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석 사 학 위 논 문

A Role of HDAC3 in Obesity-Induced Myosin Light Chain Kinase Expression and Hypertension

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이 논문을 석사학위 논문으로 제출함

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1. Introduction

Obesity is a critical cause of cardiovascular diseases, especially hypertension, becoming a leading cause of mortality worldwide (1). A key contributor to the pathogenesis of hypertension is the aberrant vascular smooth muscle cell (VSM), which plays a critical role in vasoconstriction and arterial remodelling (2).

Recent advances in epigenetic research have provided a deeper understanding of the molecular mechanisms underlying hypertension, shedding light on the regulation of related gene expression in disease progression (3). Among the various epigenetic modulators, histone deacetylases (HDACs) have garnered considerable attention due to their involvement in chromatin remodelling and transcriptional regulation. Histone deacetylase 3 (HDAC3), in particular, has emerged as an epigenetic regulator and target for treatment of various diseases including hypertension (4-6). A recent study reported that HDAC3 is responsible for hypertension development by deacetylating mineralocorticoid receptor (7). A possible role of HDAC3 was also suggested in obesity-induced hypertension via regulating methionine sulfoxide reductase A/hydrogen sulfide/VSM contraction axis (8). However, the mechanism of VSM contraction by HDAC3 remains to be elucidated.

Myosin light chain kinase (MLCK) is an essential enzyme in the regulation of blood pressure under both physiological and pathological conditions. MLCK mediates key signalling pathways for VSM contraction by phosphorylating the regulatory light chain of smooth muscle myosin (MLC₂₀), so the absence of MLCK impairs the

vasoconstriction (9). Previous research has identified that a high-fat diet (HFD) induces vascular hyper-contraction with the upregulation of MLCK (10). Recently, Jung et al. reported that broad-spectrum HDAC inhibitor suppressed HFD-induced protein expression of MLCK in mesenteric artery (MA), and attenuated obesity-induced hypertension in mice (8). Although evidence showed the link between HDAC and HFD-induced vasoconstriction and hypertension, these findings also raise important questions regarding the specific molecular pathways linking HDACs to MLCK regulation: which classes of HDACs are responsible for MLCK regulation, and whether specific HDAC such as HDAC3 could be a therapeutic target for obesity-induced hypertension.

In this study, I aimed to elucidate the role of HDAC3 in HFD-induced MLCK overexpression, increased vasoconstriction and hypertension. I also tested the antihypertensive effect of the HDAC3 inhibitor RGFP966 to provide it as a novel regimen for obesity-induced hypertension.

2. Materials and Methods

2.1. Animal Experiments:

All the animal experiments were conducted according to the guidelines of the National Institutes of Health for the Care and Use of Laboratory Animals. The experimental protocol (KM-2022-34R1) was approved by the Institutional Animal Care and Use Committee at Keimyung University School of Medicine and the study complied with all ethical regulations. Eight-week-old male C57BL/6 mice (Koatech, Gyeonggi-do, Korea) were used in this study. The mice were randomly assigned to receive either normal diet (ND) or HFD, containing 10% or 60% Kcal from fat, respectively (Harlan Laboratories, Madison, WI, USA). When the HFD-fed mice reached a hypertensive phase of over 140 mmHg systolic blood pressure (BP), they were administered RGFP966 (Cayman Chemical, MI, USA) with vehicle (Veh). RGFP966 was used at the dose of 0.5 mg/kg body weight (BW) per day via intraperitoneal injection (i.p.) for 6 consecutive days. Mice were anesthetized with pentobarbital sodium (50 mg/kg BW, i.p.). Organs were frozen in liquid nitrogen and stored at -80 °C until analysis.

2.2. Body Weight and Blood Pressure Measurement:

The BWs of the mice were recorded daily using a scale (AND KOREA, Seoul, Korea). BP of the mice were measured with a noninvasive tail-cuff system (CODA, Kent Scientific Corporation, Torrington, CT, USA) following the owner's manual (11).

2.3. HDAC3 Activity Assay:

HDAC3 activities in the arteries were measured by using a fluorogenic HDAC3 assay kit (BPS Bioscience, San Diego, CA, USA). One μg protein from the MA and aorta lysates were pipetted to a black 96-well plate. Proteins were incubated with or without an HDAC inhibitor (20 μM). The mixture included HDAC substrate, bovine serum albumin (1 mg/mL), and HDAC assay buffer, which was maintained at 37 °C for 30 min. The developer was subsequently added and the mixture was incubated at room temperature for 10 min. Fluorescence intensity was then recorded using a microplate reader (Infinite M200 pro & F200 pro, TECAN Group Ltd., Männedorf, Switzerland) with excitation and emission wavelengths at 380 nm and 460 nm, respectively. The relative HDAC3 activity was calculated and compared with the value of the non-treatment group.

