Doctorate Thesis (Abridged)

Studies on the Anti-inflammatory Mechanisms of β-Elemene
(β-Elemene の抗炎症機構に関する研究)

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As the contents of some parts of the thesis are anticipated to be published in papers in scholarly journals, they cannot be published online. The papers are scheduled to be published within 5 years.
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Background

The fundamental effect of inflammation is the delivery of leukocytes to the sites suffering from the injury. The biological process is mainly mediated via changing the local blood flow and microvasculature to assist the gathering and migration of leukocytes to the injuries. However, it is related to various chronic diseases, such as Alzheimer's disease, Parkinson's disease, cardiovascular diseases and diabetes. Aging and obesity are the two important causes of inflammatory microenvironments, which are the primary inducements for the formation of chronic diseases. β-Elemene is contained in various food substances, but its anti-inflammatory function is poorly understood. In this study, I confirmed the therapeutic effects of β-elemene on treating experimental aging- or obesity-induced chronic inflammation through regulating immune cell subsets. In addition, the RAW 264 cell line, a kind of macrophage cell line, was used to explore β-elemene's effective pathways on preventing inflammatory signals. Furthermore, the formation processes of chronic diseases are strongly associated with intestinal microbiotas abnormalities and disorders in brain function. So, I tried to explain the effect mechanism of β-elemene on treating chronic inflammation from the viewpoint of regulating the interplay between cerebral metabolites and intestinal microbiotas. The objective of the study is to explore the anti-inflammatory effect of β-elemene both *in vivo* and *in vitro*.

Chapter 1 β-Elemene Alleviates the Aging-Induced Inflammation *in Vivo* and *in Vitro*

A kind of aging mouse model was utilized in this chapter. I mainly focused on the effects of β-elemene on regulating the inflammation related cytokines of dendritic cells (DCs) and the production of regulatory T cells (Tregs) in gut-associated lymphoid tissue of aging mice. In addition, the RAW cell line was used to study the effective pathways of β-elemene on treating aging-induced inflammation.

Transforming growth factor β (TGF-β) and interleukin 10 (IL-10) are important anti-inflammatory cytokines in the immune system. The former has effects on differentiation and homeostasis of effector T cells, and the latter has the immune function of escaping from excessive immunopathology to organism self-tissues. β-Elemene was fed to aging mice for one month. Although significant regulatory effects of β-elemene were not observed in the tissue mRNA expression in the jejunum or the colon, β-elemene significantly increased the mRNA expression of TGF-β1 and IL-10 in mesenteric lymph nodes (MLN) DCs and Peyer's patches (PP) DCs of aging mice. In addition, I explored the $Foxp3^{+}CD4^{+}$ Tregs proportion in MLN and PP cells. I found that β-elemene significantly increased $Foxp3^{+}CD4^{+}$ T cells in MLNs of aging mouse. The phenomenon gave us a hint that β-elemene probably increased the ratio of Tregs in the intestinal system and helped inhibit aging-induced inflammation. Furthermore, hydroxyurea (HU) which induces DNA damage and repair, mitochondrial dysfunction and the increase of ROS level,

was used to establish a cellular model to mimic aging *in vitro*. Based on this model, I found that β-elemene could reduce HU induced signaling through MAPK pathways using western blotting in the RAW cell line.

Chapter 2 Intestinal Regulatory T Cell Induction by β-Elemene Alleviates the Formation of Fat Tissue-Related Inflammation

In this chapter, a kind of obesity mouse model was used to validate the anti-inflammation effects of β-elemene. β-Elemene was orally administrated to high fat diet (HFD)-fed obese mice for 21 days. I paid attention to Tregs production and mRNA expression of inflammation related cytokines both in the white adipose tissue and intestinal immune system of mice. I tried to elucidate the mechanism of β-elemene on treating experimental obesity-induced chronic inflammation.

