論文審査及び最終試験又は学力の確認の結果の要旨

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学位論文名	Investigating the Regulatory Mechanism of Glucose Metabolism by Ubiquitin-like Protein MNSFβ	
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論文審査の結果の要旨

申請者らは、抗原非特異的抑制因子として発見した monoclonal non-specific suppressor factor β (MNSFβ)が、ユビキチンと 57%の相同性を示すユビキチン類似タンパク質であることから、細胞内でのタンパク質翻訳後修飾機能に着目してきた。近年、MNSFβ が解糖系を抑制することを報告したことから、本研究では、MNSFβ による解糖系制御のメカニズムと MNSFβ のミトコンドリア代謝への影響を検討した。マウスのマクロファージ系細胞株である Raw264.7 において、MNSFβ siRNA によって oxygen consumption rate（OCR）と reactive oxygen species（ROS）産生量が増加し、ATP レベルが有意に減少した。また、ATP 合成酵素のインヒビターである oligomycin を添加した際の ATP レベルは、MNSFβ をノックダウンした細胞で強い減少効果を認めた。がん細胞の代謝制御に重要な転写因子である HIF-1α の発現は、MNSFβ ノックダウンにより減少した。一方、マウスの腹腔マクロファージでは、グルコース消費量、乳酸産生量、ROS 産生量に有意差は認められなかった。これらの結果より、Raw264.7 では、MNSFβ は解糖系とミトコンドリア代謝の両方に影響を与え、MNSFβ の糖代謝制御機構には HIF-1α が関与する可能性が示された。本研究は、MNSFβ のミトコンドリア代謝への影響を明らかにした初めての報告であり、がん細胞において、MNSFβ が HIF-1α を介した糖代謝リプログラミングに関与する可能性を強く示唆する。がんを含む HIF-1α を介した代謝変化を伴う疾患の研究に寄与する基礎的研究であり、ユビキチン類似タンパク質ファミリーの研究に与える影響は大きく、学位授与に値すると判断した。

最終試験又は学力の確認の結果の要旨

申請者らは、マクロファージ系列におけるユビキチン様のタンパク質(MNSFβ)の役割を解析し、解糖系とミトコンドリア代謝の両方を制御することを見出した。MNSFβはHIF-1αの転写活性の制御に関与している。MNSFβの理解は、HIF-1αを介した糖代謝リプログラミングを伴う疾患の解明に繋がる重要な研究であり、本研究は学位授与に値すると判断した。（主査：宮城聡）

申請者は、ユビキチン様タンパク質MNSFβがHIF-1αを介した糖代謝制御において解糖系とミトコンドリア代謝の調節に関与することを明らかにした。ATP合成低下の要因や細胞株特有の代謝特徴を検証し、がんや免疫疾患モデルへの応用可能性を提示した本研究は、学位授与に値すると判断した。（副査：一瀬邦弘）

申請者は、細胞株（RAW264.7）と初代培養細胞（マウスマクロファージ）を用いて、ユビキンチン様タンパク質であるMNSFβによる翻訳後修飾が細胞の代謝制御に関与することを明らかにした。代謝スイッチの制御メカニズムの理解は重要であり、今後、この翻訳後修飾の上流にあるシグナル伝達経路が解明されることで細胞内代謝における新たな制御機構の理解に寄与する可能性があり、本研究は学位授与に値すると判断した。（副査：石原孝也）

（備考）要旨は、それぞれ４００字程度とする。

学 位 論 文 の 要 旨

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論 文 内 容 の 要 旨

INTRODUCTION

Monoclonal nonspecific suppressor factor β (MNSFβ), a member of the ubiquitin-like protein family, is involved in various biological functions, such as cell proliferation, immunological responses, and apoptosis. The C-terminal glycine doublet of ubiquitin, which participates in the isopeptide bond formation during conjugation, is a conserved sequence even in MNSFβ. MNSFβ forms covalent bonds with certain lysines of specific target proteins, including endophilin II and Bcl-G. Unlike ubiquitin, MNSFβ may not be involved in the degradation of targeted proteins. While MNSFβ regulates glycolysis, its effects are related to GLUT1. Hypoxia-inducible factor-1α (HIF-1α) controls GLUT1 expression in multiple cell lines, but whether MNSFβ affects HIF-1α remains unclear.

The heterodimeric transcription factor HIF-1 increases the expression of enzymes implicated in glucose metabolism. HIF-1 comprises two subunits: an oxygen-labile HIF-1α subunit and a constitutively stable HIF-1β subunit. HIF-1α has an oxygen-dependent degradation domain (ODDD) that binds the E3 ubiquitin ligase von Hippel Lindau protein (pVHL). Prolyl hydroxylases (PHDs) hydroxylate the two proline residues within the ODDD, which recruits pVHL, and HIF-1α is ubiquitinated and degraded by the 26S proteasome. In hypoxia, PHD inhibition impedes pVHL binding. This is followed by HIF-1α stabilization, dimerization with HIF-1β, coactivator recruitment, and binding to the promoter region of target genes. The asparaginyl hydroxylase factor-inhibiting HIF-1α (FIH) suppresses HIF-1α transcriptional activity. FIH causes hydroxylation of HIF-1α in the carboxy-terminal transactivation domain

(C-TAD), which prevents HIF-1α binding to transcriptional coactivators, such as CREB-binding protein (CBP)/p300. The histone acetyltransferase CBP/p300 are relevant to HIF-1α/β heterodimer formation. Acetylated CBP/p300 interacts with the C-TAD of HIF-1α to enhance gene expression. Hypoxia, alongside many oncogenic and inflammatory stimuli, promotes HIF-1α activation and accumulation.