2.4. Primary Culture of Vascular Smooth Muscle Cells and Gene Silencing:

Vascular smooth muscle cells (VSMCs) were collected from aorta of Sprague-Dawley rat. Briefly, the aortic artery was isolated and irrigated with Dulbecco's Modified Eagle Medium (DMEM) (WELGENE, Daegu, Korea). Then, surrounding fatty tissue was removed from the aorta. The endothelial layer was scrapped gently and the de-endothelialized aorta was transferred and cultured in a collagen-coated culture dish with DMEM supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 100 U/mL

streptomycin/penicillin (WELGENE, Daegu, Korea) at 37 °C in a humidified atmosphere containing 5% carbon dioxide. HDAC3 was silenced in VSMCs using the Neon transfection system (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), according to manufactural instructions (12). The cells were treated with 20 nmol/L of rat HDAC3 small interfering RNA (5' -CAUGAUGACCAGAGUUAC-3' , Bioneer, Daejeon, Korea) or 20 nmol/L of scrambled siRNA (Bioneer, Daejeon, Korea).

2.5. Western Blot Analysis:

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis samples were prepared with MA and VSMCs (11). The samples were electrophoresed on 10 - 15% polyacrylamide gels with 0.1% SDS, transferred onto nitrocellulose membranes and subjected to immunoblotting with antibodies. Immune-reactive bands were visualized using a chemiluminescence reagent (Thermo Fisher Scientific, Waltham, MA, USA). The signal intensities bands were quantified using ImageJ (version 1.52a, National Institutes of Health, Bethesda, MD, USA). The primary antibodies used included MLCK (Santa Cruz Biotechnology, Dallas, TX, USA), phosphorylated MLC₂₀ (pMLC₂₀) (Santa Cruz Biotechnology, Dallas, TX, USA), and GAPDH (Novus Biologicals, CO, USA). Horseradish peroxidase-conjugated secondary antibodies against rabbit, mouse Immunoglobulin G (IgG) (Bethyl Laboratories, Montgomery, TX, USA), or goat IgG (Santa Cruz Biotechnology, Dallas, TX, USA) were also used.

2.6. Quantitative Reverse Transcription-Polymerase

Chain Reaction Analysis:

The total RNA of MA, aorta and VMSCs lysates were extracted using NucleoZOL RNA extraction solution (Macherey-nagelTM, Düren, Germany). Subsequently, 1 µg of RNA were used for complementary DNA synthesis using DiaStarTM RT kit (SolGent Co., Daejeon, Korea). Quantitative reverse transcription-polymerase chain reaction was performed using TOPreal SYBR Green qPCR PreMix (Enzynomics, Daejeon, Korea) and the real-time PCR detection system (Bio-Rad CFX, CA, USA) (Table 1).

2.7. Statistical Analysis:

The results were expressed as the mean \pm standard errors (SE). Statistical significance was assessed using Student's *t*-test, and differences between groups were regarded as statistically significant at a *p*-value of < 0.05 . Statistical analyses were conducted using Microsoft Excel 2016.

Table 1. Primers for Quantitative Real-Time Polymerase Chain Reaction

Gene		Sequences	
Mouse 18S rRNA	Forward:	5'-GTAACCCGTTGAACCCCATTT-3'	
	Reverse:	5'-CCATCCAATCGGTAGTAGCG-3'	
Mouse MLCK	Forward:	5'-CCAAGGACCGGATGAAGAAATA-3'	
	Reverse:	5'-CCCTGAGATCATTGCCATAGAG-3'	
Mouse HDAC3	Forward:	5'-TTCGAGTTCTGCTCCCGTTACACA-3'	
	Reverse:	5'-TAGCAGAAGCCAGAGGCCTCAAAT-3'	
Rat 18S rRNA	Forward:	5'-GTAACCCGTTGAACCCCATTT-3'	
	Reverse:	5'-CCATCCAATCGGTAGTAGCG-3'	
Rat MLCK	Forward:	5'-GTCTGCCAACTGGTACTT-3'	
	Reverse:	5'-CTCTGGTCCTTCGTGATAAG-3'	
Rat HDAC3	Forward:	5'-GGCCGCTACTATTGTCTCAA-3'	
	Reverse:	5'-CTGATAGAAGTCCACCACCTG-3'	