I found that β-elemene did not have effect on decreasing mice bodyweight, which was unexpected. However, unlike the aging mouse model, the expressions of proinflammatory cytokines tumor necrosis factor-α (TNFα), interleukin 1β (IL-1β), interleukin 6 (IL-6), interferon-γ (IFN-γ), and chemokine (C-C motif) ligand 2 (CCL2) of stromal vascular cells (SVCs) in white adipose tissue of obese mice were downregulated by oral administration of β-elemene while the anti-inflammatory cytokines TGF-β1 and IL-10 were upregulated. In addition, I found that even though the decreased $CD4^+$ T cells in lymphocytes caused by HFD in epididymal adipose tissue (EAT) and mesenteric adipose tissue (MAT) were not recovered by β-elemene treatment, the Foxp3 expression was significantly increased. These results proved that β-elemene induced $Foxp3^+$ Tregs in adipose tissue of obese mice. To evaluate the direct effect of β-elemene on the SVCs in EAT and MAT, I mimicked the inflammatory environment *in vitro* using lipopolysaccharide (LPS). However, I found that the anti-inflammatory effects were minimal even with high concentrations of β-elemene treatment. So, I hypothesized that adipose tissue inflammation was not inhibited by the direct effect of β-elemene and that other immunoregulatory functions might be executed in treating obesity-induced inflammation. Because β-elemene was orally administered, I considered the possibility that it may modulate the intestinal immune system. I found that oral administration of β-elemene increased $Foxp3^+CD4^+$ Tregs in both MLN and PP of the intestinal immune system of obese mice. Besides, β-elemene enhanced expression of TGF-β1, retinal dehydrogenase 2 (RALDH2), integrin αvβ8 and IL-10 derived from MLN-DCs and PP-DCs *in vitro*. The phenomena demonstrated that intestinal DCs were more sensitive to β-elemene than SVCs of EAT and MAT and suggested that the induction of $Foxp3^+$ T cells in SVCs observed *in vivo* was the direct effect of β-elemene on intestinal DCs. To clarify the intrinsic effect of β-elemene on the induction of $Foxp3^+$ T cells by DCs, I cultured intestinal DCs and $CD4^+$ T cells together in the absence or presence of retinoic acid (RA) and TGF-β1. I found that β-elemene promoted Tregs induction in the presence of TGF-β1. The results suggested the upregulation of RALDH2 expression by β-elemene in the coculture system, which would produce RA, and the produced RA worked together with TGF-β1 to promote the induction of $Foxp3^+$ Tregs. Based on these data, I suggest that the therapeutic effects of β-elemene on treating experimental obesity-induced chronic inflammation are by enhancing the generation of Tregs in the intestinal immune system through

modulation of DC function, and further alleviating inflammatory symptoms in the fat tissue of mice. Although feeding β-elemene did not affect body weight, it may possibly improve insulin resistance and ameliorate blood glucose levels through its anti-inflammatory effects on adipose tissue.

Chapter 3 β-Elemene Regulates Macrophages Balance Via ERK/ JNK/ P38 MAPK Signaling Pathway

In this chapter, I focused on the effects of β-elemene on balancing different phenotypes of macrophages, including M1 macrophages (classically activated macrophages) and M2 macrophages (alternatively activated macrophages) *in vitro* and *in vivo*. In addition, the RAW cell line was used to study the effective pathways of β-elemene on treating obesity-induced inflammation using immunoblotting.

It is known that obesity results in activation of the components of innate immunity such as the Toll-like receptors (TLRs). Especially TLR2 and TLR4, which mediate the downstream signaling cascades through myeloid differentiation factor 88 (Myd88), further induce the phosphorylation of MAPK kinases including the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 kinase (p38) signaling pathways. They can interfere with insulin sensitivity and are activated by pro-inflammatory cytokines and free fatty acids that have been involved in the development of insulin resistance and type 2 diabetes. I found that with an increasing concentration of β-elemene, the corresponding bands of phosphorylated p42 ERK, p46 JNK and p38 were decreased while the p42 ERK, p46 JNK and p38 unchanged, which demonstrated that the β-elemene could downregulate the LPS induced inflammatory signaling pathways in the RAW 264 cell line in a dose dependent manner. In addition, it is well known that the imbalance in the phenotypes of macrophages is often associated with chronic disease-induced inflammation. Therefore, understanding M1 macrophages and M2 macrophages interactions is essential for elucidating the molecular basis of obesity-induced inflammation progression. I found that feeding β-elemene to obese mice significantly decreased the proportion of M1 macrophages in total macrophages in the adipose tissue. At the same time, β-elemene downregulated the expression of pro-inflammatory cytokines in the macrophages, such as CCL2, IFN-γ, IL-1β and TNF-α, which are recognized as the induction molecules to switch to the M1 polarization. The results of this chapter suggested the possibility that the β-elemene may become novel macrophage-mediated therapeutic medicine.

