論 文 要 旨

1,5-anydro-D-fructose attenuates lipopolysaccharide-induced cytokine release via suppression of NF-κB p65 phosphorylation

（1,5-アンヒドロフルクトースは NF-kB の p65 のリン酸化を抑制して LPS によるサイトカインの放出を減弱する。）

孟 暁 潔

【序論および目的】（適宜、項目をたてて、必ず２頁で記載する）

Lipopolysaccharide (LPS) stimulates macrophages by activating NF-κB, which contributes to the release of tumor necrosis factor (TNF)-α and interleukin (IL)-6. 1,5-anhydro-D-fructose (1,5-AF), a monosaccharide formed from starch and glycogen, exhibits anti-oxidant activity and enhances insulin secretion. This study examined the effects of 1,5-AF on LPS-induced inflammatory reactions and elucidated its molecular mechanisms.

【材料および方法】

In this study, mice pretreated with 1,5-AF (38.5 mg/kg) and untreated mice were randomly challenged with lipopolysaccharide (LPS, 2 mg/kg) for 4 h. To determine the function of 1,5-AF in *in vitro* conditions, we pretreated RAW264.7 cells with 1,5-AF (500 μg/ml) and introduced LPS (500 ng/ml). Levels of IL-6, macrophage chemoattractant protein (MCP)-1, and TNF-α in the serum and in the cell supernatants were evaluated by ELISA. The phosphorylation of mitogen-activated kinase (MAPK), Akt, IκBα, and phosphorylation on Ser536 of the NF-κB p65 subunit in RAW264.7 cells were analyzed by western blotting. The DNA-binding activity of NF-κB and its nuclear translocalization were determined by ELISA and immunoflourescence, respectively.

【結 果】

We found that 1,5-AF pretreatment attenuated cytokine release into the LPS-challenged mice serum, including TNF-α, IL-6 and MCP-1. Furthermore, pretreatment with 1,5-AF (500 μg/ml) attenuated cytokine release, and 1,5-AF directly inhibited the nuclear translocalization of the NF-κB p65 subunit in LPS-stimulated murine macrophage-like RAW264.7 cells. This inhibition was responsible for decreased LPS-induced phosphorylation on Ser536 of the NF-κB p65 subunit, which is a posttranslational modification involved in the non-canonical

pathway.

【結論及び考察】

Collectively, these findings indicate that the anti-inflammatory activity of 1,5-AF, and the inhibitory mechanism of 1,5-AF is mediated by the inactivation of NF-κB to suppress LPS-induced translocalization and phosphorylation of the p65 subunit. These results suggest that 1,5-AF may play an important role during inflammation and may be a key molecule with therapeutic potential.

(Biochemical and Biophysical Research Communications. 2009年掲載予定)

論文審査の要旨

報告番号	総研第 60 号		学位申請者	孟 暁潔
審査委員	主査	金蔵 拓郎	学位	博士（医学）歯学・学術）
	副査	栄鶴 義人	副査	乾 明夫
	副査	久保田龍二	副査	堀内 正久

1,5-anhydro-D-fructose attenuates lipopolysaccharide-induced cytokine release via suppression of NF-κB p65 phosphorylation

（1,5-アンヒドロフルクトースはNF-kBのp65のリン酸化を抑制して
LPSによるサイトカインの放出を減弱する。）

Lipopolysaccharide (LPS) present on the outer membrane of Gram-negative bacteria is the main contributing factor to the development of inflammation and activates inflammatory immune cells including macrophages/monocytes. The activated macrophages release the proinflammatory cytokines and other inflammatory mediators such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and macrophage chemoattractant protein (MCP)-1 via transcription factor NF-κB. The increased production of these inflammatory cytokines may mediate many inflammatory diseases. Thus agents capable of inhibiting LPS stimulation are expected to possess therapeutic potential. In this study, the applicant for a degree and her coworkers demonstrated for the first time that 1,5-AF, a recently identified monosaccharide, has anti-inflammatory activity and suppresses cytokine release (TNF-α, IL-6, and MCP-1) in response to LPS stimulation in both a mouse model and murine macrophage cell lines.