HDAC: Histone deacetylase

MLCK: Myosin light chain kinase

3. Results

3.1. High-Fat Diet-Induced Increased Body Weight and Raised Blood Pressure:

Before a differential dietary regimen, BP did not differ between ND and HFD groups. After 20 weeks, systolic BP showed a significant increase in the HFD group (from 113 ± 1 to 151 ± 2 mmHg) ($p < 0.001$, ND vs. HFD), but no significant difference was observed in the ND group (Figure 1A). Likewise, diastolic BP was greatly increased in the HFD group (from 91 ± 2 to 121 ± 3 mmHg) ($p < 0.001$, ND vs. HFD), but remained unchanged in the ND group (Figure 1B). Moreover, whereas the average BW of ND and HFD groups were similar prior to diet, the BW of HFD group markedly raised compared with that of ND group after 20 weeks (ND: 39.35 ± 0.85 g and HFD: 46.95 ± 0.52 g) ($p < 0.001$, ND vs. HFD) (Figure 1C).

3.2. Inhibition of HDAC3 by RGFP966 Ameliorated High-Fat Diet-Induced Hypertension:

Mice were treated with Veh or RGFP966 for 6 days. RGFP966 did not effect the systolic and diastolic BP of ND group, while it significantly reduced systolic and diastolic BP of HFD group (from 153 ± 2 to 115 ± 2 mmHg) ($p < 0.001$, HFD Veh vs. HFD RGFP966) (Figure 2A), (from 123 ± 2 to 92 ± 1 mmHg) ($p < 0.001$, HFD Veh vs. HFD RGFP966) (Figure 2B), respectively. BW was unchanged by RGFP966 administration in both ND and HFD groups (Figure 2C).

3.3. HDAC3 Activities in Mesenteric Artery and Aorta were Increased by High-Fat Diet, Which were Reversed by RGFP966:

I evaluated the effect of RGFP966 treatment on HDAC3 activities in the MA and aorta of the mice. HFD elevated the activity of HDAC3 in MA (1.46 ± 0.05 fold) ($p < 0.001$, ND Veh vs. HFD Veh) (Figure 3A) and aorta (1.13 ± 0.03 fold) ($p < 0.05$, ND Veh vs. HFD Veh) (Figure 3B). RGFP966 treatment reduced HDAC3 activities in both the MA (1.13 ± 0.14 fold) ($p < 0.05$, HFD Veh vs. HFD RGFP966) (Figure 3A), and the aorta (1.03 ± 0.02 fold) ($p < 0.05$, HFD Veh vs. HFD RGFP966) (Figure 3B).

3.4. High-Fat Diet Increased Vasoconstriction, Which were Reversed by RGFP966:

To investigate the underlying mechanism of hypertension induced by HFD, I assessed the vasoconstriction in MA by examining the expression of MLCK and pMLC₂₀. The mRNA levels of MLCK in the MA were greatly enhanced in the HFD group compared with those in the ND group (11.55 ± 5.22 fold) ($p < 0.05$, ND Veh vs. HFD Veh), and this increase was diminished by RGFP966 administration (0.04 ± 0.02 fold) ($p < 0.05$, HFD Veh vs. HFD RGFP966) (Figure 4A). Similarly, the protein levels of MLCK in MA increased in HFD group (4.91 ± 0.53 fold) ($p < 0.01$, ND Veh vs. HFD Veh) and were lowered by RGFP966 (1.34 ± 0.69 fold) ($p < 0.05$, HFD Veh vs. HFD RGFP966) (Figure 4B). RGFP966 reduced protein levels of pMLC₂₀ in MA (0.88 ± 0.05 fold) (p

< 0.01 , HFD Veh vs. HFD RGFP966), which was induced by HFD (1.66 ± 0.07 fold) ($p < 0.05$, ND Veh vs. HFD Veh) (Figure 4C).

3.5. Silencing HDAC3 Decreased Myosin Light Chain Kinase Expression:

To investigate the involvement of HDAC3 in MLCK expression, I knockdowned (KD) HDAC3, and subsequently quantified the expressions of MLCK in the VSMC. The results showed a significant decreasing in MLCK mRNA levels following HDAC3 KD compared with those in control (Con) (0.50 ± 0.05 fold) ($p < 0.05$, Con vs. HDAC3 KD) (Figure 5A&B). Consistently, there were corresponding decreases in MLCK and pMLC₂₀ protein levels in HDAC3 KD cells (0.43 ± 0.03 fold) ($p < 0.05$, Con vs. HDAC3 KD), (0.22 ± 0.05 fold) ($p < 0.01$, Con vs. HDAC3 KD), respectively (Figure 5C-F).

