (Takara PCR Thermal Cycler MP; Takara Biomedicals, Japan). Initially, we checked PCR for 28 cycles, 30 cycles and 32 cycles and found similar results. Finally, we selected 32 cycles for reverse transcription-PCR analysis instead of amplification nature of RNA expression. PCR products were electrophoresed on 2 % agarose gel containing 0.1 % ethidium bromide. Detectable fluorescent bands were visualized by an ultraviolet transilluminator using FAS III (TOYOBO, Tokyo, Japan). The results were expressed as the ratio of the optical density to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.4. Immunohistochemically analysis of NF-κB p65 nuclear translocation in RAW 264.7 macrophages

Immunofluorescence was employed to detect translocation of the p65 component of NF-κB to the nucleus (Lebron *et al.*, 2003). Briefly, RAW 264.7 macrophages were seeded onto sterile 22 x 22-mm glass cover slips in 6-well tissue culture plates and pretreated with 30µM CAF overnight followed by LPS for 2 hours prior to fixation with 3.7% paraformaldehyde for 30 min at room temperature. We also investigated the effects of PGE2 (1µM) for 2 hours stimulation on NF-κB-p65 nuclear translocation in RAW 264.7 macrophages. After fixation, the cells were subsequently permeabilized with 0.2% Triton X-100 for 30 min at room temperature and rinsed with phosphate-buffered saline. Blocking of nonspecific binding sites was performed with 3% goat serum along with 1% BSA in phosphate-buffered saline for 60 minutes at room temperature. Rabbit polyclonal IgG against

NF-κB (p65) (Santa-Cruz Biotechnology, Tokyo, Japan) diluted 1:200 in phosphate-buffered saline (TBS) containing 3% goat serum and 1% BSA for 90 minutes at room temperature in a humidified chamber. The cover slips were washed extensively and incubated for 60 min with a 1:500 dilution of Alexa Fluor 568-conjugated anti-rabbit antibody. Nuclear staining was performed with 4', 6-diamidino-2-phenylindole (DAPI, 0.1 µg/mL, Sigma-Aldrich, Tokyo, Japan). Following extensive washing, the cover slips were mounted on slides and analyzed by fluorescence microscopy. Randomly five fields of each slide were counted and percentage of activated and non-activated cells was counted on the basis of nuclear translocation of NF-κB p65.

2.5. Drugs and reagents

γ-oryzanol was purified from Japanese rice, Koshi-hikari (Bligh *et al.*, 1959). Cycloartenyl ferulate (CAF) (Figure 1) (Wako Chemical, Osaka, Japan) was purified from rice bran γ-oryzanol. LPS from *Escherichia coli* (Sigma-Aldrich, Tokyo, Japan), CAY10404 (Cayman Chemical, Ann Arbor, MI), PD 169316 and PD 98059 (Calbiochem, Darmstadt, Germany). All other solution used was of analytical grade.

2.6. Statistical analyses

Numerical data are expressed as mean ± SEM. Statistical evaluation was carried out by Student's *t*-test and, where appropriate, one-way ANOVA; Tukey's all compare test was applied.

3. Results

3.1. Cycloartenyl ferulate inhibits activation of NF-κB-p65 in RAW 264.7 macrophages

The key regulator of inflammation as well as transcription factor, NF-κB exists mainly as a heterodimer comprised of subunits of the Rel family p50 and p65, which is normally sequestered in the cytosol as an inactive complex due to its binding with inhibitors of κB (IκBs) in unstimulated cells. Activation of NF-κB involves the phosphorylation of IκBs and the resulting free NF-κB is then translocated to the nucleus, where it binds to κB-binding sites in the promoter regions of target genes. Degradation of cytosolic IκBα enable NF-κB subunits to migrate to the nuclear compartment, where they promote gene transcription (Lebron *et al.*, 2003). Since the p65 subunit has been demonstrated to exert critical activity in the transcription of many inflammatory genes, we then investigated LPS (10ng/mL)-induced translocation of NF-κB-p65 in RAW 264.7 macrophages. Our results demonstrated that LPS (10ng/mL) induced severe inflammation in the RAW 264.7 macrophages exhibited diffuse NF-κB-p65 translocation from the cytoplasm into the nuclei of the macrophages (Figure 2). Pretreatment with 30µM CAF significantly inhibited LPS-induced nuclear translocation of NF-κB-p65 subunit in RAW 264.7 macrophages (Figure 2).