Before LPS challenge, mice were intraperitoneally injected with 1,5-AF (38.5 mg/kg). They found that 1,5-AF pretreatment attenuated cytokine release into the serum including TNF-α, IL-6 and MCP-1. Furthermore, pretreatment with 1,5-AF (500 μg/ml) attenuated cytokine release and 1,5-AF directly inhibited the nuclear translocalization of the NF-κB p65 subunit in LPS-stimulated murine macrophage-like RAW264.7 cells. This inhibition was attributable to decreased LPS-induced phosphorylation at Ser536 of the NF-κB p65 subunit, which is a posttranslational modification involved in the non-canonical pathway. These results suggest that 1,5-AF may play an important role in inflammation and may be a key molecule with therapeutic potential.

Previous work indicates that 1,5-AF increases glucose tolerance and insulin secretion in mice and has no toxicity even at a high dose of 1,5-AF (1.0 g/kg body weight). Furthermore, in the present study, they found that 1,5-AF acts as a selective inflammatory inhibitor by attenuating NF-κB p65 subunit activation; this in turn inhibits the LPS-induced cytokine overproduction without any evidence for toxicity both *in vivo* and *in vitro*. Based on these results, they proposed that 1,5-AF may be a potential therapeutic agent for inflammatory diseases especially for diabetic patients.

The present study revealed anti-inflammatory effects of a novel monosaccharide, 1,5-AF. Hence this study deserves to be a doctoral dissertation.

最終試験の結果の要旨

報告番号	総研第 60 号		学位申請者	孟 暁潔
審査委員	主査	金蔵 拓郎	学位	博士（医学・歯学・学術）
	副査	栄鶴 義人	副査	乾 明夫
	副査	久保田龍二	副査	堀内 正久

主査および副査の５名は、平成21年２月18日、学位申請者 孟 暁潔 君に面接し、学位申請論文の内容について説明を求めると共に、関連事項について試問を行った。具体的には、以下のような質疑応答がなされ、いずれについても満足すべき回答を得ることができた。

質問１） What kind of receptor recognizes LPS?

（回答） Toll like receptor 2 and 4.

質問２） What does LPS O55:B5 mean?

（回答） It means E.coli serotype.

質問３） What does EU/mg mean?

（回答） The LPS concentration is indicated in EU/mg. The unit EU (endotoxin unit) represents the biological activity of LPS. One EU is generally equivalent to 100 pg LPS.

質問４） Why is suppression of TNF-α so weak compared with IL-6 or MCP-1?

（回答） Since the transcription factors of the TNF-α gene responding to LPS treatment include activator protein (AP)-1 and nuclear factor (NF)-kappaB (κB). Based on our investigation, 1,5-AF inhibits NF-κB activation, however it has no effect on AP-1. This might be the reason for a weak effect of 1,5-AF on the release of TNF-α.

質問５） How much 1,5-AF is found physiologically in rat liver tissue?

（回答） The level of 1,5AF in the liver is 0.43 μg /g wet tissue.

質問６） Is there similarity in dose used in *in vivo* and *in vitro* study?

（回答） Yes, we used 1,5-AF about 500 μg/ml both in *in vivo* and *in vitro* studies.

質問７） Does 1,5-AF increase the production of anti-inflammatory cytokines, such as IL-10?

（回答） Yes, 1,5-AF increases the production of IL-10 in LPS-stimulated RAW264.7 cells.

質問８） Do you think the efficacy on decrease of proinflammatory cytokines is similar *in vivo* and *in vitro* studies?

（回答） Yes, I think so. Since we used same dose of 1,5-AF and similar experimental condition in *in vivo* and *in vitro* study.

質問９） Does antioxidant activity totally depend on cytokine level?

（回答） Because the paper I cited did not mention the relation between the antioxidant activity and cytokine levels, I can not tell exactly about the effect of antioxidant activity on the cytokine levels.