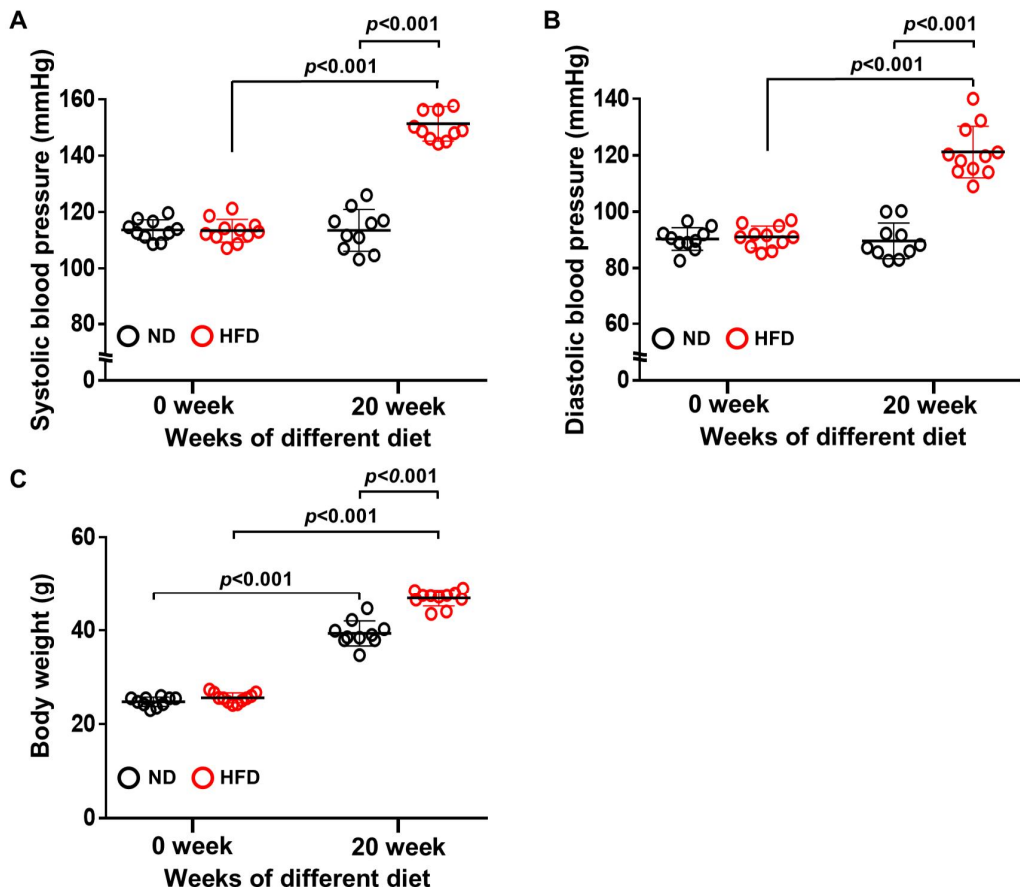


Figure 1. Changes in blood pressure and body weight by high-fat diet. Graphs show systolic blood pressure (A), diastolic blood pressure (B), and body weight (C) values before and after 20 weeks of diet regimens. Results were expressed as the mean \pm standard errors ($n = 10-11$ mice per group). Data were analyzed using the Student's t -test. ND: normal diet; HFD: high-fat diet.