3.2. Cycloartenyl ferulate downregulates LPS-induced iNOS and COX2 mRNA in RAW 264.7 macrophages

iNOS and COX-2 are considered to be important pro-inflammatory mediators during inflammation and their upregulation are associated with the transcriptional activation (Noh *et al.*, 2006). We found that unstimulated RAW 264.7 macrophages did not express iNOS mRNA but it expressed COX-2 mRNA. However, when the macrophages were stimulated with LPS (10ng/mL), iNOS mRNA significantly ($P<0.01$) upregulated at 2 and 4 hours stimulation, whereas COX-2 mRNA significantly ($P<0.01$) increased at 1, 2 and 4 hours stimulation in RAW 264.7 macrophages (Figure 3). Pretreatment with CAF (1-30 µM) overnight followed by LPS (10 ng/mL) stimulation significantly inhibited mRNA of iNOS and COX-2 in a dose dependent manner (Figure 4).

3.3. COX-2 inhibitor CAY 10404 inhibits iNOS gene but not COX-2 ined RAW 264.7 macrophages

To clarify the involvement of COX-2/PGs pathways in iNOS expression, CAY10404 (COX-2 inhibitor) was used in LPS stimulated RAW 264.7 macrophages. The cells were pretreated with CAY10404 (10µM) overnight followed by LPS (10ng/mL) stimulation for 4 hours. RT-PCR analysis demonstrated that CAY10404 partially but significantly ($P<0.05$) inhibited iNOS mRNA but not COX-2 (Figure 5). We also investigated whether PGE2 upregulates iNOS gene and NF-κB-p65 nuclear translocation in RAW 264.7 macrophages. Expectedly we found that PGE2 (1µM) for 4 hours stimulation significantly ($P<0.05$) upregulated iNOS mRNA in macrophages (Figure 6A-B). However, PGE2 (1µM) for 2 hours stimulation did not show any remarkable effects on NF-κB-p65 nuclear translocation in RAW 264.7 macrophages (Figure 6 C).

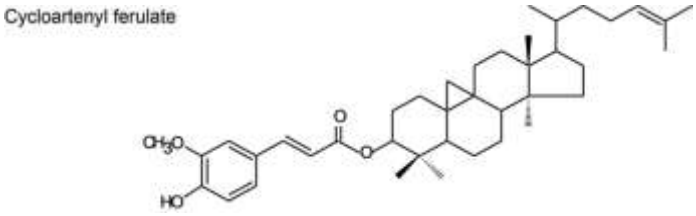

Figure 1. Chemical structure of cycloartenyl feulate (CAF) showing ferulic acid moiety and sterol moiety.

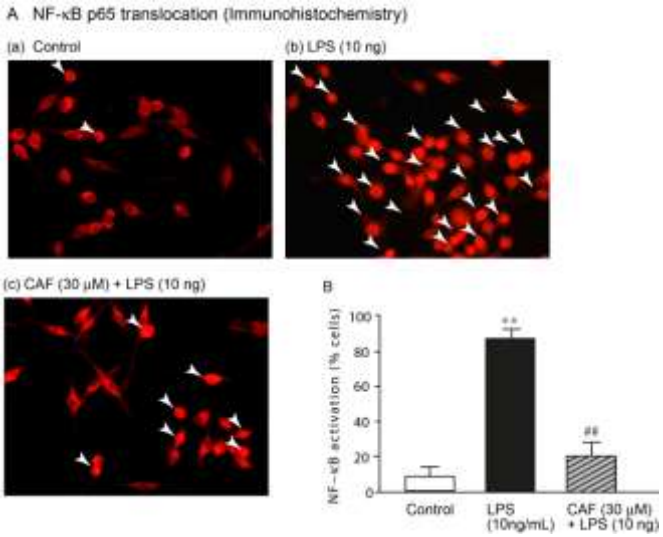

Figure 2. Effects of Cycloartenyl ferule on NF-κB p65 nuclear translocation in RAW 264.7 macrophages. (A-a) Control, (A-b) LPS (10ng/mL) and (A-c) CAF (30μM) + LPS (10ng/mL). The data represent the mean ± SEM. ** *P*<0.01, compare between control and LPS treated macrophages. ## *P*<0.01, compare between LPS and CAF + LPS groups. Arrows indicate nuclear translocation of NF-κB p65 in macrophages. n=5.

