主論文の要旨

Inhibition of cyclooxygenase-1 by nonsteroidal anti-inflammatory drugs demethylates MeR2 enhancer and promotes *Mbnl1* transcription in myogenic cells

非ステロイド性抗炎症薬によるシクロオキシゲナーゼ-1 の抑制は、
MeR2 エンハンサーの脱メチル化を介して、
筋原細胞での *Mbnl1* 転写を促進する

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【Background】

Muscleblind-like (MBNL) is a multifunctional RNA binding protein that modulates diverse RNA metabolisms, including splicing, polyadenylation, stability, and localization of mRNA. MBNL1 is highly expressed in skeletal muscle, and abnormally expanded CUG-repeats in the *DMPK* gene cause myotonic dystrophy type 1 (DM1) by sequestration of MBNL1 to nuclear RNA foci. Downregulation of MBNL1 availability leads to aberrant regulation of alternative splicing of hundreds of genes, which causes various manifestations of DM1.

Several therapeutic strategies for DM1 are currently under investigation. Induction of MBNL1 expression is one of the promising therapies for DM1. Indeed, overexpression of *Mbnl1* rescues disease-associated muscle hyperexcitability and myotonia in a transgenic mouse model of DM1. In addition, we previously reported that phenylbutazone, a non-selective nonsteroidal anti-inflammatory drug (NSAID), ameliorates muscle weakness and muscle pathology by enhancement of MBNL1 expression. Phenylbutazone augments transcription of *Mbnl1* mRNA by demethylation of MeR2 (the methylated region 2), an enhancer element in *Mbnl1* intron 1.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for inflammatory diseases including neurodegenerative and neuromuscular diseases. NSAIDs inhibit cyclooxygenase (COX) that catalyzes the conversion of arachidonic acid to prostanoids. There are two COX isoforms: COX-1 is constitutively expressed in most tissues, and COX-2 is inducibly expressed in response to inflammation. Selective inhibitors for each COX isoform have been developed, although most classical NSAIDs inhibit both COX isoforms.

【Methods】

C2C12 cells were incubated with 100 μM each of 29 NSAIDs (Prestwick Chemical Library) for 24 hours, and *Mbnl1* mRNA and MBNL1 protein levels were quantified by real-time RT-PCR and Western blot analysis, respectively. For knocking down COX-1 or COX-2, siRNAs were introduced into these cells using Lipofectamine RNAiMax (ThermoFisher). Luciferase vectors were introduced into C2C12 myoblasts using Lipofectamine 3000 (ThermoFisher), and luciferase assay was performed using the Dual Luciferase Reporter Assay System kit (Promega). To generate the MeR2-knockout (MeR2-KO) C2C12 cell line, MeR2 region was deleted by CRISPR/Cas9 system using two single-guide RNAs (sgRNAs) targeting the upstream and downstream sites of MeR2.

【Results】

We first screened 29 NSAIDs to examine a class effect of NSAIDs on enhancement of *Mbnl1* expression. Our screening identified 13 drugs that increased *Mbnl1* mRNA, in which four out of six COX-1-selective NSAIDs and two out of seven COX-2-selective

NSAIDs increased *Mbnl1* mRNA (Fig. 1). Wilcoxon rank sum test showed that COX-1-selective NSAIDs increased *Mbnl1* mRNA more than COX-2-selective NSAIDs (p = 0.022). These results suggest that a substantial number of NSAIDs can increase MBNL1 expression, in which COX-1-mediated pathway plays a pivotal role.

To dissect the regulation of MBNL1 expression through COX-mediated pathways, we knocked down COX-1 and COX-2 (Fig. 2a-d), and examined *Mbnl1* mRNA levels and MBNL1 protein levels in C2C12 cells. We found that knockdown of COX-1 upregulated expression of *Mbnl1* mRNA while knockdown of COX-2 downregulated *Mbnl1* mRNA in C2C12 cells (Fig. 2e, f). Western blotting analysis also showed elevation of MBNL1 protein expression in COX-1-knocked down cells (Fig. 2g, h). Similar results were observed in myotubes differentiated from DM1 patient-derived iPSCs (induced pluripotent stem cells) (Fig. 3). These suggest that MBNL1 expression is suppressed through the COX-1-mediated pathway in myogenic cells.

