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研究課題名（和文）Vitamin D - vitamin D receptor axis affects muscle differentiation and improves muscle regeneration

研究課題名（英文）Vitamin D - vitamin D receptor axis affects muscle differentiation and improves muscle regeneration

研究代表者

ＭＡＲＩＡ　ＴＳＯＵＭＰＲＡ（TSOUMPRA, MARIA）

国立研究開発法人国立精神・神経医療研究センター・神経研究所　遺伝子疾患治療研究部・科研費研究員

研究者番号：８０７５６１９８

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研究成果の概要（和文）：1,25-水酸化ビタミンD3(VD3)は、核内受容体のビタミンD受容体(VDR)に結合し、標的遺伝子のvitamin D response element: VDREに結合することにより遺伝子発現を調節する。
本研究では、ジストロフィン類似蛋白であるアルファジストロブレビン(Dtna)発現調節におけるVD3/VDR系の作用を解明した。正常および筋ジス細胞において、VD3はDtnaやVDR遺伝子、蛋白の発現を促進した。部位特異的変異誘発とクロマチン免疫沈降法により、マウスDtna遺伝子筋肉特異的プロモーターにVDREを同定し、VD3がVDR依存性に本プロモーター活性を促進することを見出した。

研究成果の学術的意義や社会的意義
VD3投与により、マウスの骨格筋細胞の筋原性因子の転写調節とともにVDRの発現亢進がおこることから、VD3は筋肉に有益な作用を有すると想定される。これまでにもVD3の筋肉に対する作用は報告されていたものの、その機序や標的遺伝子には不明な点が残されていた。本研究により、VDR依存性のDtna発現調節機構が明らかとなった。今後Dtnaの筋肉における詳細な機能や作用機序を明らかにすることにより、本成果は骨格筋疾患においてジストロフィン結合蛋白複合体を安定化させることを目的とする治療の開発に役立つ可能性がある

研究成果の概要（英文）：The active metabolite of vitamin D, 1,25-dihydroxyvitamin D3 (VD3), exerts its tissue-specific actions through binding to its intracellular vitamin D receptor (VDR) which functions as a heterodimer with retinoid X receptor (RXR) to recognize vitamin D response elements (VDRE) and activate target genes. We have elucidated the regulatory role of VD3/VDR axis on the expression of dystrobrevin alpha (DTNA), a protein homologue of dystrophin. In healthy and dystrophic murine-derived myogenic cells, Dtna and Vdr gene and protein expression were upregulated by VD3 in presence of VDR/RXR only. Through site-directed mutagenesis and chromatin immunoprecipitation assays, we validated a VDRE site in Dtna murine muscle-specific promoter and proved that the positive regulation of Dtna by VD3 is VDR-mediated and specific. Our study reveals a novel mechanism of VDR-mediated regulation for Dtna, which may be positively explored in treatments aiming to stabilize dystrophin in musculoskeletal diseases.

