Adrenic acid as an inflammation enhancer in non-alcoholic fatty liver disease

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学 位 論 文 の 内 容 要 旨

Adrenic acid as an inflammation enhancer in non-alcoholic fatty liver disease

アドレン酸は非アルコール性脂肪性肝疾患において炎症促進因子として作用する

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Epidemiological studies have indicated that the prevalence of non-alcoholic fatty liver disease (NAFLD) is rising in both Western countries and other regions of the world. A subset of NAFLD patients, which are considered to have non-alcoholic steatohepatitis (NASH), are at increased risk of liver cirrhosis, hepatocellular carcinoma, and liver-related mortality. Therefore, the early identification of NASH is very important for preventing liver complications. Disease progression in NAFLD is a complex process involving several factors, including lipid metabolism. Despite extensive investigation of the molecular mechanisms responsible for lipid-related liver damage in NAFLD, the available quantitative data about the use of lipid profiles for differentiating steatohepatitis from simple steatosis are still inconclusive. So far, lipidomic studies have suggested that at least 500 lipid species are present in plasma, and over 1,000 lipid species are found within cells. Hence, we hypothesized that there might be other lipid species that are pathologically related to NAFLD, and some of them might be useful for differentiating steatohepatitis from simple steatosis. Thus, our aim is to identify novel links between lipid species and the progression of NAFLD.

In this study, we first examined the lipid profiles of genetically obese db/db mice and used a choline deficient-L-amino acid defined-high fat diet (CDAHFD) to induce steatohepatitis in the mice. The CDAHFD induced hepatic steatohepatitis and fibrosis, which are comparable to the pathology of human NASH, in db/db mice. CDAHFD-fed db/db mice also showed obesity and hypercholesterolemia, two metabolic features commonly found in human NAFLD. The mRNA expression level of ACOX1 (peroxisomal beta-oxidation) was decreased in CDAHFD-fed db/db mice and there were no significant changes in mRNA expression of CYP2E1 and CYP4A14 (microsomal omega oxidation) compared to standard diet (SD)-fed db/db mice. The mRNA expression level of FAS (de novo lipogenesis), FATP2, FATP5 (fatty acid uptake) were also decreased but no significant changes in mRNA levels of MCAD, LCAD (mitochondria beta oxidation) and MTTP (triglyceride export). The mRNA levels of GPx1 (antioxidative enzyme) was decreased, while UCP2 (mitochondria uncoupling protein) increased. Overall, these data suggest that liver steatosis in our steatohepatitis model could be due to impaired peroxisomal beta-oxidation while liver injury in part related to reactive oxidative stress (ROS).

Lipid analysis was performed using liquid chromatography/mass spectrometry (LC/MS). LC/MS can profile lipid species accurately from a small minimally pre-treated sample within a short period of time. Based on LC/MS analysis, CDAHFD-fed db/db mice

showed significantly higher hepatic and plasma levels of free adrenic acid ($p<0.05$). Because adrenic acid is less examined in NAFLD, we next focused on elucidating its potential role in the progression of the disease. The higher free adrenic acid was not related to higher intake because the diet did not contain adrenic acid. Therefore, we analyzed mRNA expression of ELOVL2 and 5, two enzymes responsible for elongation of arachidonic acid to adrenic acid. Basal expressions of ELOVL2 and 5 were lower in SD-fed db/db mice but increased in CDAHFD-fed db/db mice. Combining these results with gene expression analysis of steatosis-related genes, we concluded that increased level of free adrenic acid in CDAHFD-fed db/db mice was due to impaired peroxisomal beta-oxidation and enhanced elongation of arachidonic acid.

The mRNA level of CD68, a marker for monocyte/macrophage, was increased in livers of CDAHFD-fed db/db mice. This suggests that there is a recruitment of inflammatory cells. Chemokines can attract immune cells to inflammatory sites. Therefore, we hypothesized that adrenic acid could influence disease progression by enhancing chemokine gene expressions. Using HepG2 as a hepatocyte model we found that under treatment with recombinant human (rh) TNFα, adrenic acid-pretreated HepG2 cells expressed higher mRNA levels of TNFα, IL8, MIP1β and MCP1. The similar findings were observed after treatment with rhIL1β, except for MCP1. Therefore, the upregulating effect of adrenic acid on MCP1 mRNA expression during inflammation is cytokine-specific. The inflammatory activities of TNFα and IL1β are mediated by type 1 TNF receptors (TNFR-1) and type 1 IL-1 receptors (IL1R-1), respectively. On the other hand, type 2 IL-1 receptors (IL1R-2) act as "decoy receptors" and inhibit IL1β activity The mRNA levels of TNFR-1 in adrenic acid-pretreated HepG2 cells was not upregulated, while mRNA levels of IL1R-1 were significantly decreased under cytokine stimulation. On the other hand, mRNA levels of IL1R-2 were increased. These results indicated that: (i) negative regulation by IL1R-2 inhibits IL1β-induced inflammation, and (ii) adrenic acid might modify TNFα or IL1β signaling pathways at the post-receptor level. Overall, from invitro experiment, we concluded that adrenic acid could enhance chemokine gene expressions.

We next confirmed our experimental findings in the setting of human NAFLD. The previous study showed that changes in the serum ALT levels of NASH patients were correlated with liver inflammation. Therefore, we divided our patients into three groups based on their ALT levels (different cut-off values were employed for males and females). In our data analysis, we found that the mean plasma adrenic acid level was higher in group 3 (ALT ≥60 IU/L for

males, ≥40 IU/L for females) than in group 1 (ALT <30 IU/L for males, <20 IU/L for females) and group 2 (30 IU/L ≤ ALT < 60 IU/L for males, 20 IU/L ≤ ALT < 40 IU/L for females). These intergroup differences and the associated linear trend were statistically significant. Overall, these findings support our in vitro results. We also obtained a similar finding with regard to the plasma level of docosapentaenoic acid (22:5n6) in our human data set. Since the formation of docosapentaenoic acid (22:5n6) involves β-oxidation in peroxisomes, this might indicate that our patients had mild disease that did not involve significant changes in peroxisome β-oxidation, as shown by the mouse model. The limitations of our human sample analysis include the small number of patients, the lack of histological examinations, and the fact that it only included patients from one medical center.

