主論文の要約

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学 位 論 文 題 目

Studies on antitumor and anti-cachectic activities of eicosapentaenoic acid-enriched phospholipids derived from marine echinoderms

（棘皮動物に豊富な EPA 結合型リン脂質の抗腫瘍ならびに抗悪液質作用に関する研究）

During the past 30 years, n-3 PUFAs, especially EPA, has received much attention because of its beneficial effects on human health, including its use in the prevention and treatment of cancer and cachexia. However, most of these clinical studies have been carried out using EPA bound to triacylglycerol or ethyl esters. Little information has been known on the effects of EPA bound to phospholipids (PLs). With their increasing bioavailability, new evidence have emerged on the biological activities of EPA-enriched phospholipids (EPA-PL). The marine echinoderms such as starfish *Asterias amurensis* and sea cucumber *Cucumaria frondosa* are good natural sources of EPA-PL. Thus, in the present study, the starfish PLs (SFP) and sea cucumber PLs (SCP) were extracted and their antitumor and anti-cachectic activities were investigated both *in vitro* and *in vivo*.

In chapter 1, the transport and uptake effects of liposomes consisted of marine complex lipids derived from starfish *A. amurensis* and sea cucumber *C. frondosa* or soy-PL in small intestinal epithelial cell models were investigated. It was found that SFP and SCP contained 42 % and 47.9 % EPA, respectively. The average particle sizes of liposomes prepared in this study were from 169 to 189 nm. The results revealed that the transport of all the liposomes across M cell monolayer model were much higher than Caco-2 cell monolayer model. The present data also demonstrated that the liposomes consisted of EPA-PL showed significantly higher transport and uptake than soy-PL liposomes in both Caco-2 and M cell monolayer models, suggesting the higher absorption of EPA-PL in small intestine epithelial cells. It indicates that the acyl chain composition of phospholipids should greatly affect their absorption in small intestine. In addition, the findings in this chapter exhibited that treatment with 1 mM liposomes composed of EPA-PL for 3 h tended to increase the

EPA content while co-incubation with soy-PL liposomes increased the accumulation of linoleic acid in phospholipids fraction of both differentiated Caco-2 and M cells. It suggests that an increase in unsaturation of phospholipids fraction in small intestine epithelial cells may be responsible for the higher transport and uptake effects of liposomes composed of EPA-PL across the small intestinal epithelial cell models in comparison with soy-PL liposomes. Moreover, the present data also revealed that liposomes consisted of EPA-PL decreased the TEER values of Caco-2 and M cell monolayers for up to 3 h as compared to control but no obvious differences were observed between the Caco-2 and M cell monolayers, whereas soy-PL liposomes did not decrease the TEER values during the experimental period. Furthermore, it was also observed that the hybrid liposomes consisted of SFP/SFC/Chol exhibited higher transport across the M cell monolayer in comparison with other liposomes. Similarly, the uptake effect of the SFP/SFC/Chol liposomes was much higher than the other liposomes both in the Caco-2 and M cell monolayer models. Treatment with SFP/SFC/Chol liposomes could decrease the TEER values of Caco-2 and M cell monolayers notably. These data suggest that the higher transport and uptake effects of hybrid liposomes consisted of SFP/SFC/Chol may be due to opening the tight junctions of Caco-2 and M cell monolayers. The present data also showed that the cell viability of differentiated Caco-2 and M cells were not reduced after treatment with 1mM marine complex lipid or soy-PL liposomes for 3 h. This indicates that the transport and uptake effects of marine complex lipid or soy-PL liposomes in small intestinal epithelial cell models are independent issue to cell viability.

The findings in chapter 2 manifested that liposomes composed of EPA-PL extracted from starfish *A. amurensis* and sea cucumber *C. frondosa* exhibited potent antitumor effects on several kinds of cancer cells and in S180 ascitic tumor-bearing mice. SFP and SCP liposomes showed a dose-dependent inhibitory effects on the viability of S180, P388, HepG2 and HeLa cells. These results indicate that SFP and SCP exert anti-proliferative activity against several kinds of cancer cells. In S180 ascitic tumor-bearing mice, a rapid increase in ascitic fluid with tumor cells growth and high early mortality rate were observed. Dietary SFP and SCP liposomes (100 mg/kg/day) for totally 14 consecutive days prolonged the life span of tumor-bearing mice. In addition, it was also found that oral administration of SFP and SCP liposomes decreased the ascitic fluid volume and the ascitic tumor cells viability in tumor-bearing mice. It may be concluded that EPA-PL increase the life span of S180 ascitic tumor-bearing mice via decreasing ascitic fluid volume and killing ascitic tumor cells. The