In this study, MNSFβ alters both glycolysis and mitochondrial metabolism, markedly affecting glucose metabolism and cytokine production, and HIF-1α may be involved in this regulatory mechanism.

MATERIALS AND METHODS

We used two types of cells, the macrophage-like cell line Raw264.7 and peritoneal macrophages harvested from female BALB/c mice. Peritoneal macrophages were harvested 4 days after intraperitoneal injection of 1.5 ml of sterile 4% Brewer's thioglycollate medium. The cells were collected by centrifugation at 400 ×g for 5 min, washed and seeded. All experiments with animals in this study were approved by the Animal Care and Use Committee of Shimane University.

Cells were transfected with siRNAs for 48 h or cDNAs for 18 h and were used in various experiments. HIF-1α mRNA expression was analyzed by RT-PCR. The DNA binding activity of HIF-1a was evaluated by EMSA. Western blotting was performed to detect HIF-1α, acetyl-p300/CBP, FIH and PHD2 protein expression. Intracellular ROS was evaluated with a ROS Assay Kit -Highly sensitive DCFH-DA- (Dojindo). Intracellular ATP was detected using an ATP Assay Kit-Luminescence (Dojindo). The mitochondrial oxygen consumption rate (OCR) was evaluated using an Extracellular OCR Plate Assay Kit (Dojindo). Glucose and lactate in the supernatant were detected using a glucose assay kit-WST (Dojindo) and glycolysis cell-based assay kit (Cayman). The cytokine expressions in cell culture supernatants were detected using a Proteome Profiler Mouse Cytokine Array Kit, Panel A (R&D Systems).

Statistical significance was analyzed by Student's t-test and expressed as P values. $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

MNSFβ knockdown increased OCR and ROS production in Raw264.7 cells, which showed a markedly decreased ATP level upon oligomycin treatment. In addition, previous studies show that MNSFβ siRNA reduces lactate in the culture supernatant and glucose consumption in Raw264.7 cells, suggesting that MNSFβ knockdown shifts the primary ATP production pathway from glycolysis to OXPHOS.

HIF-1α and autoacetylation activate CBP/p300, which is involved in various gene

expression. MNSFβ siRNA decreased HIF-1α expression and transcriptional activity and acetyl-CBP/p300 expression. The decreased HIF-1α transcriptional activity probably resulted from decreased HIF-1α and acetyl-CBP/p300 expression. It is unlikely that MNSFβ is involved in protein degradation, as previously reported. CBP/p300, which binds to HIF-1α, is a histone acetylase that acetylates DNA, thereby reducing the binding between histones and DNA and recruiting transcription factors to the binding site. Therefore, the decrease in acetyl-CBP/p300 may inhibit the binding of HIF-1α to DNA. Further experiments are needed to determine how MNSFβ affects HIF-1α and acetyl-CBP/p300 expression.

Our evaluation of the effects of MNSFβ on the regulation of glucose metabolism in murine peritoneal macrophages, which are not cancer cells, revealed no changes in lactate secretion, glucose consumption, or ROS production. Notably, HIF-1α expression in peritoneal macrophages remained at a low level in the unstimulated state. These results suggest HIF-1α mediation of these MNSFβ-induced metabolic changes at least in Raw264.7 cells. The MNSFβ knockdown-triggered changes may have been difficult to observe in unstimulated peritoneal macrophages because these cells primarily depend on OXPHOS for ATP production. In macrophages, LPS promotes the glycolytic pathway. The MNSFβ knockdown-triggered reduction in LPS-stimulated HIF-1α protein expression in peritoneal macrophages suggests that MNSFβ can regulate the glycolytic pathway in LPS-stimulated peritoneal macrophages. The present study implicates MNSFβ in glucose metabolism and inflammatory responses. As the effect of MNSFβ on glycolysis and mitochondrial metabolism is a new finding in the regulation of glucose metabolism, MNSFβ can be a potential target for the treatment of diseases, such as cancer, that involve metabolic changes due to HIF-1α activation. MNSFβ also alters cytokine production pattern, suggesting its involvement in inflammatory processes through HIF-1α signaling. This may help in the elucidation of new mechanisms in inflammatory diseases. Detailed molecular mechanisms of how MNSFβ specifically regulates HIF-1α and how this regulation affects metabolic pathways and cytokine productions might require further elucidation. Overall, MNSFβ may be an important ubiquitin-like protein that regulates multiple functions of macrophages.

CONCLUSION

MNSFβ affects both glycolysis and mitochondrial metabolism, suggesting HIF-1α involvement in the MNSFβ-regulated glucose metabolism in Raw264.7 cells.

The study primarily utilizes the Raw264.7 cell line, which may not fully represent the behavior of human macrophages. Thus, caution should be exercised when extrapolating these results to humans.