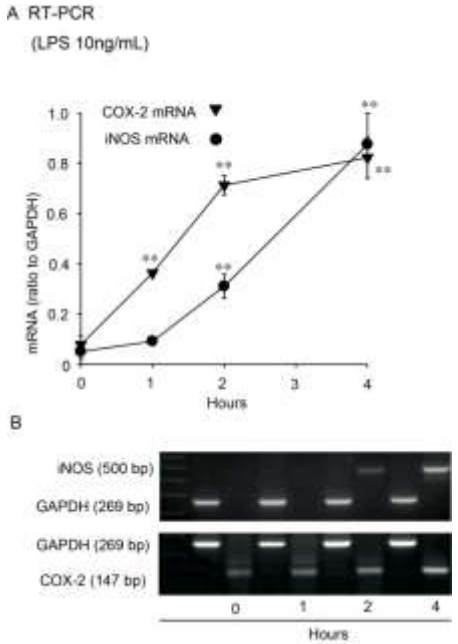

Figure 3. LPS (10 ng/mL) stimulates iNOS and COX-2 gene in RAW 264.7 macrophages. (A) Time dependent (1-4 hours) expression of iNOS and COX-2 mRNA in RAW 264.7 macrophages. (B) Representative agarose gel image showing the iNOS and COX-2 amplicons. The results were expressed as the ratio of the optical density to GAPDH. The data represent the mean ± SEM. ** *P*<0.01, compare between 0 hour and 1-4 hours respectively. n=5.

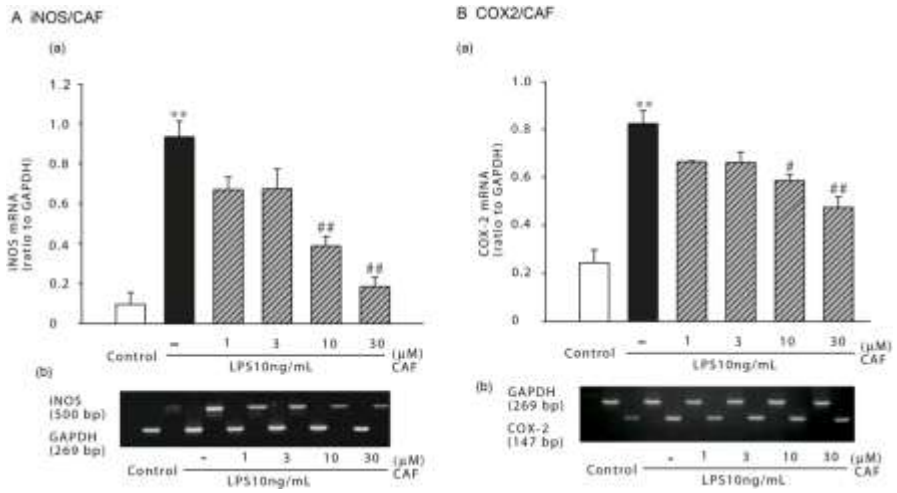

Figure 4. Cycloartenyl ferulate inhibits iNOS and COX-2 mRNA expressions in RAW 264.7 macrophages. Raw macrophages were pretreated with 1,3,10 and 30μM CAF respectively over night followed by LPS (10ng/mL) stimulation for 4 hours. (A-a) RT-PCR analysis of iNOS mRNA, (A-b) Representative agarose gel image showing the iNOS mRNA amplicons. (B-a) RT-PCR analysis of COX-2 mRNA, (A-b) Representative agarose gel image showing the COX-2 mRNA amplicons. The results were expressed as the ratio of the optical density to GAPDH. The data represent the mean ± SEM. ∗ *P*<0.05 and ∗∗ *P*<0.01 compare between control and LPS treated macrophages. # *P*<0.05 and ## *P*<0.01, compare between LPS and CAF+LPS treated macrophages. n=5.