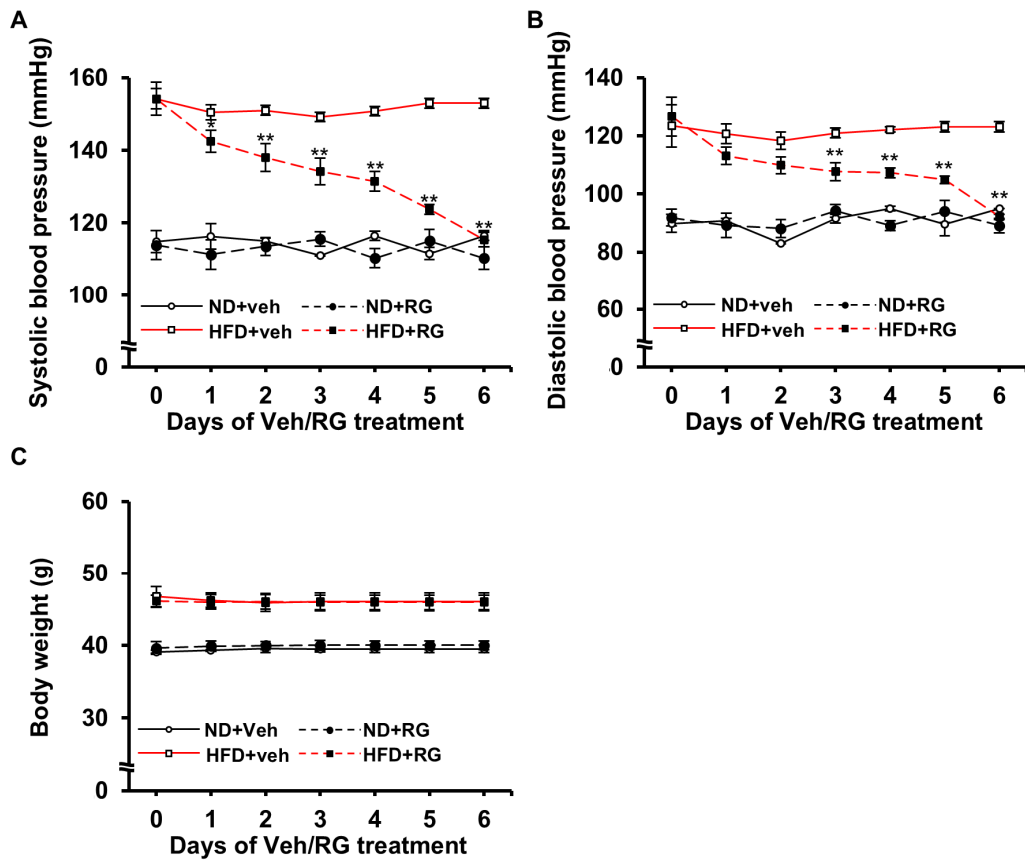


Figure 2. Effects of high-fat diet and RGFP966 on blood pressure. Graphs show systolic blood pressure (A), diastolic blood pressure (B), and body weight (C) during 6 days of vehicle or RGFP966 treatment (0.5 mg/kg body weight per day). Results were expressed as the mean \pm standard errors ($n = 3-5$ mice per group). Data were analyzed using the Student's *t*-test. ND: normal diet; HFD: high-fat diet; Veh: vehicle; RG: RGFP966. *: $p < 0.05$ HFD Veh vs. HFD RGFP966; **: $p < 0.001$ HFD Veh vs. HFD RGFP966.

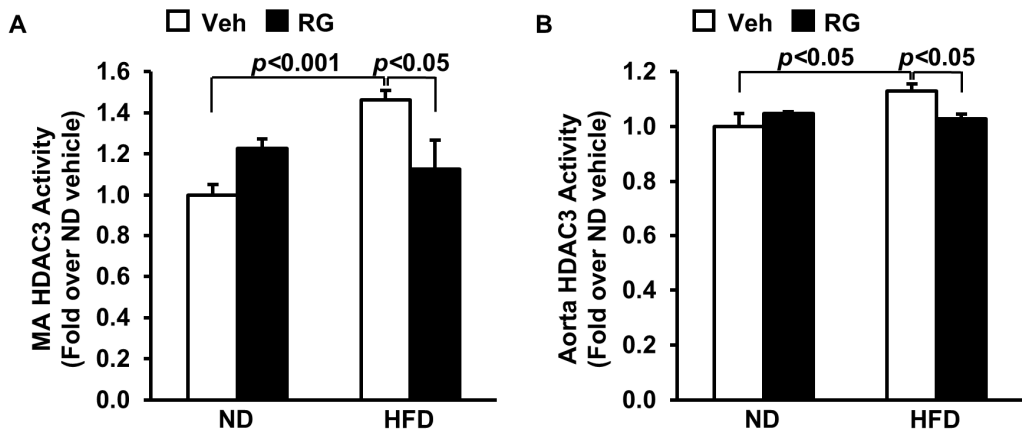


Figure 3. Effects of high-fat diet and RGFP966 on the activities of HDAC3 in mouse mesenteric artery and aorta. HDAC3 activity in mesenteric artery (A) and aorta (B) of ND- or HFD-fed mouse after vehicle or RGFP966 treatment. Results were expressed as the mean \pm standard errors (n = 3 - 4 mice per group). Data were analyzed using the Student's *t*-test. ND: normal diet; HFD: high-fat diet; Veh: vehicle; RG: RGFP966; HDAC: histone deacetylase; MA: mesenteric artery.

