

氏 名	ふじあんてい Fujianti
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学 位 論 文 名	バソプレッシン V1a 受容体欠損マウスにおける加齢変化促進の機序解明と応用
論 文 審 査 委 員	(委員長) 教 授 黒 尾 誠 (委 員) 教 授 尾 仲 達 史 教 授 秋 元 哲

## 論文内容の要旨

### 1 研究目的

Lipofuscin is an autofluorescent pigment that accumulates in aging cells. The first description of this lipofuscin in the literature was by Hueck in 1912 and Hannover in 1842. In addition to a value for a convenient marker of aging, lipofuscin is often involved in age-related diseases. It has been known that the adrenal cortex of rodents and humans accumulates lipofuscin with age. How this aging process proceeds is not known. We previously found that lipofuscin deposition was accelerated in the adrenal cortex of mice lacking V1a vasopressin receptor gene (V<sub>1a</sub>KO). In the adrenal cortex, arginine vasopressin (AVP) exerts two main effects by acting on the V<sub>1a</sub> receptor subtype: it increases the mitogenic activity of the zona glomerulosa and stimulates aldosterone and glucocorticoid secretion in mice, rats, and humans. However, besides these hormonal functions of AVP, it remains unknown whether AVP plays a role in the production and accumulation of aging pigments. This study was conducted to clarify the time course of lipofuscin accumulation in the adrenal cortex of V<sub>1a</sub>KO mice. We also identified accompanying changes in the gene expression profiles by comparing the adrenal cortices of V<sub>1a</sub>KO and wild-type (WT) mice.

### 2 研究方法

#### Experiment I

The aim of this experiments was to measure the distribution of lipofuscin in the adrenal cortices of V<sub>1a</sub>KO and WT mice. We utilized two groups of mice of both sexes at age of 2, 6, and 12 months. To quantitate the amount and timing of lipofuscin deposits, we have collected 60 adrenal glands. Rhodamine phalloidine and DAPI were applied to membrane and nucleus staining, respectively. The slides were examined under confocal microscope and digital images were recorded. The area and localization of lipofuscin in adrenal slices were examined by Image-J software.

#### Experiment II

Gene expression profile was analyzed by using cDNA microarray in the adrenal cortex of male mice at 2 months age. The experiments were performed as previously described and according to the manufacturer's

instructions. The expression of selected genes was confirmed by quantitative RT-PCR. The total RNA was isolated from adrenal gland ( $n = 10$ ) and cDNA was synthesized by SuperScript III first strand synthesis reagent kit. Activity of MMP-14 (membrane-type 1 matrix metalloproteinase) in the adrenal cortex were analyzed according to the manufacturer's instructions (AnaSpec Inc., CA, USA).

#### Experiment III

To investigate adrenal  $V_{1a}$ -mediated cellular signaling, we analyzed mouse Y1 adrenocortical cell line. The cells at a density of  $5 \times 10^5$  cells/35-mm glass base dish were treated with buffer (control) or 50nM AVP (treatment) for 0, 3, 6, and 12 hours. The total RNA was isolated from Y1 cells and cDNA was synthesized. The expression of target genes was measured by qRT-PCR. RT-PCR was performed to exam the expression levels of  $V_{1a}$ ,  $V_{1b}$  or oxytocin receptors in Y1 cell.

### 3 研究成果

#### Experiment I

At 2 months of age, lipofuscin autofluorescence was detected in the adrenal cortex of  $V_{1a}$ KO male mice, but not of WT mice. High-magnification images showed that lipofuscin accumulation began in the cytoplasm and augmented up to the age of 12 months. Importantly, significantly larger deposits were detected in  $V_{1a}$ KO mice. These findings suggest that  $V_{1a}$ KO mice showed accelerated aging changes that were observed in the normal aging process.

#### Experiment II

From the expression profiles, we found two up-regulated genes, *Xafl* and *Serpina1b*, and down-regulated genes, *Adi1* and *Abhd1*, in the adrenal cortex of  $V_{1a}$ KO. Our qRT-PCR analysis performed on new adrenal samples confirmed the results of microarray experiments. The MMP14 activity was significantly higher in the adrenals of  $V_{1a}$ KO mice than those of WT mice. Together with the result of reduced *Adi1* expression level in the KO adrenals, it can be speculated that *Adi1* regulates cellular morphology and migration of adrenocortical cells in  $V_{1a}$ KO through the activation of the MMP14.

