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学位論文題目

Studies on the Compounds having Suppressive Effects on the Activation of Macrophages and Mast Cells
（マクロファージ及び肥満細胞の活性化抑制作用を示す化合物に関する研究）

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論 文 内 容 要 旨

Macrophages play a major role in host defense against infection and tumor development. During inflammatory process, activation of macrophages by inflammatory stimuli causes release of various inflammatory cytokines and chemical mediators. Lipopolysaccharide (LPS), one of the major component of outer membrane of gram-negative bacteria, stimulates immune cells, mainly macrophages, to release various mediators such as tumor necrosis factor (TNF)- α, interleukin (IL)-1, IL-6, IL-10, prostanoids, and nitric oxide (NO). Sepsis is a clinical syndrome frequently mediated by LPS, and in patients with sepsis, TNF- α produced by LPS plays a major role in the associated systemic toxicity. TNF- α also induces the secretion of cytokines such as IL-1 and activates other immune cells including T cells. Among the chemical mediators released from activated macrophages, the arachidonate metabolite prostaglandins (PGs) control pain, fever and inflammatory responses, and NO generated by NO synthase (NOS) induces tissue injury at the inflammatory site. Mast cells also play a central role in the initiation of the immune response associated with allergic disorders and asthma. Mast cells express Fc RI, a high-affinity receptor for IgE, on their surface. Cross-linkage with the antigen of the IgE-Fc RI complex activates mast cells, and leads to degranulation and the production of cytokines and arachidonate metabolites. Histamine stored in secretory granules is rapidly released in response to the antigen, and induces the contraction of smooth muscles, an increase in vascular permeability and the secretion of mucus. The antigen also induces the production of arachidonic acid metabolites such as PGD_2, a chemoattractant for eosinophils, and leukotriene (LT) B_4, a chemoattractant for eosinophils and neutrophils, and peptide-LTs that increase vascular permeability. Among the cytokines produced in response to the antigen, IL-4 and IL-13 induce production of IgE by B cells and induce Th2 cell development. Therefore, it is suggest that modulation of macrophages and mast cell activation might be therapeutic targets for controlling inflammatory diseases.

The medicinal plants have been used for the treatment of various diseases since ancient times. Therefore, medicinal plants have been recognized as a source of new therapeutic candidate compounds, and various pharmaceutical agents have been isolated from the medicinal plants. For example, salicylic acid was identified in 1839 as an active component in a number of plants known for their analgesic activity, and was first synthesized in 1853. It led to the development of aspirin, which is the most widely used synthetic drug today. Still now, a variety of studies has been carrying out to find lead compounds to develop more potent and less toxic drugs from the medicinal plants. On the basis of this research current, I have studied to find lead compounds for anti-inflammatory drugs from natural resources and analyzed their mechanism of action. In cultures of macrophages and mast cells, I have studied effects of four kinds of natural products, furanocoumarins isolated from the roots of *Angelica dahurica*, lignans isolated from the roots of *Acanthopanax chiisanensis*, synthesized flavonoids 2'-hydroxychalcone derivatives, and stilbene resveratrol, a constituent of *Vitis vinifera* extract, on the production of inflammatory chemical mediators and cytokines.

1. Furanocoumarin imperatorin Among various fractions of the roots of *Angelica dahurica*, n-hexane fraction and methylene chloride fraction showed potent inhibitory effects on the LPS-induced PGE_2 production in rat peritoneal macrophages. From the fractions, five furanocoumarins were isolated and their semi-synthesized compounds were prepared. Among the isolated furanocoumarins and semi-synthesized compounds, phellopterin, imperatorin and isoimperatorin completely inhibited the LPS-induced PGE_2 production, and imperatorin suppressed the LPS-induced expression of cyclooxygenase (COX)-2 and membrane-associated PGE_2 synthase (mPGES)-1, without affecting expression of COX-1 and cytosolic PGES. These results suggest that the inhibition by imperatorin of the LPS-induced PGE_2 production is mediated through suppression of induction of COX-2 and mPGES-1. Furthermore, imperatorin inhibited the LPS-induced TNF-α production in the murine macrophage cell line RAW 264.7 cells. To clarify its inhibitory mechanism of action for PGE_2 and TNF-α production, effects of imperatorin on the LPS-induced activation of mitogen-activated protein kinases (MAPKs) and transcription factor nuclear factor (NF)-κB which are two major signaling molecules for the induction of the proinflammatory proteins such as COX-2, inducible NO synthase (iNOS) and TNF-α in LPS-stimulated macrophages were analyzed. Imperatorin did not affect the LPS-induced phosphorylation of ERK, p38 MAPK and JNK, but reporter gene assay and electrophoretic mobility shift assay revealed that the LPS-induced activation of NF-κB was suppressed by imperatorin. Furthermore, the inhibition by imperatorin of the activation of NF-κB is induced through direct inhibition of the binding of NF-κB to DNA, without affecting the LPS-induced degradation of IκB-α. It is reported that cysteine 38 and 62 of NF-κB bind to DNA by forming hydrogen bond with sugar/phosphate backbone of the κB-DNA motif, and several studies have been reported that α,β-unsaturated carbonyl compounds react directly with NF-κB. Imperatorin also contains α,β-unsaturated carbonyl moiety and the imperatorin-mediated inhibition of NF-κB activation was reversed by disulfhydryl compound dithiothreitol, suggesting that imperatorin may modify a sulfhydryl group in NF-κB. From these results, it was suggested that the inhibition by imperatorin of PGE_2 and TNF-α production in LPS-stimulated macrophages is mediated by suppression of the activation of NF-κB.