Chapter 4 β-Elemene's Effect on Regulating Obesity-Induced Imbalance of Brain-Gut Axis

To examine the effect of β-elemene on obesity-induced imbalance of brain-gut axis, the cerebral metabolites and intestinal microbiota of each mice group were examined. Firstly, the metabolite data of prefrontal cortex (PFC), hippocampus (HIP) and hypothalamus (HYP) of the mouse brain were collected by ^{1}H nuclear magnetic resonance (NMR) spectroscopy. After quantitative analysis, cluster analysis and principal component analysis (PCA) based on SPSS 22.0 were used to classify all the mice cerebral metabolites. Secondly, 16S rDNA sequencing was used to analyze the microbial community structure of

mice stool samples. Finally, the cerebral metabolites and intestinal microbiota at phylum level were connected by Pearson correlation.

It has been proved that one of the intrinsic mechanisms of obesity related to brain dysfunction is the gut microbiota, which seems like as a linkage between the environmental pressures (such as lifestyle or diet) and the host physiology regulating neural pathways. In this chapter, I paid attention to PFC, HIP and HYP exploration, which control the behavior, memory and hormones regulation of mice respectively. I found that HFD caused region-dependent changes of cerebral metabolites, such as myo-Inositol and taurine, the important metabolites which attend neurodegeneration-associated inflammatory process. Both of them represented a large decrease in the PFC, but a significantly increase in the HIP and HYP of obese mice. Based on the cluster analyses and the PCA, I found that β-elemene had effects on recovering the HFD-induced changes of metabolites occurring in the mice brain, especially PFC and HIP, and thus could possibly alleviate the disorders of neuronal metabolism, promote the repair of damaged brain tissue and maintain normal osmoregulation of brain cells. In addition, I examined the composition of intestinal microbiota in the mice at different levels, including the phylum, class, order, family and genus. I found that β-elemene intervention could downregulate the population of *Firmicutes*, but no obvious changes could be observed concerning the abundance of the *Bacteroidetes.* Except for the two dominant microbiotas mentioned above, *Actinobacteria* presented obvious changes under different treatments. Although it's in low proportion in the microbiotas of mice, *Actinobacteria* was testified as important phylum for the maintenance of gut homeostasis. In addition, at the family and genus level, even though different strains of intestinal microbiota have different degrees of sensitivity toward to β-elemene, the cluster analyses clearly demonstrated that this compound could reverse the HFD-caused changes of composition and contents of mice gut bacteria. After that, I explored the interaction between the cerebral metabolites and intestinal microbiotas using Pearson correlation. I found that *Firmicutes* was possibly controlled by the cerebral inflammation and inhibitory neurotransmitters. *Bacteroidetes* in mice intestinal bacteria were related to the cerebral aerobic respiration and glucose cycle. Besides, *Actinobacteria* may affect the cerebral energy metabolism.

Conclusions

As two important causes of inflammatory microenvironments, the aging and obesity were explored in the present study. I found that β-elemene was more effective on treating inflammation induced by obesity than that induced by aging. Taken together, this study highlights the therapeutic effects of β-elemene on treating experimental obesity-induced chronic inflammation by adjusting the balance of immune cell populations in fat tissue through enhancing the generation of Tregs in the intestinal immune system and maintaining homeostasis of gut-brain axis.