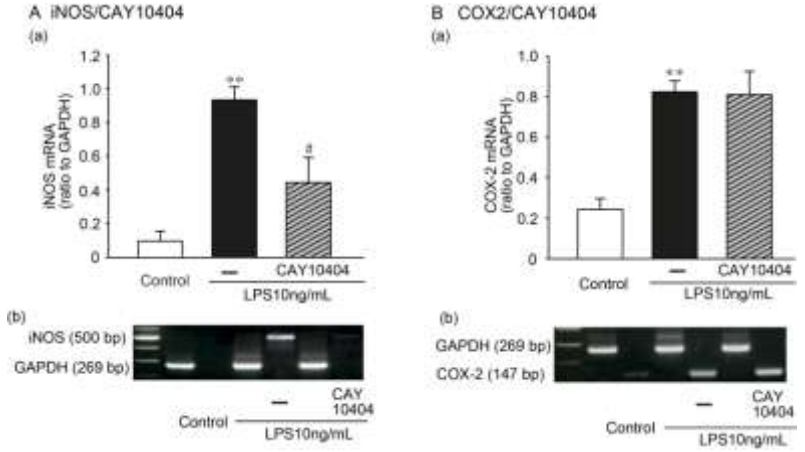

Figure 5. Effects of CAY 10404 on iNOS and COX-2 mRNA in LPS stimulated RAW 264.7 macrophages. RAW 264.7 macrophages were pretreated with CAY10404 (10μM) overnight followed by LPS (10ng/mL) stimulation for 4 hours. (A-a) iNOS mRNA, (A-b) Representative agarose gel image showing the iNOS amplicons. (B-a) COX-2 mRNA expressions, (B-b) Representative agarose gel image showing the COX-2 amplicons. The data represent the mean ± SEM. ∗∗ *P*<0.01, compare between control and LPS treated macrophages. # *P*<0.05, compare between LPS and LPS +CAY 10404 groups. n=5.

3.4. Effects of MAP kinase inhibitors on iNOS and COX-2 gene in RAW 264.7 macrophages

As the MAPKs are important signaling pathways for iNOS and COX-2 gene regulation, the effects of PD169316 (a p38 MAP kinase inhibitor) and PD98059 (a p44/42 MAP kinase inhibitor) were investigated on iNOS and COX-2 gene expression in RAW 264.7 macrophages. The macrophages were pretreated with PD169316 (20μM) or PD98059 (20μM) overnight followed by LPS (10ng/mL) stimulation for 4 hours. RT-PCR results demonstrated that PD98059, significantly (*P*<0.05) inhibited iNOS mRNA but not COX-2 (Figure 7A-B). On the other hand, PD169316 neither inhibited iNOS nor COX-2 mRNA in LPS treated macrophages (Figure 7C-D).

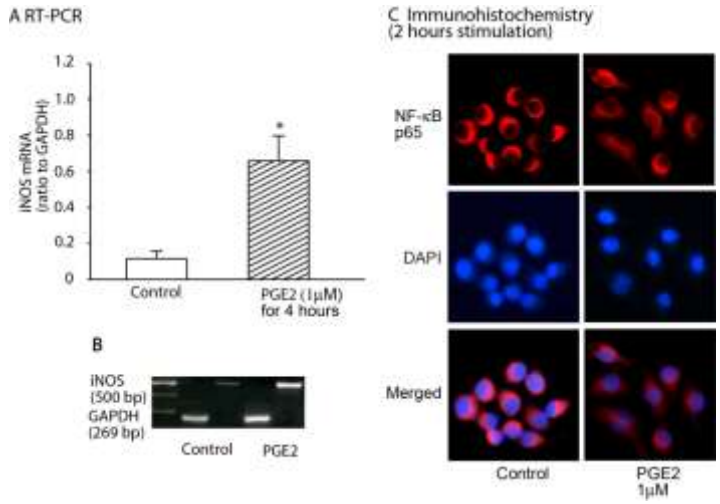

Figure 6. Effects of PGE2 on iNOS mRNA and NF-κB p65 nuclear translocation in RAW 264.7 macrophages. RAW 264.7 macrophages were treated with PGE2 (1μM) for 4 hours. (A) iNOS mRNA; control and PGE2 treated macrophages. (B) Representative agarose gel image showing the iNOS amplicons. The data represent the mean ± SEM. ∗ *P*<0.05, compare between control and PGE2 treated macrophages. n=5. (C) Effects of PGE2 on NF-κB-p65 nuclear translocation in RAW 264.7 macrophages. Cell were treated with PGE2 (1μM) for 2 hours, fixed and stained. No remarkable change was found between control and treated macrophages in nuclear translocation of NF-κB-p65. n=5.

