CHAPTER 4

Discussion and conclusion

The compounds that could diminished the production of pro-inflammatory cytokines and mediators, have been considered as an efficient therapeutic agents for preventing prolonged inflammatory disorders, including atherosclerosis and cardiovascular diseases, diabetes, cancer, and arthritis (3,5). Recently, pharmacological activities of crebanine have been reported, such as anti-oxidant, anti-arrhythmic, anti-microbial activities, as well as improvement of neurodegenerative diseases (13,14). Previous studies have shown that crebanine diminished cancer cells proliferation and induced cell apoptosis. Furthermore, crebanine also suppressed human lung adenocarcinoma cells invasion through the inhibition of NF-κB activation (15). However, anti-inflammatory effect of crebanine on LPS-activated macrophages has not been investigated.

Macrophages are the major inflammatory cells which play a role in inflammation process. Since macrophages are activated by inflammatory stimuli leads to increase the overproduction of inflammatory mediators and cytokines, causing inflammatory development to chronic inflammation (7). For example, they are the major inflammatory cells that infiltrated in cancer, which play critical roles in the cancer progression (2). In the initiation stage, macrophages releases a lot of inflammatory mediators to create an inflammatory environment and promote tumor growth. When tumors progress to malignancy, macrophages contributes to the angiogenesis, invasion, and metastasis of tumors (29). This study, RAW 264.7 macrophages were used as an outstandingly experimental model for considering the anti-inflammatory activities and molecular inflammatory mechanism of crebanine. The present study, we found for the first time that crebanine displayed an effective anti-inflammatory activities through reducing the production of pro-inflammatory cytokines, including, IL-6 and TNF-α in LPS-stimulated RAW 264.7 macrophages. Moreover, crebanine could suppressed iNOS and COX-2

protein expressions in LPS-induced RAW 264.7 cells that subsequently decreased significant mediators, NO and PGE_2 productions.

Several signal transduction cascades and transcription factors are associated with the expression of inflammatory genes in macrophages. MAPKs is a family of serine/threonine protein kinases that facilitate many cellular responses to inflammatory stimuli (32). Many reports have indicated that LPS can trigger MAPKs signaling protein, this activation is implicate in the up-regulated of inflammatory cytokines production at the transcriptional and translational levels (53-55). PI3-K/Akt signaling pathway is a further inflammatory cascade that plays a crucial role as upstream signaling molecules in inflammatory responses (56,57). PI3-K is activated after ligands bind to receptors, then activates the protein kinase Akt. PI3-K/Akt signaling has been presented to control the expression of inflammatory genes induced by endotoxin in human monocytic cell model (37). Therefore, the modulation of MAPKs and PI3-K/Akt phosphorylation are the potential purpose for anti-inflammatory treatment in LPS-activated macrophages. It was found that the phosphorylation of ERK1/2, JNK, p38 MAPK and Akt by LPS was significantly suppressed by crebanine treatment in LPS-induced RAW 264.7 cells. Additionally, we ensure that the suppression of inflammatory mediator expressions are involved with the down-regulation of MAPKs and PI3-K/Akt pathways. The effect of MAPKs inhibitors including, p38 MAPK (SB202190), JNK (SP600125), ERK1/2 (PD98059) and PI3-K/Akt (LY294002) on the production of IL-6 and NO were determined and found that the MAPKs and PI3-K/Akt inhibitors considerably inhibited LPS-stimulated IL-6 and NO productions. These results were correlated with previous study that the activation of ERK1/2, JNK, p38 MAPK and Akt signaling molecules are implicated in LPS-stimulated the level of IL-6 and iNOS expression (58-60). Taken together, the results demonstrated that crebanine might inhibit the production of pro-inflammation cytokines and mediators by suppressing the activation of MAPKs and PI3-K/Akt signaling pathway in LPS-induced RAW 264.7 macrophages.