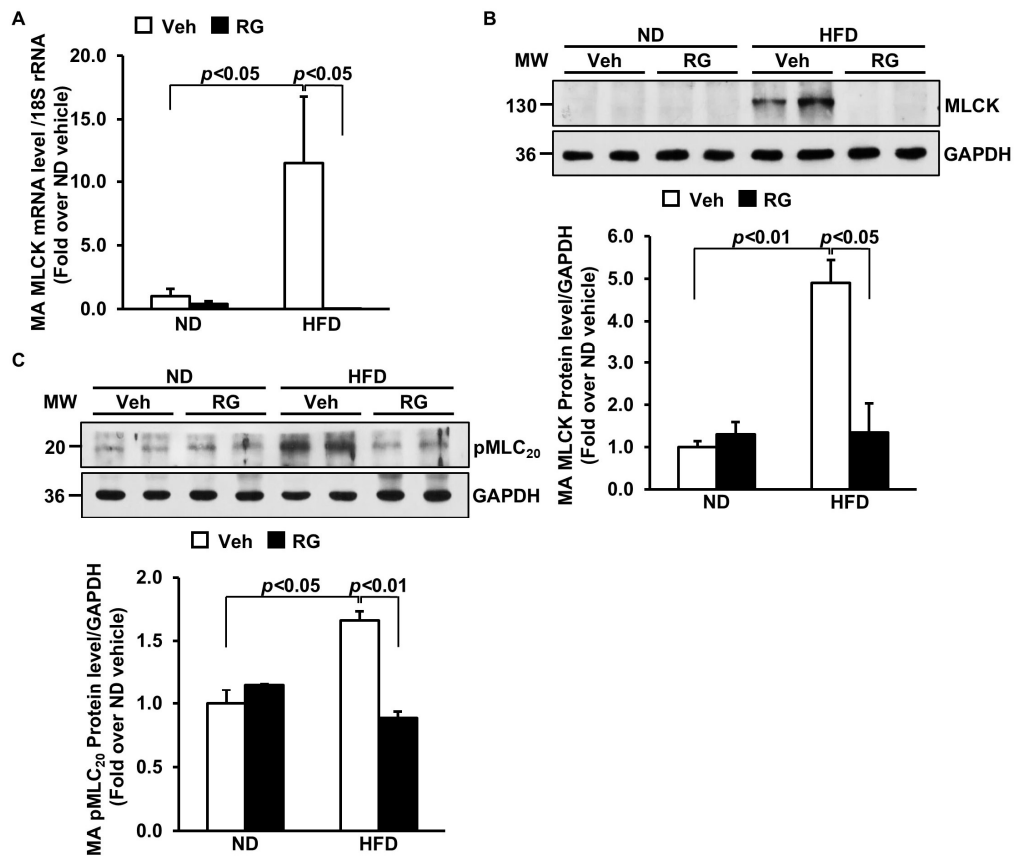


Figure 4. Effects of RGFP966 on high-fat diet-induced vasoconstriction in mouse mesenteric artery. Graphs summarize the mRNA, protein levels with representative western blots for MLCK (A&B) and protein levels with representative western blots for pMLC₂₀ (C) in mouse mesenteric artery of ND- or HFD-fed mouse following vehicle or RGFP966 treatment. Results were expressed as the mean \pm standard errors ($n = 3 - 4$ mice per each group). Data were analyzed using the Student's t -test. ND: normal diet; HFD: high-fat diet; Veh: vehicle; RG: RGFP966; HDAC: histone deacetylase; MA: mesenteric artery; MLCK: myosin light chain kinase; pMLC₂₀: phosphorylation of myosin light chain 20.

