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学位論文名	**喫煙は血管平滑筋細胞での新規細胞死フェロトーシスを誘導し、大動脈瘤形成に寄与する**
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論文内容の要旨

1　研究目的

Cigarette smoking is a major risk factor for aortic aneurysm (AA) and dissection (AD); however, no causative link between smoking and these aortic disorders has been proven. Although the loss of medial vascular smooth muscle cells (VSMCs) at the arterial wall is considered one of the key pathophysiologic event in AA and AD, the underlying mechanism is unclear. Cigarette smoking extract (CSE), a gas phase extract, induces cytotoxic effects in certain cell types. Previous studies have suggested that the major cytotoxic compounds in CSE react with intracellular glutathione (GSH) and subsequently deplete GSH content. Ferroptosis, a novel form of regulated cell death, has been reported to be involved in various diseases and attracted much attention in recent years. Ferroptosis is caused by the accumulation of iron-dependent lipid peroxides due to a loss of intracellular glutathione peroxidase 4 (GPX4) activity directly or indirectly by a decrease in GSH levels. Because GSH reduction is the starting point for the CSE-induced cytotoxicity, we hypothesized that smoking could induce ferroptosis in VSMCs and contribute to AA formation. The purpose of this study was to explore the mechanism responsible for cigarette smoke-induced VSMC death and its role in AA formation.

2　研究方法

CSE induced cell death was assessed in rat primary VSMCs, rat VSMC line A7r5, and human endothelial cells (EC) line EAhy926 by SYTOX Green and LDH assay. The effects of various cell death inhibitors and relative pathways (Ferrostatin-1, [Fer-1, ferroptosis inhibitor], Liproxstatin-1 [Lip-1, ferroptosis inhibitor], Deferoxamine [DFO, iron chelator], Z-VAD [pan-caspase inhibitor], Z-YVAD [caspase-1 inhibitor], Nec-1 [Necrostatin-1, necroptosis inhibitor], N-acetyl cysteine [NAC, antioxidant], and diphenyleneiodonium chloride [DPI, NADPH inhibitor]) on CSE-induced VSMC death were evaluated. Lipid peroxidation and ferroptosis were assessed by C11-Bodipy staining and Ptsg2 mRNA expression, respectively.

The expression of proinflammatory cytokines and matrix metalloproteinases were also assessed by real-time PCR analysis. Regarding the effect of the major antioxidant enzyme glutathione peroxidase 4 (GPX4) and GSH, we established GPX4-overexpressing A7r5 cells and tested the effect of CSE on ferroptosis in these cells. We also measured the GSH content after CSE stimulation. Furthermore, the effects of CSE on VSMC loss were evaluated in ex vivo cultured murine aortic rings in the presence or absence of Fer-1. Histologic changes were also evaluated by Elastic Van Gieson (EVG) staining and electron microscopic analysis.

3 研究成果

CSE markedly induced cell death in A7r5 cells and primary rat VSMCs, but not in EAhy926 cells, which was completely inhibited by specific ferroptosis inhibitors (Fer-1 and Lip-1) and DFO. CSE-induced VSMC death was partially inhibited by NAC and DPI, but not by inhibitors of Z-VAD, Z-YVAD, or Nec-1. CSE also upregulated mRNA levels of L-1β, IL-6, TNF-α, MMP-2, MMP-9, and TIMP-1 in A7r5 cells, which was inhibited by Fer-1. Furthermore, CSE increased mRNA level of Ptgs2, lipid peroxidation, and depleted intracellular GSH, all of which are key features of ferroptosis. VSMC ferroptosis was induced by acrolein (ACR) and methyl vinyl ketone (MVK), major cytotoxic constituents of CSE. Furthermore, CSE caused medial VSMC loss in ex-vivo aortas. Electron microscopy analysis showed mitochondrial damage and fragmentation in the medial VSMCs of CSE-treated aortas. All of these manifestations were partially restored by Fer-1. These findings demonstrate that ferroptosis is responsible for CSE-induced VSMC death and suggest that ferroptosis is a potential therapeutic target for preventing AA and AD.

4 考察

Cigarette smoking has a substantial impact on the development of AA and AD. However, the mechanism by which smoking induces AA and AD is not yet fully understood. In the present study, we found that CSE induced a novel form of regulated cell death termed ferroptosis in VSMCs. We observed that CSE-induced cell death was not inhibited by inhibitors of apoptosis or other forms of cell death, such as pyroptosis and necroptosis. On the other hand, CSE-induced cell death was significantly inhibited by DFO, NAC, and DPI, in accordance with the notion that iron-dependent lipid peroxidation plays a critical role in the initiation of ferroptosis.