#### Experiment III

PCR analysis revealed that  $V_{1a}$  subtype was the main vasopressin/oxytocin receptor in Y1 cells. In fact, application of AVP ( $10^{-7}$  M) stimulated a prompt increase in intracellular  $Ca^{2+}$  concentrations  $[Ca^{2+}]_i$  in Y1 cells. This  $[Ca^{2+}]_i$  was suppressed by the  $V_{1a}$  antagonist SR49059. Furthermore, when  $V_{1a}$  receptor was stimulated by AVP, *Xafl* expression in Y1 cells was significantly suppressed. These results indicate that the AVP and  $V_{1a}$  receptor system plays an important role in regulating the expression of aging-related genes.

### 4 考察

#### Experiment I

$V_{1a}$ KO lipofuscin can be detected as early as at 2 months of age. Accumulation of lipofuscin was augmented in both of mice groups especially in 12 months, but larger deposits were detected in the  $V_{1a}$ KO mice group. Notably, the calculated area of lipofuscin fluorescence in the adrenal sections of male  $V_{1a}$ KO increased 6.8 times from 2 to 12 months of the age. Unexpectedly, this number in the WT adrenal sections was the similar level of 6.7 times. These results suggested that the effect of  $V_{1a}$  deletion already started in

the 2 months of age and continued throughout our observation period.

## Experiment II

The transcripts of differentially expressed four genes were significantly altered between the adrenal cortices of WT and V1aKO mice. Xaf1 and serpin1b expression in V1aKO mice were increased than WT mice. Xaf1 increases p53 and plays an important role in cell fate between apoptosis and survival. Serpin1b is one of five  $\alpha$ 1-protease inhibitor proteins known to regulate neutrophil elastase. In contrast, Adil encodes a multi-functional protein with significant homology to bacterial acireductone dioxygenase and regulates mRNA processing in the nuclei. Adil is also known as the membrane-type 1 matrix metalloproteinase MMP-14-binding protein. We examined the possibility of altered enzymatic activity of MMP14 in adrenal cortex of V1aKO mice. The exact role of Abhd1 is not known. However, a family protein, Abhd5, is involved in lipid metabolism in the liver. Among these four genes, we focused on Xaf1, because this gene was reported to interact with the p53 tumor suppressor protein and enhance the non-tumor related functions of p53, such as senescence.

## Experiment III

RT-PCR analysis revealed that V<sub>1a</sub> is a main vasopressin receptor in Y1 cells. The application of AVP ( $10^{-7}$  M) stimulated prompt increase in intracellular  $\text{Ca}^{2+}$  concentrations [ $\text{Ca}^{2+}$ ] in Y1 cell, which was suppressed by V<sub>1a</sub> antagonist. Finally, when V<sub>1a</sub> was activated by AVP, Xaf1 gene expression in Y1 cells was significantly suppressed. These results indicated that V<sub>1a</sub> and AVP system plays an important role in regulating transcripts for aging-related genes.

## 5 結論

In summary, this study delineated an enhanced aging phenotype in the adrenal cortex of V1aKO mice by examining the time course of lipofuscin accumulation. The V1a receptor in the adrenal cortex significantly impacts the gene regulatory network. The four genes selected in this study are likely candidates for the enhanced aging phenotype in V1aKO adrenal glands. Responsible gene(s) for adrenal aging should be identified in further studies.