In conclusion, the combination of increased polyunsaturated fatty acids and elongase expression and impaired peroxisomal β-oxidation cause the accumulation of adrenic acid in experimental steatohepatitis, but not in simple steatosis. Under inflammatory conditions, adrenic acid might exacerbate inflammation by enhancing the expression of chemokine genes in hepatocytes. Increased plasma adrenic acid levels are associated with high ALT levels in NAFLD patients. Taken together, adrenic acid accumulation contributes to disease progression in NAFLD. A further study is needed to determine whether adrenic acid measurements could be used as a diagnostic biomarker of steatohepatitis in NAFLD.

神戸大学大学院医学(系)研究科（博士課程）

論文審査の結果の要旨

受付番号	甲 第2713号	氏名	SAUT HORAS HATOGUAN NABABAN
論文題目 Title of Dissertation	アドレン酸は非アルコール性脂肪性肝疾患において炎症促進因子として作用する Adrenic acid as an inflammation enhancer in non-alcoholic fatty liver disease		
審査委員 Examiner	主査 Chief Examiner 小川 渉 副査 Vice-examiner 古屋敷智之 副査 Vice-examiner 福本巧		

（要旨は１，０００字～２，０００字程度）

本研究は、NAFLD の発症や進行に関与する脂質種の同定を目的としたものである。本研究では、肥満モデルマウスである db/db マウスにコリン欠損アミノ酸制限高脂肪食（CDAHFD）を摂取させることで、NASH を誘発させた。CDAHFD 摂取 db/db マウスは、肥満を呈し、高コレステロール血症を発症した。分子レベルでの検討を行った結果、CDAHFD 摂取 db/db マウスでは、ペルオキシソーム β 酸化に関わる ACOX1 の mRNA レベルの発現が減少したが、ミクロソーム ω 酸化に関わる CYP2E1 と CYP4A14 の mRNA レベルの発現は、CDAHFD 摂取 db/db マウスと標準食摂取 db/db マウスとの間で、有意な差がなかった。de novo 脂質合成に関わる FAS や脂肪酸取り込みみに関わる FATP2、FATP5 の mRNA 発現量も減少したが、ミトコンドリア β 酸化に関わる MCAD、LCAD、中性脂肪排出に関わる MTTP の mRNA 発現量には大きな変化は見られなかった。抗酸化酵素である GPx1 の mRNA の発現量は減少したが、ミトコンドリアの脱共役タンパク質である UCP2 は増加した。

次に、液体クロマトグラフィー質量分析（LC/MS）により脂質種の分析が行われ、CDAHFD 摂取 db/db マウスの肝臓中と血漿中の遊離アドレン酸レベルが、標準食摂取 db/db マウスと比較して、統計学的に有意な高値を示した。アラキドン酸からアドレン酸への変換（炭素鎖伸長）に関係する 2 つの酵素 ELOVL2 と ELOVL5 の mRNA 発現レベルを分析した結果、対照 C57BL/6J マウスと比較して、標準食摂取 db/db マウスの ELOVL2 と ELOVL5 の mRNA 発現レベルは低下したが、CDAHFD 摂取 db/db マウスでは増加した。以上より、CDAHFD 摂取 db/db マウスにおけるアドレン酸レベルの増加は、ペルオキシソーム β 酸化の悪化とアラキドン酸の炭素鎖伸長亢進によるものと考えられた。

CD68 の mRNA レベルは、CDAHFD 摂取 db/db マウスの肝臓中で増加し、CDAHFD 摂取 db/db マウスの肝臓における炎症細胞の動員によるものと考えられた。また、HepG2 細胞を用いた実験の結果、アドレン酸を前処理した HepG2 細胞にリコンビナントヒト TNFα を作用させることで、TNFα、IL8、MIP1β、MCP1 の mRNA レベルが増加した。また、アドレン酸を前処理した HepG2 細胞において、TNFR-1 の mRNA レベルは増加せず、サイトカイン刺激下での IL1R-1 の mRNA レベルは有意に減少した。一方で、IL1R-2 の mRNA レベルは増加した。これらの結果は、次に示す可能性を示す。1) IL1R-2 による負の制御は、IL1β が誘発する炎症反応を抑制し、2) アドレン酸は受容体より下流において、TNFα、あるいは、IL1β 関連シグナルを制御する。

最後にヒト NAFLD 患者に対する実験が行われた。ALT 濃度に基づいて、患者を 3 つのグループに分けた結果、血漿中アドレン酸レベルは、グループ 3（男性 ALT ≥ 60 IU/L, 女性 ≥ 40 IU/L）の方が、グループ 1 （男性 ALT < 30 IU/L, 女性 < 20 IU/L）やグループ 2 （男性 30 IU/L ≤ ALT < 60 IU/L, 女性 20 IU/L ≤ ALT < 40 IU/L）と比較して、高値を示した。我々は、また、ドコサペンタエン酸（22:5n6）の血漿レベルにおいても同様の結果が得られた。

本研究は NASH 発症における脂質の役割を解析し、アドレン酸の炎症惹起における役割を示したものである。これは、従来認識されてこなかったアドレン酸と NASH 発症との関係性を示唆する知見として価値ある集積であると認める。よって、本研究者は、博士（医学）の学位を得る資格があるものと認める。