論文の内容の要旨

獣医学専攻
平成17年度博士課程　入学
氏名　MD　シャフィクル　イスラム
指導教員名　　尾崎　博

Studies on antioxidant and anti-inflammatory effects of rice bran phytosteryl ferulates

(米糠由来植物性ステロールフェルラ酸エステルの抗酸化および抗炎症作用の研究)

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. Rice is one of the world's most important food crops and more than half of the people in the world eat rice as the main part of their diets. γ-oryzanol (γ-ORZ), a phytosteryl ferulate extracted from rice bran oil. γ-ORZ has been using as a medicine in Japan since 1962. Each year Japan manufactures approximately 7,500 tons of γ-ORZ from 150,000 tons of rice bran. Imagine a single compound that possesses the multiple health benefits properties, the compound with such wide-ranging power is γ-ORZ. This is of particular importance that γ-ORZ is effective in the treatment of a broad range of gastrointestinal disorders, including peptic ulcer, gastritis, the irritable bowel syndrome, and nonspecific gastrointestinal complaints. Moreover, γ-ORZ is widely used as an anabolic drug by bodybuilding athletes, hypolipedimic agent for both hypercholesterolaemic and hypertriglyceridaemic patients, for the treatment of chronic schizophrenia, postmenopausal syndrome in women and lowering the risk of colon cancer as well. Considering the aforementioned background, research on rice bran phytochemicals was designed

based on structure-antioxidant and anti-inflammatory potentiality both in *in vivo* and *in vitro* models.

Chapter 1: Antioxidant, free radical scavenging and NF-κB activity of phytosteryl ferulates: structure-activity studies

There is emerging interest in the use of naturally occurring antioxidants for the management of a number of pathophysiological conditions, most of which involve free radical damage. In this study, initially antioxidant and free radical scavenging activities were measured using TBARS and DPPH assays, respectively. The results demonstrated that γ-ORZ, CAF, 24-mCAF, and β-SF and also FA had strong free radical scavenging and antioxidant potency, which was comparable to α-tocopherol. However, the sterol moiety such as CA does not have both activities. Therefore, it is suggested that the common ferulic acid-derived structure of γ-ORZ, CAF, 24-mCAF and β-SF with phenolic-hydroxyl group under the similar situation exhibited the antioxidant potency and scavenging activity.

There are numerous sources of ROS production in the cell complex defense mechanism which cannot be ruled out with the chemical reactions in solution. Therefore, cell based ROS production system was employed. CAF and eFA, a membrane permeable FA, greatly inhibited the ROS production in NIH 3T3 fibroblast cells induced by H_2O_2 in this system. It is notable that sterol moiety alone (CA) also significantly inhibited the intracellular ROS production.

NF-κB has been shown to play an important role in regulating the expression of many genes involved in cell survival, immunity, and inflammation. NF-κB activity was analyzed by measuring translocation of NF-κB p65 in LPS activated RAW 264.7 macrophages, and found that γ-ORZ, CAF, 24-mCAF, β-SF as well as eFA and CA, all these chemicals significantly inhibited the NF-κB activity. CAF was also investigated on LPS activated macrophages for iNOS and COX-2 gene evaluation. The results demonstrated that CAF significantly inhibited the LPS-stimulated increased iNOS and COX-2 mRNA in a dose (10 and 30μM) dependant manner in RAW 264.7 macrophages.

Recently, great attention has been focused on the relationship between NF-κB and ROS. Inflammatory stimuli such as LPS triggers ROS production via the activation of NADPH-oxidase system in macrophages and ROS enhance the signal transduction pathways for NF-κB activation in the cytoplasm and translocation into the nucleus. In addition, there is evidence that exogenous oxidant such as H_2O_2 induces NF-κB activation in some cell lines and high concentration of antioxidants abolish NF-κB activation in response to LPS or hypoxia, which suggests that ROS is involved in NF-κB activation. However, the relationship between NF-κB activation and ROS is still controversial.

Chapter 2: Effects of phytosteryl ferulates in experimental colitis in mice

Colorectal cancer is an important public health problem, one of the most fearsome complications of inflammatory bowel diseases (IBDs) mainly ulcerative colitis (UC) but also Crohn's colitis which affect millions of people worldwide. The conventional medical treatment of IBD relies on the use of aminosalicylates, corticosteroids, immunosuppressive drugs (azathioprine, 6-mercaptopurin, methotrexate, cyclosporin) and antibiotics. However, aminosalicilates (5-amino salicylic acid (5-ASA) derivatives) and/or glucocorticoids remain the principals of therapy for IBD at different stage of disease. However, the clinical effects are often transitory and frequently induce side effects. Therefore, there is a need for better therapeutic agents that effectively induce remission and alter the natural course of the disease with minimum or no side effects of the treatment.