present data also revealed that oral administration of SFP and SCP liposomes significantly restored the GSH levels as well as SOD and CAT activities in the liver of S180 ascitic tumor-bearing mice as compared to the model control group, suggesting that antitumor properties of EPA-PL may also be partly attributed to their potent antioxidant activities. Moreover, the observation of AO and EB staining indicated that EPA-PL killed S180 ascitic tumor cells via induction of apoptosis. In chapter 2, the data revealed that the mRNA and protein expression of pro-apoptotic factor Bax were up-regulated, whereas the anti-apoptotic factor Bcl-2 were down-regulated in S180 ascitic tumor cells after treated with SFP or SCP liposomes. The upregulation of caspase 9 and caspase 3 mRNA levels as well as cleaved-caspase 9 and cleaved-caspase 3 protein expression in tumor cells were also observed in the SFP and SCP liposomes-treated group. These data imply that the mechanism of EPA-PL in inducing apoptosis of S180 ascitic tumor cells must involve activation of mitochondrial apoptotic pathways.

In chapter 3, the anti-cachectic activity of EPA-PL in mice bearing S180 ascitic tumor was investigated. The results manifested that oral administration of SFP and SCP liposomes (100 mg/kg/day) for totally 14 consecutive days could suppress the cachectic body weight loss in mice bearing S180 ascitic tumor. Excessive loss of adipose tissue and significant increase of serum NEFA level in CAC mice were also observed in this study, but these changes were suppressed by SFP and SCP administration, suggesting the involvement of lipolysis in tumor-induced weight loss and the amelioration of EPA-PL for weight loss by inhibiting lipolysis in CAC mice. However, dietary SFP and SCP could not ameliorate the anorexia of CAC mice. Thus, EPA-PL prevented cachectic weight loss in CAC mice, most likely by preservation of adipose tissue through inhibiting lipolysis. It was also found that the serum levels of TNF-α and IL-6 increased dramatically in CAC mice but these increases were suppressed markedly after oral administration of SFP and SCP liposomes. Reduction of pro-inflammatory cytokines secretion by EPA-PL might be attributed to prominent anti-inflammatory properties of EPA. In addition, the present data exhibited that the mRNA expression of ZAG in WAT of CAC mice was up-regulated dramatically in the WAT of CAC mice but dietary SFP and SCP decreased its mRNA level significantly. The findings in this chapter also demonstrated that oral treatment of SFP and SCP could markedly suppress the increased expression of ATGL and HSL in CAC mice. These results suggest that the anti-cachectic effect of EPA-PL in CAC mice is associated with regulation of lipolysis. The present findings also

showed that the mRNA levels of PGC-1α and UCP2 in WAT were up-regulated significantly in CAC mice resulting in the enhancement of the mitochondrial activities converting WAT into BAT and then increasing lipid utilization. However, dietary SFP and SCP could reverse these increases, suggesting the ameliorating effect of EPA-PL in CAC mice through suppressing the lipid over-utilization. On the other hand, the data in this chapter revealed that oral administration of SFP and SCP did not affect the mRNA expression of key lipogenic transcription factors in WAT of CAC mice except for C/EBP α. The present results demonstrated that the mRNA expression of GLUT4 was dramatically inhibited in the WAT of CAC mice, but treatment of SFP and SCP liposomes could suppress the decline of the mRNA level of GLUT4. Furthermore, in this chapter, it was demonstrated that the genes which regulate the intracellular accumulation of lipids such as VLDLr, LPL and FABP4 were dramatically down-regulated in the WAT of CAC mice. Treatment of SFP and SCP liposomes could suppress the decrease of LPL mRNA expression of LPL. These results indicate that EPA-PL can ameliorate CAC by recovering the function of FAs uptake into adipocytes. As has been mentioned above, all of these data suggest that the performance of EPA-PL to preserve adipose mass in CAC mice could also contribute at least in part to recover lipogenesis.