研究分野：Endocrinology

キーワード：vitamin D　vitamin D receptor　dystrobrevin　dystrophin　muscular dystrophy

１．研究開始当初の背景
（Background）

The biologically active metabolite of vitamin D, 1-alpha -25-dihydroxyvitamin D3 (VD3), is a nuclear hormone that exerts tissue-specific pleiotropic effects mainly by binding to its intracellular vitamin D receptor (VDR) (Carlberg and Polly, 1998). Subsequently, VDR dimerizes with a class of retinoid X receptor (RXR) and the VDR-RXR heterodimer can translocate to the nucleus to recognize promoter localized-vitamin D response elements (VDRE) and either activate (most commonly) or suppress target gene transcription (Dilworth and Chambon 2003; Glenville et al. 1998; Umesono et al. 1991). Solid evidence towards the VD3/VDR axis interference in transcriptional downregulation of key myogenic factors during the murine skeletal muscle development has been earlier reported by our Tokushima university collaborators (Endo *et al.* 2003), whereas more recent studies documented that a complete (Girgis et al. 2015) or myocytic-specific (Girgis et al. 2019) VDR ablation in mice accounts for the observed reduced grip strength. In both VDR ablation cases, the muscle-related changes mimicked an atrophic phenotype and were attributed to a reduced expression in genes associated with calcium handling channels. It is thus highly probable that the literature-documented VDR-mediated muscle-specific effects were generated by homeostatic dysregulation and aberrant signaling that was originated in tissues other than muscle or even by non-genomic mechanisms related to the disrupted calcium uptake and thus are not a direct effect of muscle VDR ablation *per se*. The identification of VDR expression in muscle tissue hints presence of a direct pathway for VD3 to impact on skeletal muscle-related genes and muscle function that is yet to be discovered.
In muscle, presence of a large transmembrane complex, termed dystrophin associated protein complex (DAPC), maintains the integrity of muscle membrane and facilitates signal propagation and muscle force generation and transduction (Constantin 2014). Mutations in the gene that encodes for the key core protein of DAPC, dystrophin, lead to destabilization of DAPC in Duchenne muscular dystrophy (DMD) or Becker muscular dystrophy (BMD) patients (Straub and Campbell, 1997; Hoffman *et al.* 1987). Dystrobrevin alpha (Dtna) is a homologue of dystrophin and is strongly associated with the C terminus of dystrophin through a specific coiled-coil motif interaction (Sadoulet-Puccio et al. 1997) whereas its N-terminal domain directly interacts with sarcoglycan and sarcospan subcomplex in the DAPC (Yoshida et al. 2000). Dtna levels are significantly reduced in muscle specimens obtained from *mdx* mice (a commonly used murine DMD model) (Blake 2002) or mice lacking the sarcoglycan complex (Strakova *et al.* 2014), as well as in muscle of DMD human patients and limb-girdle muscular dystrophy patients with mutations in sarcoglycan components. We can thus deduce that absence of Dtna may contribute to the pathogenesis in muscular dystrophies, through disruption of DAPC signalling. However the exact function and mode of transcriptional regulation of Dtna in muscle is not fully elucidated.

２．研究の目的
We aimed to discover muscle-specific genes that are positively regulated by VD3/VDR axis. We focused our research on genes that are members of the DAPC, because a potential fortification of DAPC in the absence of dystrophin by VD3 administration may aid the stabilization of this complex in DMD patients. We were also keen to discover whether the VD3/VDR axis is actively participating in the modulation of the expression of fast (type II) or slow (type I) fiber markers during the *in vitro* myogenic differentiation. Because the presence of fast fibers is associated with higher ability of individuals to quickly adjust to posture perturbations (Paillard 2017), their abundance may be beneficial for the frail and elder population, whereas an increase in slow fibers has therapeutic benefits in dystrophic patients (Boyer *et al.* 2019). Herein we have shown that the VD3/VDR axis positively regulates gene and protein expression of Dtna gene in healthy and dystrophic cells.

３．研究の方法

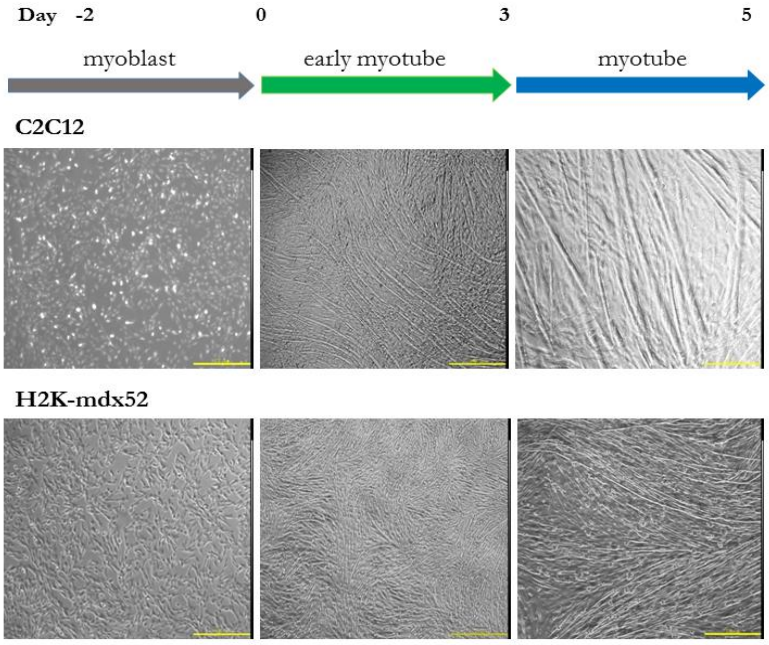
We have induced *in vitro* myogenic differentiation by switching cell cultures from the full (10 % BSA supplemented) medium to low horse serum medium in two different murine myogenic cell lines: the C2C12, a well-established in *vitro* model for studying myogenic differentiation (Yaffe & Saxel 1977; Burattini *et al.* 2004) and the H2K-mdx52 that are immortalized cells derived from the humanized *mdx52* mouse model (Araki *et al.* 1997). We first assessed the Dtna and Vdr gene and protein modulation by VD3 administration at the three distinct phases of myogenic differentiation (myoblasts, early myotubes and myotubes) using real-time quantitative PCR analysis (RT-qPCR) and western blot analysis respectively. We further validated the VD3-VDR axis induced upregulation of Dtna by *Dtna1*, *MyoD* and *Vdr* silencing in C2C12 cells or VDR-RXR overexpression in H2K-mdx52 cells, that possess low levels of VDR. We have cloned the three murine individual Dtna promoters as described in Holzfeind *et al.* 1999 into the promotless pGL4 vector and identified the muscle-specific (Dtna-M), versus neuronal/brain specific (Dtna-B) promoter via dual luciferase assays performed in C2C12 cells or neuro-2A cells. In order to validate the functionality of the multiple *in silico* identified putative VDRE binding sites in Dtna-M we have generated truncations of the full-length promoter or point mutations in putative VDREs. Finally, we have confirmed the functionality of our newly identified VDRE by ChIP assay using primers to amplify a 100 bp fragment of Dtna-M promoter encompassing the (-1474/-1461) VDRE region and three different VD3 concentrations.

