論 文 の 内 容 の 要 旨

応用生命化学　専攻
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論文題目

Studies on biological functions of flavonoids that affect the transcription of genes related to lipid metabolism
（脂質代謝関連遺伝子の転写を調節するフラボノイド類の機能解析研究）

Chapter 1 Introduction

A worldwide increase in obesity and related chronic diseases is a big issue to be solved. Globally, the prevalence of obesity increased from 4.8% in 1980 to 9.8% in 2008 for men, and from 7.9% to 13.8% in women. Obesity is usually accompanied by cardiovascular diseases, metabolic syndrome, and diabetes. Therefore it is crucial to find an approach to treat the diseases caused by obesity.

Apolipoprotein B (Apo B) concentrations, which reflect the number of small, dense LDL particles in plasma, are a significant predictor of cardiometabolic risk. Over-secretion of Apo B is closely associated with metabolic syndrome, type 2 diabetes, elevated risk of coronary heart disease, and the development of atherosclerosis. Thus, inhibition of Apo B secretion has the potential as one of approaches to treat the diseases caused by obesity.

Flavonoids are common dietary components of vegetables, fruits, wine, and tea, with the function of anti-cancer, anti-allergic, anti-inflammatory, antioxidant, and anti-microbial. Epidemiological studies show that the dietary intake of flavonoid was inversely associated with the mortality from coronary heart disease. In addition, food scientific researches indicate that consumption of flavonoids in foods and beverages may decrease the risk of atherosclerosis by improving the lipid metabolism. However, the precise mechanism is largely unknown.

In this study, I have developed in vitro assay systems for screening food components and natural substances that suppress the transcription of microsomal triglyceride transfer protein (MTP), which plays an important role for the assembly and secretion of Apo B containing lipoproteins. Then I focused on the function of Luteolin, which was identified as the strongest

flavonoid that suppressed MTP transcription. On the other hand, it was reported that Quercetin intake was inversely correlated with plasma total and LDL-cholesterol. I analyzed Quercetin functions and found its inhibitory effect on Apo B synthesis.

Chapter 2 Luteolin inhibited Apo B secretion through lowering MTP transcription, which is dependent on reduced HNF4α activity

MTP plays an obligatory role in the stage of Apo B assembly and secretion. Firstly, we screened 156 kinds of food components that suppress the transcription of MTP using the human MTP gene promoter (-204~+33) in HepG2 cells. The results showed that flavones (Luteolin, Apigenin, Acacetin, Chrysin) and flavonols (Fisetin, Kaempferol) reduced MTP transcription activity, but not isoflavones (Genistein, Daidzein). Among all the components, Luteolin showed the strongest inhibition activity. In addition, Luteolin also decreased the MTP mRNA level and Apo B secretion in HepG2 and Caco-2 cells. These results suggested that Luteolin inhibited Apo B secretion by lowering MTP transcription. Next the mechanism of Luteolin was investigated.

The MTP promoter used in the screening system contained a pair of functional responsive elements for HNF4 and HNF1. Mutant analyses revealed that suppression of MTP transcription by Luteolin was observably declined when the MTP promoter had a mutation at the HNF4α-B or HNF1α site. Because HNF1α is a target gene of HNF4α, it is postulated that Luteolin inhibited MTP transcription via decreased HNF4α activity.

To verify the hypothesis, the effect of Luteolin on HNF4α activity was studied. It was observed the luciferase activity increment by overexpression of HNF4α was decreased with the treatment of Luteolin. Besides Luteolin depressed the mRNA level of HNF4α target genes (Apo B, PEPCK, G6Pase, and HNF1α). Surprisingly, Luteolin also lowered HNF4α mRNA and protein levels, resulting from the inhibition of HNF4α self-regulation factors (COUP-TFII and FXR). In order to determine the relationship between HNF4α activity and Apo B secretion, siHNF4α was used in HepG2 cells. The results showed that Apo B secretion reduced by HNF4α knockdown was further decreased with Luteolin treatment. These results suggest that Luteolin decreased MTP transcription by inhibiting HNF4α activity. In contrast, it is different to neglect distinct pathways other than an HNF4α pathway for the inhibitory effect of Luteolin on Apo B secretion.

Chapter 3 Luteolin directly bound to HNF4α to decrease the activity through the effect on DNA binding activity of HNF4α and co-factor recruitment

In this chapter, the mechanism of inhibition of HNF4α activity by Luteolin was studied. It is postulated that Luteolin inhibited HNF4α activity from outside of cells through a specific receptor (indirect action) or through their direct interaction within cells (direct action). When HepG2 cells

were cultured with one of Luteolin glucosides (Luteolin-7-glucoside and Isoorientin), which are thought to be poorly taken up by cells, both of them did not decrease MTP transcription, HNF4α activity, and Apo B secretion. These results suggest Luteolin functioned inside cells. Hence, it was thought Luteolin inhibited HNF4α activity through a direct interaction.