## 論文審査の結果の要旨

申請者は、「バゾプレッシン V1a 受容体欠損マウスの副腎にはリポフスチンの沈着が目立つ」という観察に基づき、バゾプレッシン (AVP) -V1a 受容体シグナルの障害が副腎の老化を加速する、という仮説を立てた。この仮説の検証を試みたのが本学位論文である。

リポフスチンは加齢に伴って様々な組織に沈着する自家蛍光物質で、その形成機序は明らかではないが、老化の指標の一つとして知られている。本学位論文では、以下の 3 つの実験を行っている。

【実験 1】バゾプレッシン V1a 受容体欠損マウス (V1aKO マウス) および野生型マウスの副腎におけるリポフスチン沈着量の比較。

V1aKO マウスと野生型マウスのオスとメス、それぞれ 2、6、12 ヶ月齢の副腎を採取し、共焦点顕微鏡を用いて、自家蛍光を発するリポフスチンの沈着面積を定量して比較したところ、

V1aKO マウスでリポフスチンの沈着量が多いという結果が得られた。

【実験 2】バゾプレッシン V1a 受容体欠損マウス (V1aKO マウス) および野生型マウスの副腎における遺伝子発現パターンの比較。

V1aKO マウスと野生型マウス (2 ヶ月齢オス) の副腎から RNA を抽出し、マイクロアレイを用いて V1aKO マウスで発現が増強している遺伝子を 2 つ (Xaf1 と *Serpina1b*)、減少している遺伝子を 2 つ (*Adi1* と *Abhd1*) を同定し、定量的 RT-PCR で確認した。また、*Adi1* 蛋白は MMP-14 と結合して酵素活性を抑制することが知られているため、副腎抽出液中の MMP-14 活性を測定し、V1aKO マウスで活性亢進が認められることを示した。Xaf1 は p53 と結合してその活性を制御することや、p53 の活性化が細胞老化を誘導することが知られており、V1aKO マウスにおける副腎の老化の加速に Xaf1 の発現増強が重要な役割を果たす可能性があると考え、解析の対象を Xaf1 に絞った。

【実験 3】マウス副腎腫瘍由来細胞株 Y1 の AVP 刺激に対する反応の解析。

培養 Y1 細胞には V1a 受容体が発現していること、AVP の添加で細胞内カルシウム濃度の一過性の上昇を認めることを確認した。また、AVP 刺激で Xaf1 遺伝子の発現が抑制されることを RT-PCR で確認した。

以上の結果から、AVP-V1a 受容体シグナルの阻害による遺伝子発現の変化が副腎の老化を加速する、と結論した。

V1a KO マウスの副腎にリポフスチンの沈着が目立つことは、申請者の指導教官である興水らによって 2012 年に報告されているが、その分子機構について解析したのは本学位論文が初めてであり、この点で新規性・独創性があると考えられる。

一方、「V1a KO マウスでは副腎の老化が促進している」と結論するには、リポフスチンの沈着亢進と Xaf1 の発現亢進以外にも、例えば p53, p21, p16 など普遍的な老化の指標を検討することが今後の課題と考えられる。また、V1a KO マウスで発現が大きく変化した Xaf1 以外の遺伝子 (*Adi1*, *Abhd1*, *Serpina1b*) がリポフスチンの沈着亢進との関連の有無についても今後の検討が待たれる。

学位論文に関しては、研究の背景、方法、結果、議論の各セクションが分かりやすく記載されており、図表も適切に使用されている。以上の評価を踏まえ、本学位論文は、以下の軽微な修正の後、合格と判定する。

1) マウスの月齢、試薬の濃度が要旨、本文、図の説明の間で一致していないところがあるので、修正すること。

2) Introduction で、マウスの副腎には網状層 (zona reticularis) がないと述べているが、【実験 1】ではリポフスチンの沈着部位として reticular layer を挙げており、整合性をとる必要がある。

上記項目およびタイプミスを修正したものが 2022 年 2 月 18 日審査委員長に提出され、同 2 月 21 日に全て修正されていることを確認した。

## 最終試験の結果の要旨

申請者の発表は、研究の背景、目的、方法、結果、考察について、それぞれ分かりやすく簡潔

にまとめられており、時間配分も適切であった。

主査や副査の質問に対しても適切に回答し、今回の研究の限界と今後の課題についても十分認識していた。

リポスチンやバゾプレッシンに関する一般的な知識も、研究の背景を説明する際に要領よくまとめて発表した。

以上、最終試験において申請者の研究能力および科学的素養は学位に値するものと考えられる。