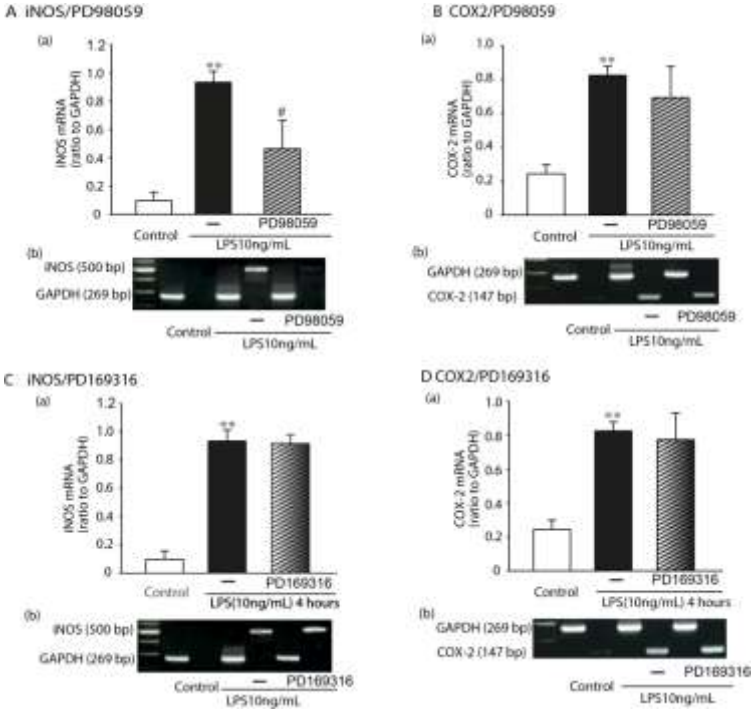

Figure 7. Effects of MAPKs inhibitor on iNOS and COX-2 mRNA in LPS stimulated RAW 264.7 macrophages. RAW 264.7 macrophages were pretreated with PD98059 (20μM) or PD169316 (20μM) overnight followed by LPS (10ng/mL) stimulation for 4 hours. Total RNA extracted for the RT–PCR analyses. In case of PD98059, (A-a) iNOS mRNA, (A-b) Representative agarose gel image showing the iNOS amplicons; (B-a) COX-2 mRNA, (B-b) Representative agarose gel image showing the COX-2 amplicons. In case of PD169316, (C-a) iNOS mRNA, (C-b) Representative agarose gel image showing the iNOS amplicons; (D-a) COX-2 mRNA and (D-b) Representative agarose gel image showing the COX-2 amplicons. The data represent the mean ± SEM. ∗∗ *P*<0.01, compare between control and LPS treated macrophages. # *P*<0.05, compare between LPS and LPS + PD98059 groups. n=5.

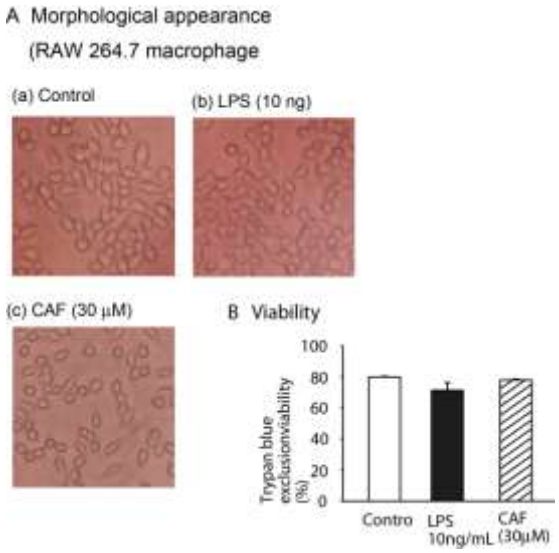

Figure 8. Cytotoxicity and viability of cycloartenyl ferulate. Phase-contrast microscopy showing morphology of RAW 264.7 macrophages incubated overnight with CAF (30μM), LPS (10ng/mL) or control respectively for 4 hours. Representative images; (A-a) control, (A-b) LPS and (A-c) CAF treated macrophages. B Graphical representation of trypan blue exclusion viability (%) of control, LPS and CAF treated macrophages. n=5.

3.5. Viability and cytotoxicity of CAF in RAW 264.7 macrophages

Cell viability and cytotoxicity assessment is an important and essential part of *in vitro* research. We also investigated the effects of LPS and CAF on RAW 264.7 macrophages. RAW 264.7 macrophages were cultured in a 6-well tissue culture plates pretreated with CAF (30μM) overnight or LPS (10 ng/mL) for 4 hours. Morphological analysis was done under phase-contrast microscope. Typical morphology of cells and 70-80 % trypan blue exclusion viability were found both in CAF or LPS treated groups respectively indicating no cytotoxic effects (Figure 8).