In chapter 4, the antilipolytic effects of SFP and SCP on basal lipolysis in 3T3-L1 adipocytes were not observed. However, SFP and SCP could prevent the TNF-α and IL-6-stimulated lipolysis after treatment for 48 h. The data also showed that an anti-diabetic drug rosiglitazone (an agonist of PPAR γ) attenuated both basal and TNF-α-stimulated lipolysis significantly. In addition, the present study revealed that supplementation of SFP and SCP to medium increased the accumulation of EPA into phospholipids fraction of 3T3-L1 adipocytes in the absence or presence of TNF-α. And this change may have effect on the metabolic rate and lipolysis in 3T3-L1 adipocytes through affecting the membrane fluidity of cells. The present data also showed that antilipolytic effects of SFP and SCP were boosted notably in the presence of the inhibitor of ERK 1/2, PD 98059. It indicates that the inhibitory effect of EPA-PL on TNF-α-induced ERK 1/2 activation may account for its antilipolytic action in adipocytes. In addition, the results revealed that AICAR-induced AMPK activation could suppress the basal and TNF-α-stimulated lipolysis in 3T3-L1 adipocytes. Moreover, the antilipolytic actions of SFP and SCP on TNF-α-stimulated lipolysis were blocked by the inhibitor of AMPK, compound C, suggesting that the antilipolytic effects of SFP and SCP in adipocytes are also regulated by the activation of AMPK. The present data also indicated that

treatment of TNF-α induced a significant decrease on both ATGL and G0S2 gene expression levels in 3T3-L1 adipocytes. However, treatment with SFP and SCP could inhibit the decrease of G0S2 mRNA level significantly. In addition, treatment of SFP and SCP could up-regulated the mRNA and protein levels of perilipin in 3T3-L1 adipocytes as compared to TNF-α-treated cells. However, treatment with EPA-PL did not alter the mRNA levels of ATGL and HSL in 3T3-L1 adipocytes in this study. Therefore it can be speculated that the antilipolytic effect of EPA-PL is predominantly due to regulation of G0S2 and perilipin. The findings in this chapter also showed that treatment with SFP and SCP could not inhibit the down-regulation of the mRNA expression of the key transcription factors in 3T3-L1 mature adipocytes in the presence of TNF-α. It suggests that the antilipolytic action of EPA-PL is not responsible for ameliorating the function of lipolysis in adipocytes. Moreover, the results exhibited that the mRNA levels of LPL and FABP4 were dramatically down-regulated in adipocytes after treated with 10 ng/mL TNF-α for 48 h in this chapter. But co-incubation with SFP and SCP could elevated the mRNA expression of LPL and FABP4 as compared to TNF-α-treated cells. These results imply that EPA-PL can ameliorate TNF-α-stimulated lipolysis by recovering the function of FAs uptake in 3T3-L1 adipocytes. Furthermore, the effect of EPA-PL on glucose consumption and uptake in 3T3-L1 adipocytes were also investigated. The data showed that treatment with SFP and SCP could increase the glucose consumption and uptake of adipocytes significantly in the absence or presence of TNF-α, suggesting that EPA-PL may up-regulate glucose utilization. The findings in this chapter also exhibited that the presence of PI3K inhibitor LY294002 abrogated the increase of glucose consumption and uptake in 3T3-L1 adipocytes after treated with SFP and SCP in the absence or presence of TNF-α. It indicates that the effect of EPA-PL on glucose metabolism could be mediated via activation of PI3K. In addition, it was also found that SFP and SCP could increase the mRNA expression of GLUT4 in 3T3-L1 adipocytes as compared to control. Moreover, the TNF-α-induced decline of the GLUT4 mRNA level in adipocytes was also up-regulated after treated with EPA-PL. Taking into consideration all together, these results demonstrate that EPA-PL treatment may also ameliorate the glucose metabolism and attenuate TNF-α-induced insulin resistance in 3T3-L1 adipocytes.

In conclusion, based on all data in this study, it is suggested that the EPA-PL derived from starfish *A. amurensis* and sea cucumber *C. frondosa* exert potent antitumor and anti-cachectic

activities both *in vitro* and *in vivo*. Although further study on human intervention trial is needed and further investigations are also warranted to confirm the structure-activity relationship of PLs types and the variety of n-3 PUFAs, these encouraging findings may provide the basis for marine PLs supplementation in cancer and cancer-associated cachexia therapy.