Moreover, CSE exhausted the intracellular GSH level even before the cellular morphologic changes occur, suggesting that GSH depletion is the primary mechanism involved in the induction of VSMC ferroptosis. The electron microscopic findings showing prominent alterations in mitochondrial morphology also support the idea that ferroptosis is involved in CSE-induced cell death. In this study, we clearly showed that CSE induced VSMC ferroptosis in vitro and caused medial VSMC loss in ex vivo aortas; however, the in vivo effect of CSE on the development of AA or AD remains to be examined. Moreover, molecular mechanisms

of CSE-induced lipid peroxidation and its subcellular location (e.g., plasma membrane and mitochondria) still need to be clarified.

5　結論

In conclusion, we demonstrated that ferroptosis plays an essential role in CSE-induced VSMC cytotoxicity through intracellular GSH depletion in vitro. CSE also induced medial VSMC damage and loss in ex vivo aortas. These findings provide new insights into the mechanism of cigarette smoking-related cytotoxicity and suggest that ferroptosis is a potential therapeutic target for preventing AA and AD.

論文審査の結果の要旨

本学位論文は、「喫煙が大動脈瘤の危険因子である」という事実に着目し、喫煙が大動脈瘤形成の原因となる可能性について検討したもので、１）タバコの煙の抽出物（Cigarette Smoke Extract; CSE）が培養血管平滑筋細胞にフェロトーシスを誘導すること、２）CSE がマウス大動脈リング標本の平滑筋に *ex vivo* で障害をもたらすこと、を明らかにした。フェロトーシスとは、最近明らかとなった細胞死の一形態で、鉄依存性の過酸化脂質の蓄積によって誘導される。申請者は、CSE が平滑筋細胞内の還元型グルタチオンを枯渇させることで脂質の酸化が亢進し、フェロトーシスが誘導されることを示した。実験のデザインも適切で、得られたデータは上記結論を支持するのに十分であった。

CSE の細胞毒性に関する先行研究は多数存在する。CSE が肺胞や気管支上皮細胞に酸化ストレスやアポトーシスを誘導することは知られているが、血管平滑筋細胞にフェロトーシスを誘導するという申請者の知見は新規性が高い。本研究は、必ずしも喫煙が *in vivo* における大動脈瘤形成の原因であることを示したものではないが、喫煙と大動脈瘤の関係を理解する上で重要な手掛かりになるものと評価できる。

原著論文としての完成度は高く、既に *Am J Physiol Heart Circ Physiol* に publish されている。ただし、学位論文としては、実験データだけでなく、グルタチオン代謝や脂質酸化、それらに関わる酵素、鉄の役割、実験で用いたさまざまな inhibitor の作用点、フェロトーシスにつながるメカニズムについての概念図なども提示し、予備知識が少ない読者にも理解できるような工夫があれば、より良いと思われる。

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）フェロトーシスの概念図を introduction に追加すること。

２）大動脈の組織学的構造（内膜、中膜、外膜）の記載は、初出時に the tunica intima (intima), the musculoelastic tunica media (medica), the fibrous tunica adventitia (adventitia)と記載し、以後 intima, medica, adventitia を用いることで統一すること。

３）Reference の書式を統一すること（雑誌名を省略形にするのかしないのか、タイトルを記載するのかしないのか、スペースやカンマ、ピリオド、コロン、セミコロンの使い方など)。

最終試験の結果の要旨

申請者のプレゼンテーションは、まず研究の背景となった大動脈瘤の臨床的特徴、フェロトーシスのメカニズム、タバコの煙の抽出物（CSE）の作成法、およびそれらに関連する既知の知見を整理し、次に既知の知見に基づいて立てた仮説を説明、仮説を検証するための実験とその結果を提示し、最後に実験結果から導かれる結論とその学術的・臨床的意義、今回の研究の限界などを述べ、全体的に非常に良く分かりやすくまとめられていた。

主査や副査の質問に対する回答も適切であった。予想される質問（フェロトーシスはどのような疾患を引き起こすか、喫煙が危険因子とされる疾患の中でフェロトーシスが重要な役割を果たす疾患は何か、など）に対する回答のスライドも用意してあり、十分に準備してプレゼンテーションに臨んでいることがうかがえた。本学位論文のテーマである大動脈瘤、フェロトーシス、喫煙に関連する疾患などについての周辺知識も十分であった。

以上から、申請者の研究能力、科学的素養・態度は学位に値するものと考えられる。