To examine whether NSAIDs promote MBNL1 expression by upregulating the activity of the MeR2 enhancer, we inserted the MeR2 enhancer upstream of the SV40 promoter and the firefly luciferase cDNA (pGL3P-MeR2; Fig. 4a, b). C2C12 myoblasts were introduced with pGL3P-MeR2 or pGL3P, and were treated with one of the 29 NSAIDs at 100 μM for 24 h. Luciferase assay revealed that 10 out of the 29 NSAIDs significantly increased the luciferase activity of pGL3P-MeR2 (Fig. 4c), while no drugs affected that of pGL3P (Fig. 4e). Furthermore, fold inductions of *Mbnl1* mRNA expression by NSAIDs (Fig. 1) were correlated well with those of luciferase activity of pGL3P-MeR2 (Fig. 4d), but not those of pGL3P vector (Fig. 4f). In addition, knockdown of COX-1, but not of COX-2, upregulated the luciferase activity of pGL3P-MeR2 (Fig. 4g, h). These results suggest that the MeR2 enhancer is suppressed through the COX-1-mediated pathway.

To investigate the role of the MeR2 enhancer more directly, we deleted the genomic MeR2 region in C2C12 myoblasts by CRISPR/Cas9 system (Fig. 5a). We obtained one MeR2-knockout C2C12 cell line (MeR2-KO C2C12), where MeR2 was deleted in both alleles (Fig. 5b). We confirmed that knockdown of COX-1 had no effect on *Mbnl1* mRNA expression in MeR2-KO C2C12, but upregulated *Mbnl1* mRNA expression in the control cell line (WT C2C12), which retained MeR2 (Fig. 5c). Thus, COX-1 requires the MeR2 enhancer to suppress *Mbnl1* mRNA expression.

Next, we analyzed COX-1-dependent regulation of MeR2-methylation in C2C12 myoblasts using bisulfite sequencing. As expected, knockdown of COX-1 (Fig. 6), but not of COX-2, significantly suppressed methylation of MeR2 in C2C12 cells. These results suggest that COX-1 inhibition suppresses methylation of MeR2 and enhances *Mbnl1* transcription in C2C12 myoblasts.

Finally, we investigated the possible enzymes involved in the regulation of DNA methylation in COX-1-knocked down myogenic cells. Methyltransferase 3α (DNMT3a),

which is necessary for *de novo* DNA methylation, and ten-eleven translocation (TET) family proteins, which promote DNA demethylation through oxidation of 5-methylcytosine, play important role in DNA methylation. We observed that knockdown of COX-1, but not of COX-2, upregulated mRNA expressions of all three members of TET (*Tet1, Tet2, and Tet3*). In contrast, knockdown of neither COX-1 nor COX-2 affected *Dnmt3a* mRNA expression (Fig. 7). These suggest a role of TET in the COX-1-dependent suppression of MeR2-methylation in C2C12 cells.

【Discussion】

Enhancement of MBNL1 expression is one of the attractive therapeutic modalities for DM1. Our study shows that nearly a half of the screened NSAIDs enhanced *Mbnl1* expression in myogenic cells, suggesting possible application of NSAIDs for DM1 patients. Indeed, phenylbutazone, a non-selective COX inhibitor, ameliorates muscle weakness and muscle pathology in DM1 mouse model. Previous researches have shown that the beneficial effects of NSAIDs on skeletal muscles are not limited to DM1. Chronic administration of NSAIDs, such as ibuprofen and acetaminophen, increases muscle volume and muscle strength in healthy aged adults. Furthermore, several NSAIDs have been reported to prevent muscle damage and preserve the number and function of muscle cells in a mouse model of Duchenne muscular dystrophy. Although further analyses are required, we expect that COX-1-selective NSAIDs and possibly their derivatives will become one of therapeutic options for DM1 patients.

【Conclusion】

I have shown that *Mbnl1* transcription is suppressed by COX-1-mediated pathway, and propose that COX-1-selective NSAIDs are potential drugs for DM1 patients.