質問10） Can cytokines be completely inhibited if the dose of 1,5-AF is increased?

（回答） Although we did not investigate the effect of higher dose 1,5-AF on cytokines release, we examined the effect of 1,5-AF (800 μg/ml) on iNOS expression. It could inhibit iNOS expression completely.

質問11） What kind of medical condition can be treated by 1,5-AF?

（回答） Inflammatory diseases such as chronic inflammatory condition of diabetic patients.

質問12） After phosphorylation of serine 536, IκBα is dissociated from p50 and p65 complex, how is IκBα processed?

（回答）According to the figure 3B in my study, IκBα was degraded after phosphorylation of serine 536.

質問 13）In figure 3B, lane 2, why is IκBα band not changed after stimulation with LPS?

（回答）Compared with the control, the total amount of IκBα is decreased because of degradation.

質問 14）Does non-canonical pathway play an important role under LPS stimulation judging from your paper?

（回答）Yes, I think so. In our experiments, it seems that non-canonical pathway plays an important role when the cells were stimulated with LPS.

質問 15）Does 1,5-AF exist in blood?

（回答）No, 1,5-AF could not be detected in the rat blood.

質問 16）Does 1,5-Anhydroglucitol (1,5-AG) have similar effect to 1,5-AF in suppressing cytokines release?

（回答）Yes, it does. However, there is a difference between 1,5-AF and 1,5-AG. For example, 1,5-AG can suppress IL-1β production but 1,5-AF can not.

質問 17）Is 1,5-AF used as energy?

（回答）The data of 1,5-AF pathway in mammalian tissues are still fragmentary. We are not sure about it.

質問 18）How does 1,5-AF affect IKK-γ?

（回答）While we did not address this issue, we suppose that 1,5-AF has no effect on IKK-γ because there is no effect of 1,5-AF on IKKα/β, that is regulated by IKK-γ.

質問 19）What is an origin of 1,5-AF you used?

（回答） We used the 1,5-AF derived from starch.

質問 20）Does 1,5-AF from different sources have exactly same structure and function?

（回答）Yes, 1,5-AF have the same structure and function even from different sources.

質問 21）In figures 1 and 2, what is the basis for deciding the concentration of 1,5-AF?

（回答）We have used the current concentration of 1,5-AF based on our preliminary experiments

質問 22）In supplemental figure 3, HMGB1 is used as a loading control. Isn't it affected by LPS?

（回答）HMGB1 is a late phase pro-infalmmatory mediator. It is released after 16h stimulation with 500 ng/ml LPS in RAW264.7 cells. Because we stimulated cells for 1hour with LPS in our experiment, it does not affect HMGB1 level and we used it as a nuclear protein control.

質問 23）In figures 1 and 2, MCP-1 and TNF-α are detected to some extent but IL-6 is completely not detected under normal condition, can you explain this difference?

（回答）The similar results have been observed in other papers as well.

質問 24）In figure 3, panel C, p65 phosphorylation is remarkably inhibited by 100 μg/ml 1,5-AF, but in figure 2, the inhibition by the same dose is very weak. How can this discrepancy be explained?

（回答）After LPS stimulation, the inflammatory mediators release by macrophages are not only dependent on NF-κB but also on AP-1 which plays a role in inflammation. Since there is no effect of 1,5-AF on AP-1 after LPS stimulation, a canonical pathway also plays role in inflammation, therefore p65 phosphorylation is remarkably inhibited by 100 μg/ml 1,5-AF.

質問 25）COX-2 inhibitor, SC236, suppresses translocation of p65, does this means COX2 has ability to activate NF-κB?

（回答）We cited the reference "Suppression of RelA/p65 nuclear translocation independent of IkappaB-alpha degradation by cyclooxygenase-2 inhibitor in gastric cancer" in our paper. It was mentioned that SC236 has anti-NF-κB effects independent of COX-2 in gastric cancer.

以上の結果から５名の審査員は申請者が大学院博士課程修了者としての学力識見を有しているものと認め、博士（医学）の学位を与えるに足る資格を有するものと認定した。