Inflammation process is closely regulated by NF-κB and AP-1. Many studies reported that the NF-κB and AP-1 are a key transcription factors that modulates the expression of various pro-inflammatory genes including iNOS, COX-2, IL-6 and TNF-α in

inflammatory conditions (61-63). The canonical pathway of NF-κB activation is triggered by pro-inflammatory cytokines including TNF-α and IL-1 and pathogens such as LPS via TLR4 (64). Thereby, TLR4 activation induces the recruitment of many adapter proteins, these adapter molecules afford a structural platform, to which downstream kinases are recruited resulting in the transduction of downstream signaling. Upon LPS stimulation, TLR4 associates with MyD88 lead to recruitment of IRAK4 and TRAF6, to form a complex with TAK1. The activated TAK1 then induces the activation of MAPKs and IKK, which initiates IκB-α phosphorylation (65). The phosphorylation allows free NF-κB to translocate into the nucleus and binds to enhancer elements of many inflammatory genes, including iNOS and COX-2 genes (62). The effect of crebanine on the activation of NF-κB was examined. The results shown that, crebanine inhibited LPS-induced p65 phosphorylation at serine536, but did not influence on LPS-induced IκB-α degradation and nucleus translocation of p65. Base on this discovering suggest that crebanine did not effect on classical pathway of LPS-stimulated TLRs mediated IKK-IκB-α pathway.

Consistent with several research have indicated that the IκB-α degradation and nuclear translocation of p65 are the key steps of NF-κB activation (66-68). However, phosphorylation of NF-κB subunit p65 have also reported to regulated NF-κB transcription activity. Previous studies showed that DNA binding and tran-activating capacity of NF-κB are regulated by phosphorylation on serine536 residue of p65, which is independent of IκB-α degradation (69,70).

AP-1 transcription factor is a dimeric protein complex mainly consist in Jun, Fos protein family and ATF protein. It known to be modulated the expression of inflammatory genes because the promoter of TNF-α, IL-6, iNOS and COX-2 genes contains the AP-1 binding site (71,72). Thus, the effect of crebanine on the nucleus translocation and phosphorylation of c-Jun, a main part of the AP-1 in LPS-stimulated RAW 264.7 macrophages was determined. Crebanine also decreased LPS-stimulated translocation to the nucleus and the phosphorylation on serine63 of c-Jun. These results proposed that crebanine may inhibit LPS-induced inflammatory effects via the inhibition of NF-κB and AP-1 transcriptional activity.

Current evidence suggest that MAPKs and PI3-K/Akt signaling pathways play the role as upstream signaling molecules in inflammatory responses (73,74). NF-κB transcriptional activity was depend on the activation of MAPKs signaling protein and also PI3-K/Akt signaling pathway in LPS stimulate inflammation (58,75). Moreover, Akt is also involve with LPS-stimulated phosphorylation of NF-κB and AP-1 (76,77). In accordance with reference data, to consider whether the activation of MAPKs and PI3-K/Akt signaling pathways are complicated in the phosphorylation of NF-κB and AP-1 (78-80), the level of phosphorylated NF-κB p65 and c-Jun were examined. Our data shown that MAPKs and PI3-K/Akt specific inhibitors significantly inhibited LPS-stimulated phosphorylation of p65 on serine536. Besides, JNK and ERK1/2 inhibitors also inhibited the LPS-stimulated phosphorylation of c-Jun on serine63. This data indicated that crebanine treatment prevented the LPS-stimulated phosphorylation of the inflammatory transcription factor via the modulation of MAPKs and PI3-K/Akt signaling cascades.

In conclusion, the present study revealed that crebanine exerts inhibitory effect on LPS-stimulated pro-inflammatory cytokines production such as IL-6 and TNF-α. Furthermore, crebanine suppresses LPS-stimulated iNOS and COX-2 expressions lead to the reduction of NO and PGE_2 productions. The inhibitory mechanisms might be involved with the modulation of NF-κB and AP-1 transcriptional factors through the blockade of downstream signaling molecules in TLR4-induced MAPKs and PI3-K/Akt signaling pathways. The results from this study suggests that crebanine might be used as an alternative agent in the development of therapeutic treatment for inflammatory-involved disorders. However, the safety and pharmacokinetic studies of crebanine in animal and clinical models would be needed to be further investigated. In addition, crebanine may be considered as a broad range inhibitor of MAPKs and PI3-K/Akt signaling pathways.