4. Discussion

The anti-inflammatory efficacy of phytosteryl ferulate, CAF is investigated in murine RAW 264.7 macrophages. LPS activates intracellular signaling pathways are largely unresolved which involve a series of events resulting in the transmission of the signal from the plasma membrane through the cytoplasm to the nucleus where gene expression is up-regulated (Chen *et al.*, 1999). In spite of unresolved, most of the scientific reports suggest that LPS activates the IκB kinase (IKK)-NF-κB pathway, extracellular signal-regulated kinases (ERK) 1 and 2, c-Jun N-terminal kinase (JNK) and p38 in human monocytes (Guha *et al.*, 2001) and macrophages (Sanghera *et al.*, 1996). In our study we found that LPS activated NF-κB indicated by nuclear translocation of NF-κB-p65 in RAW 264.7 macrophages and pretreatment with CAF (30μM) expectedly inhibited the nuclear translocation of NF-κB-p65 in macrophages. Like NF-κB activation, LPS also increased both iNOS and COX-2 mRNA in RAW 264.7 macrophages in a time dependent manner. Interesting, expression of COX-2 gene was faster than that of iNOS gene. Like NF-κB-p65 nuclear translocation inhibition, CAF (1-30μM) dose dependently inhibited both iNOS and COX-2 mRNA. CAF 1 and 3μM showed negligible effects both on iNOS and COX-2 gene. But, CAF 10 and 30μM significantly inhibited iNOS and COX-2 mRNA and 30μM strongly inhibited iNOS and COX-2 mRNA. In comparison the gene level, iNOS was strongly inhibited than COX-2 gene. Other scientific also suggested that there are many intracellular signaling pathways where iNOS gene is inhibited strongly but not COX-2.For example, CKD712 (S-1-α-naphthylmethyl-6, 7-dihydroxy-1, 2, 3, 4-tetrahydroisoquinoline), a newly synthesized tetrahydroisoquinoline and an enantiomer (S form) of YS 49 (a derivative of higenamine) completely inhibited NF-κB activation and iNOS gene, but not COX-2 in LPS activated RAW 264.7 macrophages, suggesting that another mechanism may be involved in the induction of COX-2 by LPS in addition to the NF-κB pathway (Tsoyi *et al.*, 2008). In order to explain why the iNOS gene was downregulated strongly than COX-2, we investigated the effect of compounds associated with the signaling cascade such as COX-2 inhibitor, p38 MAP kinase inhibitor, and p44/42 MAP kinase inhibitor on iNOS and COX-2 gene expression in LPS activated macrophages. Interestingly, CAY10404 (COX-2 inhibitor) more strongly inhibited iNOS gene than COX-2 gene, PD98059 (p44/42 MAP kinase inhibitor) partially but significantly (P<0.05)

inhibited iNOS mRNA but not COX-2 gene. On the other hand, PD169316 (p38 MAP kinase inhibitor) neither inhibited iNOS nor COX-2 mRNA. These findings underscore the concept that there may be the involvement of some other COX-2 signaling pathways of macrophage pathophysiology that involved in the induction of COX-2 by LPS in addition to the NF-κB pathway. In accordance with our findings, other scientific reports also suggested the similar anti-inflammatory mechanism in macrophages such as shikonin/alkannin derivatives (analogs of naphthoquinone pigments) significantly inhibited iNOS and prevented phosphorylation of ERK; however, there was no effect on p38 or pJNK in LPS stimulated RAW 264.7 macrophages (Cheng *et al.*, 2008). One of the key enzymes that play a pivotal role in tumor promotion and progression is COX-2, a rate-limiting enzyme in prostaglandin biosynthesis. Now day, scientific evidence suggests that inappropriate induction of COX-2 mediates cell proliferation, tumor growth, suppress the immune response to malignant cells (Liang *et al.*, 1999). It has been reported that activation of MAP kinase phosphorylation transduces signals to activate the transcription of NF-κB-mediated pro-inflammatory cytokines in which the activation of ERK is involved (Cheng *et al.*, 2008; Hambleton *et al.*, 1996). We also speculated that CAF inhibits iNOS through ERK1/2-MAPKp38 signaling pathway. For that reason we investigated the effects of PGE2 on RAW 264.7 macrophages. We only found that PGE2 significantly upregulated iNOS gene, but no remarkable effects on NF-κB-p65 nuclear translocation in macrophages. More precise study needs to identify the signaling pathways that involve CAF in the anti-inflammatory action.

5. Conclusions

The anti-inflammatory effects of CAF might be mediated through inhibition of LPS-induced iNOS and COX-2 genes via the suppression of NF-κB activation at the nuclear translocation level.

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Conflict of interest

None to declare.

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