2. Lignan taiwanin C Among various fraction of the roots of *Acanthopanax chiisanensis*, chloroform fraction showed a potent inhibitory activity against the protein kinase C activator 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced PGE_2 production in rat peritoneal macrophages. Among five lignans, *l*-sesamin, savinin, helioxanthin, taiwanin C and *cis*-dibenzylbutyrolactone isolated from chloroform fraction, taiwanin C had the most potent inhibitory activity on the TPA-induced PGE_2 production. Taiwanin C also potently suppressed the endomembrane Ca^{2+}-ATPase inhibitor thapsigargin- or the non-specific protein kinase inhibitor staurosporine-induced PGE_2 production, indicates that the inhibitory activity of taiwanin C for PGE_2 production is not specific to TPA. Taiwanin C did not affect the TPA-induced expression of COX-2, and the release of radioactivity from [^{3}H]arachidonic acid-labeled macrophages. *In vitro* determination of COX activity demonstrated that taiwanin C inhibited enzymatic activities of both COX-1 and COX-2 (IC_{50} values; 1.06 μM and 9.31 μM,

respectively), reflecting the inhibition of both COX-1 and COX-2-dependent PGE_2 production. These findings suggested that taiwanin C inhibits PGE_2 production by the direct inhibition of COX similar to the mechanism of action of NSAIDs.

3. Flavonoid 2'-hydroxy-4'-methoxychalcone Among four 2'-hydroxychalcone derivatives, 2'-hydroxy-4'-methoxychalcone (compound 1), 2',4-dihydroxy-4'-methoxychalcone (compound 2) and 2',4-dihydroxy-6'-methoxychalcone (compound 3) suppressed the LPS-induced production of nitrite and TNF-α in RAW 264.7 cells, but 2'-hydroxy-4,4'-dimethoxychalcone (compound 4) showed no effect. These findings indicated that the substitution of -OH with $-OCH_3$ at position 4 (compound 4) decreased the inhibitory activity of compound 2. The reduction of the inhibitory activity caused by the substitution of 4-OH with 4-OCH_3 was also observed in our previous study on the suppression of TPA-induced PGE_2 production in rat peritoneal macrophages. These findings suggest that the inhibition of LPS-induced production of nitrite and TNF-α by 2'-hydroxychalcone derivatives is induced by a similar mechanism to the inhibition of TPA-induced PGE_2 production. Compound 1 inhibited the LPS-induced activation of NF-κB via inhibition of the LPS-induced degradation of IκB-α. Besides NF-κB, the promoter region of both *iNOS* and *TNF-α* genes also contains a binding site for the transcription factor activator protein (AP)-1, and compound 1 also inhibited the LPS-induced activation of AP-1 and phosphorylation of JNK. It is reported that the redox regulation is involved in the activation of NF-κB and AP-1, and antioxidant reagent pyrrolidine dithiocarbamate and *N*-acetylcysteine suppressed the production of nitrite and TNF-α by inhibiting the activation of NF-κB and AP-1. It is also reported that 2'-hydroxychalcone shows potent antioxidant activity. Therefore, it is possible that compound 1 inhibited the activation of NF-κB and AP-1 through its antioxidant property. In addition, it is reported that various flavonoids including 2'-hydroxychalcone, genistein and quercetin have inhibitory activities against protein tyrosine kinases. Since activation of tyrosine kinases such as c-Src by LPS leads to both activation of NF-κB and phosphorylation of MAPKs, it is possible that the inhibition of NF-κB and AP-1 by compound 1 is mediated through their inhibitory activities against protein tyrosine kinases. Although the clarification of exact role of compound 1 in inhibiting the activation of NF-κB and AP-1 is needed, it is suggested that its inhibitory effects on the production of nitrite and TNF-α is mediated by suppression of the activation of NF-κB and AP-1.