The anti-inflammatory effects of γ-ORZ, CAF, FA, a possible metabolite of γ-ORZ were investigated *in vivo*, on dextran sulfate sodium (DSS)-induced colitis in mice by monitoring disease activity index (DAI), histopathology score, tissue myeloperoxidase (MPO) activity, mRNA expressions of cytokines and COX-2, colon length, and nuclear factor-κB (NF-κB) activity in colitis tissue. Both DAI and histopathology score revealed that DSS induced a severe mucosal colitis, with a marked increase in the thickness of the muscle layer, distortion and loss of crypts, depletion of goblet cell and infiltration of macrophages, granulocytes and lymphocytes. MPO activity was correlated with the development of colonic inflammation, and administration of γ-ORZ significantly ameliorated these conditions. γ-ORZ significantly reduced the up-regulated IL-1β, IL-6, TNF-α and COX-2 mRNA expressions in DSS-induced colitis mice. The nuclear NF-κB p65 significantly increased and IκB-α in the cytoplasmic extract significantly decreased in the DSS-induced colitis tissue, which was significantly ameliorated by the treatment with γ-ORZ. CAF had a similar ameliorative potency as γ-ORZ did.

It is documented that reactive oxygen species activates NF-κB which leads to the generation of pro-inflammatory cytokines and inducible enzymes, such as COX-2 and iNOS, in leukocytes and macrophages. Conversely, the pro-inflammatory cytokines causes oxidative stress by promoting the release of reactive oxygen species by immune cells and also by non-immune cells. Thus, inflammation and oxidative stress are involved in the spiraling vicious cycle that contributes to the severity of the intestinal inflammation. Role of reactive oxygen species in initiating and controlling the phosphorylation cascades that result in NF-κB activation is still controversial. The redox-sensitive mechanism responsible for the regulation of NF-κB, however, is still not fully understood, and further studies are necessary to solve the precise mechanism.

Chapter 3: Inducible nitric oxide synthase and cyclooxygenase-2 signal transduction in LPS activated RAW 264.7 macrophages

LPS activated intracellular signaling pathways are largely unresolved. 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 and macrophages. Intracellular signaling cascade involved in pro-inflammatory response such as iNOS and COX-2 expression were investigated in LPS activated RAW 264.7 macrophages. LPS (10ng/mL) stimulation of the macrophages increased iNOS and COX-2 gene differently in a time-dependant manner (1-4 hours), where, COX-2 expression started to increase earlier than iNOS. Treatment with CAY 10404 (a COX-2 inhibitor) significantly inhibited LPS-mediated iNOS induction but not the induction of COX-2 gene. In addition, PGE2 analogs ONO-AE1259 (an EP2 agonist) and ONO-AE1329 (an EP4 agonist) also significantly upregulated iNOS gene in RAW 264.7 macrophages.

There are many intracellular signaling pathways that can not be explained precisely and logically. Finally, involvement of MAP kinase pathways for the iNOS and COX-2 gene upregulation in LPS activated macrophages were investigated. PD98059 (a p44/42 MAP kinase inhibitor) inhibited iNOS mRNA level partially but significantly ($p<0.05$) but not COX-2 gene in LPS activated macrophages. However, PD169316 (a p38 MAP kinase inhibitor) neither inhibited iNOS nor COX-2 gene in LPS activated macrophages. Therefore, ERK and p38 inhibitors differently affect iNOS and COX-2 expression in activated macrophages. 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.

Conclusion

The effects of phytosteryl ferulates on antioxidation may come via ROS scavenging and inhibition of ROS production in the living cells. The anti-inflammation may come from the iNOS/NO rather COX-2 pathway via the inhibition of LPS -induced NF-kB activation. Rice bran phytosteryl ferulates having potential anti-inflammatory, antioxidant and free radicals scavenging activities could be new potential therapeutic and/or preventive agents for gastrointestinal inflammatory diseases and disease induced by oxidative stress. Further study needs for more precise conclusion.