HNF4α protein purified from E.coli BL21strain was incubated with Luteolin and subjected to gel filtration. Fractionated HNF4α protein turned yellow, due to the interaction with yellow colored Luteolin, whereas fractionated HNF4α without Luteolin remained colorless. Purified HNF4α protein was also analyzed by the methods of absorption spectrum, trypsin cleavage and BIACORE. Absorption spectrum analysis showed that there was an absorption peak in about 400nm (near the absorption spectrum peak of free Luteolin) only when HNF4α was incubated with Luteolin. Trypsin cleavage analysis showed that Luteolin treated HNF4α protein was resistant to trypsin hydrolysis. BIACORE analysis revealed HNF4α protein interacted with Luteolin. Using the Luteolin conjugated beads, it was observed that HNF4α from HepG2 bound to Luteolin. All the results indicate Luteolin is capable of being associated with HNF4α directly.

Subsequently, ChIP assay showed the DNA binding activity of HNF4α to the target gene promoter region was reduced by Luteolin. GAL4 luciferase assays revealed that Luteolin decreased the HNF4α transcription activity via its ligand-binding domain. This suggests that Luteolin may hinder the recruitment of co-factor(s) required for induction of HNF4α transcription activity.

Chapter 4 Luteolin improved glucose and lipid metabolism in high fat diet induced obese mice

In this chapter the effect of Luteolin in vivo on lipid metabolism was studied. 5-week-old C57BL/6 mice were fed with high fat diet (HFD) for 11 weeks to gain weight. Then the mice were separated into 3 groups (n=8) based on their body weights and blood glucose levels, and were fed with their respective diet: HFD diet, HFD+0.6% Luteolin diet, and HFD+1.5% Luteolin diet. 1.5% Luteolin diet markedly reduced total body, liver, abdominal fat, visceral fat, and subcutaneous fat weights. Serum levels of cholesterol (total, VLDL, LDL, and HDL), triglyceride (total, LDL, and HDL), and Apo B (Apo B 100, Apo B 48) were also significantly decreased by 1.5% Luteolin diet. Additionally, 1.5% Luteolin significantly lowered hepatic cholesterol and triglyceride levels. The rise in serum glucose and insulin caused by HFD was significantly suppressed by 1.5% Luteolin while improving glucose tolerance. These results indicate Luteolin improved glucose and lipid metabolism in high fat diet induced obese mice.

Chapter 5 Quercetin decreased Apo B secretion via C/EBPβ

Based on the finding that Quercetin intake was inversely correlated with plasma total and LDL-cholesterol, it was postulated Quercetin may affect gene expression of MTP and Apo B, which are tightly related to plasma cholesterol levels. Firstly, Quercetin declined MTP and Apo B mRNA levels in differentiated Caco-2 and HepG2 cells. Experiments on mRNA stability using actinomycin D (a transcription inhibitor) revealed no effect of Quercetin. Hence, it seems likely that Quercetin may decrease MTP and Apo B transcription. The luciferase assay using the human MTP gene promoter (-204~+33) or the human Apo B gene promoter (-1800~+42) were performed. Quercetin significantly lowered Apo B transcription.

Using several versions of deleted reporter genes, it was found that the region of human Apo B promoter between -73 and -29b was required for Quercetin-mediated suppression of Apo B gene expression. This region contained a C/EBP-binding motif that was conserved among human, mouse, and rat. It has been reported that both C/EBPα and C/EBPβ are crucial factors for Apo B transcription in liver and intestine, respectively. Overexpression of C/EBPα or C/EBPβ significantly increased the luciferase activity of Apo B reporter gene and Quercetin suppressed it. These results suggest that Quercetin inhibit C/EBPα or C/EBPβ activity.

When HepG2 or Caco-2 cells were cultured with Quercetin-3-glucuronide, which is thought to be poorly taken up by cells, no decline in Apo B mRNA level was observed. This result implies that Quercetin, which is efficiently taken up by cells, affects C/EBPβ activities within cells. Using Quercetin-conjugated beads it was observed C/EBPβ from Caco-2 cells bound to Quercetin directly.

Next the mechanism of the inhibitory effect of Quercetin on C/EBPβ activity was investigated by ChIP assay and GAL4 luciferase system. ChIP assay using differentiated Caco-2 cells showed the C/EBPβ association with its binding motif in the Apo B promoter was not affected by Quercetin treatment. In contrast, GAL4 luciferase assay revealed that Quercetin reduced C/EBPβ transcriptional activity in a dose-dependent manner. These results indicate that Quercetin hinders the recruitment of co-factor(s) required for induction of C/EBP transcriptional activity.

Chapter 6 Conclusion

In this research, biological functions of flavonoids that affect the transcription of genes related to lipid metabolism were studied. It is concluded that Luteolin inhibited Apo B secretion through an HNF4α pathway and improved glucose and lipid metabolism in vivo. In contrast, the mechanism by which Luteolin directly inhibited Apo B secretion remains to be further investigated. Besides, Quercetin inhibited Apo B gene expression through a C/EBP pathway in vitro. These results would provide us a new approach for preventing life style-related diseases and open an avenue for potential application of flavonoids to health maintenance.