4. Stilbene resveratrol In the rat basophilic leukemia cell line RBL-2H3 cells, resveratrol suppressed the antigen dinitrophenol-conjugated human serum albumin (DNP-HSA)-induced degranulation, and production of IL-13 and LTC_4. Resveratrol down-regulated DNP-HSA-induced phosphorylation of Akt, c-Jun, and ERK which participate in degranulation, IL-13 production, and eicosanoid release, respectively. The activation of protein tyrosine kinases such as the Src family kinases Lyn and Fyn, and the Syk/Zap family kinase Syk is one of the earliest signaling events induced by the aggregation of Fc RI on mast cells. Among these protein tyrosine kinases, subsequent trans- and auto-phosphorylations of Syk result in an increase in catalytic activity and consequential tyrosine phosphorylation of downstream signaling kinases. Based on structural similarity to the Syk inhibitor

piceatannol (*trans*-3,3',4,5'-tetrahydroxystilbeben) which has one more hydroxy group on 3'-position than resveratrol (*trans*-3,4,5'-tetrahydroxystilbeben), I postulated that one possible mechanism by which resveratrol inhibits mast cell activation is the inhibition of Syk. Resveratrol suppressed DNP-HSA-induced tyrosine phosphorylation of Syk with a similar potency to that of piceatannol. From these results, it was suggested that the inhibition by resveratrol of the antigen-induced degranulation and production of IL-13 and LTC_4 is mediated by suppressing tyrosine phosphorylation of Syk in mast cells.

In conclusion, it is suggested that the furanocoumarin imperatorin, the lignan taiwanin C, the flavonoid 2' -hydroxy-4'-methoxychalcone, and the stilbene resveratrol might be lead compounds for novel anti-inflammatory drugs having suppressive effects on the activation of macrophages, and mast cells, respectively. In addition to the further clarification of their mechanism of action for anti-inflammatory activity of these compounds, it is expected that a better understanding of the structure-activity relationship, the drug metabolism and the molecular modeling may provide the approaches to the development of the new anti-inflammatory drugs.

審 査 結 果 の 要 旨

本研究は，抗炎症活性を示す生薬の作用機序を解明する一環として，生薬中に含まれる成分を単離し，それらの化合物あるいはその誘導体についてマクロファージあるいは肥満細胞の活性化を抑制する作用があるかどうか解析し，さらにその作用機序について解析したものである。

はじめに，解熱・鎮痛作用がある生薬 *Angelica dahurica* の根から単離した5種類の furanocoumarin (byakangelicin, phellopterin, imperatorin, isoimperatorin, oxypeucedanin methanolate) 及び化学合成したそれらの誘導体についてラット腹腔マクロファージの活性化に対する作用を解析し，imperatorin, phellopterin 及び isoimperatorin にはマクロファージを lipopolysaccharide (LPS) で刺激することにより亢進する prostaglandin (PG) E_2 産生を抑制する作用があることを明らかにした。その作用機序について解析した結果，imperatorin には LPS による cyclooxygenase (COX)-2 及び membrane-associated PGE synthase-1 蛋白の誘導を抑制することによって PGE_2 産生亢進を抑制する作用があることを明らかにした。また，LPS 刺激による tumor necrosis factor (TNF)-α 産生亢進も imperatorin によって抑制されることを明らかにした。その作用機序は，転写因子 nuclear factor (NF)-κB の阻害蛋白質である inhibitory protein of NF-κB (IκB)-α の分解促進を抑制するためではなく，NF-κB の DNA への結合を直接的に阻害することにより TNF-α mRNA の発現亢進を抑制するためであることを明らかにした。

次に，抗炎症活性がある生薬 *Acanthopanax chiisanensis* の根から単離した5種類の lignan (*l*-sesamin, helioxanthin, savinin, taiwanin C, *cis*-dibenzylbutyrolactone) について，ラット腹腔マクロファージを 12-*O*-tetradecanoylphorbol 13-acetate (TPA) で刺激することにより亢進する PGE_2 産生に対する作用について調べ，taiwanin C には強い PGE_2 産生抑制作用があることを明らかにした。その作用機序について解析した結果，taiwanin C には TPA による細胞膜リン脂質からのアラキドン酸遊離を抑制する作用はなく，また TPA による COX-2 蛋白の誘導を抑制する作用もなく，COX-1 及び COX-2 の酵素活性を直接抑制することによって PGE_2 産生を抑制する作用があることを明らかにした。

次に，マウスマクロファージ様細胞株 RAW264.7 細胞を LPS で刺激することにより亢進する nitrite 及び TNF-α の産生に対する flavonoid である 2'-hydroxychalcone の各種誘導体の作用について解析し，2'-hydroxy-4'-methoxychalcone が最も強く nitrite 及び TNF-α の産生を抑制する作用があることを明らかにした。また，その作用機序は転写因子 NF-κB 及び activator protein-1 の活性化を抑制するためであることを明らかにした。

最後に，stilbene である resveratrol には，ラット肥満細胞株 RBL-2H3 細胞を抗原刺激することにより亢進する脱顆粒反応，interleukin-13 及び leukotriene C_4 の産生亢進を抑制し，その作用機序は抗原刺激による Syk の tyrosine リン酸化を抑制することにより，その下流の Akt, ERK 及び c-Jun のリン酸化の抑制するためであることを明らかにした。

本研究により，抗炎症活性を示す生薬の作用機序が一部解明され，またマクロファージ及び肥満細胞の活性化を抑制する作用機序をもつ抗炎症薬としてのリード化合物が数多く発見された。

よって，本論文は博士（薬学）の学位論文として合格と認める。