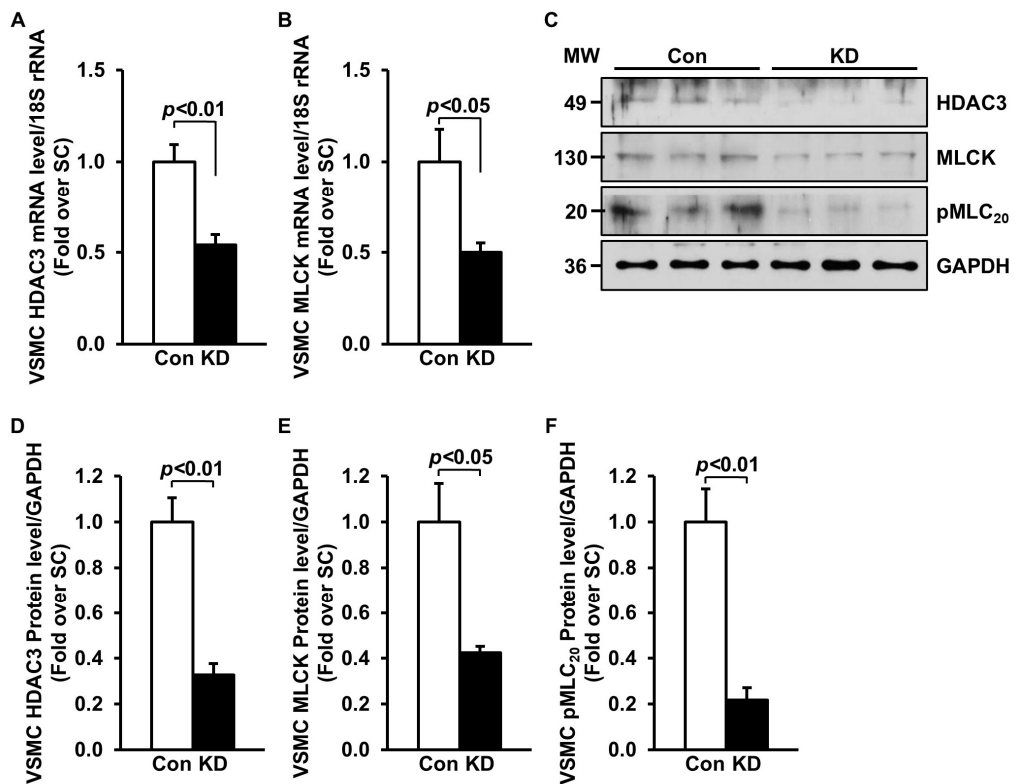


Figure 5. Effects of HDAC3 knock down on myosin light chain kinase expression and vascular smooth muscle contraction. Graphs show the mRNA level of HDAC3 and MLCK of HDAC3 knockdown in VSMC (A&B). Representative pictures of western blot for HDAC3, MLCK, pMLC₂₀ and internal control GAPDH (C). Graphs summarize the protein levels of HDAC3 (D), MLCK (E), pMLC₂₀ (F), respectively. Results were expressed as the mean \pm standard errors ($n = 3$ mice per group). Data were analyzed using the Student's *t*-test. Con: control; KD: knockdown; HDAC: histone deacetylase; VSMC: vascular smooth muscle cell; MLCK: myosin light chain kinase; pMLC₂₀: phosphorylation of myosin light chain 20.

4. Discussion

This study revealed the novel role of HDAC3 in obesity-induced hypertension, through the upregulation of MLCK gene expression. The HDAC3 inhibitor RGFP966 administration significantly inhibited both obesity-induced hypertension and increase in MLCK mRNA and protein levels. Furthermore, targeted knockdown of the HDAC3 gene in primary VSMC culture resulted in a notable reduction in MLCK expression. These findings suggest the possible repurposing of RGFP966 as a therapeutic option for obesity-induced hypertension.

Smooth muscle MLCK, responsible for MLC_{20} phosphorylation, is an essential enzyme for smooth muscle contraction and vascular tone increase (13,14). Consequently, MLCK plays a critical role in the pathogenesis of hypertension. Numerous studies have reported increased levels of both MLCK mRNA and protein across various hypertension models, including spontaneously hypertensive rats, angiotensin II-treated hypertensive mice, and HFD-treated hypertensive mice (10,15). In addition, aiming to reduce MLCK expression, whether through inhibitors or gene deletion, has shown a promising effect on managing hypertension. He et al. indicated that attenuation of MLCK was able to abolish deoxycorticosterone acetate salt-induced hypertension (13). Other research also showed that MLCK inhibitor decreased rat pulmonary hypertension (16). Moreover, it has also been reported that pan-HDAC inhibitor ameliorated HFD-induced hypertension through downregulation of MLCK (8). Consistent with these findings, this study revealed that hyper-activated HDAC3 in the arteries increased MLCK, resulting in increased phosphorylation of MLC_{20} .

The dose of RGFP966 I used here corresponds to 1.4-5% of the dose

used by other researchers for diabetes and cancer (17,18). However, it was sufficient to normalize both systolic and diastolic BP to normal levels. These data underscore the potential of RGFP966 as an innovative antihypertensive therapeutic regimen.

In conclusion, this study not only highlights the regulatory role of HDAC3 on MLCK, but also opens a promising avenue for therapeutic interventions targeting obesity-induced hypertension. Further investigations on the link between HDAC3 and MLCK expression may provide a novel target and strategy.

5. Summary

This study emphasizes the critical role of HDAC3 in facilitating hypertension induced by HFD via its regulation of MLCK gene expression. The HDAC3 inhibitor RGFP966 administration markedly attenuated both obesity-induced hypertension and the corresponding elevations in MLCK mRNA and protein levels. Furthermore, selective knockdown of HDAC3 in VSMC significantly suppressed MLCK expression. These results highlight HDAC3's regulatory impact on MLCK and its potential as a promising therapeutic target for managing obesity-induced hypertension.

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A Role of HDAC3 in Obesity-Induced Myosin Light Chain Kinase Expression and Hypertension

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(Abstract)

Obesity and its associated hypertension are becoming a crucial intimidation to global health. Myosin light chain kinase (MLCK) has been identified to play a vital role in vascular smooth muscle contraction and blood pressure regulation. Previous studies have reported a potential association between histone deacetylase (HDAC) activity and the upregulation of MLCK in high-fat diet (HFD)-induced hypertension. In this study, I investigated the roles of HDAC3 in obesity-induced hypertension using a selective HDAC3 inhibitor RGFP966 and small interfering RNA, to elucidate a pathological signalling axis between HDAC3 and MLCK expression. Nine-week-old male C57BL/6 mice were fed either a normal diet or HFD for 20 weeks. When HFD-fed group reached the hypertensive phase, each group of mice was intraperitoneally

injected with vehicle or RGFP966 (0.5 mg/kg/day) for 6 days. HFD induced hypertension with increased HDAC3 activity in the arteries. RGFP966 treatment effectively reversed HFD-induced hypertension through inhibited vascular smooth muscle contraction by suppressing the increases in MLCK mRNA and protein levels. Silencing of HDAC3 in vascular smooth muscle cells led to a reduction in MLCK expression. These findings suggest that HDAC3 plays a critical role in MLCK gene upregulation and that targeting HDAC3 could be a promising therapeutic approach for obesity-induced hypertension.

비만으로 인한 Myosin Light Chain Kinase 발현 및 고혈압 발생에서 HDAC3의 역할

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(지도교수 김지인)

(초록)

비만성 고혈압은 전 세계적으로 심각한 건강문제로 부각되고 있습니다. 미오신 경쇄 키나아제(MLCK)는 혈관 평활근 수축과 혈압 조절에 중요한 역할을 하는 것으로 알려져 있으며, 고지질 식이에 의한 비만성 고혈압에서 히스톤 탈아세틸화효소(HDAC) 활성과 MLCK 상향 조절 사이의 잠재적 연관성이 보고된 바 있습니다. 이에 본 연구자는 선택적 HDAC3 억제제 RGFP966을 사용하여 고지질 식이에 의한 비만성 고혈압에서 HDAC3의 역할과 HDAC3와 MLCK 발현 사이의 병리학적 기전을 조사하였습니다. 본 실험에서는 생후 9주 된 수컷 C57BL/6 쥐에 20주 동안 정상 식이 또는 고지질 식이를 제공하였으며, 고지질 식이 그룹이 고혈압 단계에 도달했을 때 각 그룹의 쥐에게 6일 동안 RGFP966(체중 kg 당 0.5 mg) 또는 생리식염수를 복강 내 주사로 하루에 한 번 투여하였습니다. 20주 동안의 고지질 식이는 동맥에서 HDAC3의 활성을 증가시키고 MLCK를 증가시켜 혈관수축

증가와 함께 고혈압을 유발하였습니다. RGFP966 처리는 고지질 식이에 의한 고혈압을 효과적으로 치료하였으며, MLCK mRNA 및 단백질 발현 증가를 억제함으로써 혈관 평활근 수축을 억제하였습니다. 추가적으로, 일차 배양한 혈관 평활근 세포에서 HDAC3 siRNA 처리를 통한 HDAC3의 발현 억제는 MLCK 발현을 감소시켰습니다. 이 결과들은 HDAC3가 MLCK 유전자 상향 조절과 고혈압에 중요한 역할을 하며 HDAC3를 표적으로 삼는 것이 고지질 식이로 인한 비만성 고혈압에 대한 효과적인 치료 전략이 될 수 있음을 시사합니다.