４．研究成果

(1) Successful induction of myogenic differentiation in C2C12 and H2K mdx52 cells.

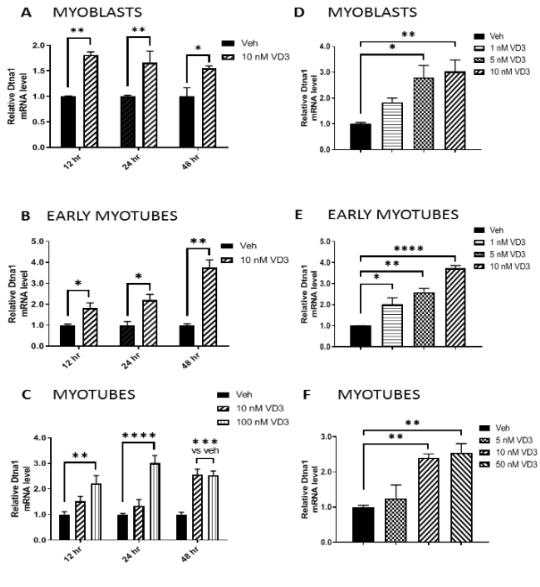
Upper panel: C2C12 differentiation in the presence of 1.5 % HS medium. Lower panel: H2K-mdx52 cell differentiation in the presence of 2.5 % HS medium. In both cases, cells were placed in DM when culture reached 80 % of confluence (day 0). After 48 hrs, early myotubes were visible and on day 5 mature myotubes were visible in the culture. Scale bar 200 μm

F1: Images of C2C12 and H2K-mdx52 cells obtained at the three phases of myogenic differentiation.

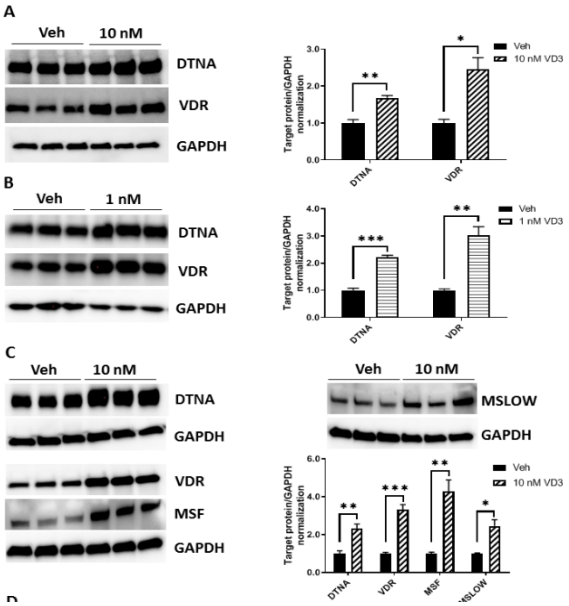


(2) VD3 increases Dtna1 gene and protein levels during different stages of C2C12 myogenic differentiation. We demonstrated that mRNA levels of endogenous Dtna1 were significantly upregulated upon administration of 10 nM VD3 in myoblasts (1A) and early myotubes (1B) or 100 nM VD3 in myotubes (1C) at all intervals examined. A VD3-dose dependent upregulation of Dtna1 was ascertained for all three phases examined (1D-F). Western blot analysis in C2C12 myoblasts (2A) and early myotubes (2B) demonstrated significant upregulation of DTNA and VDR, whereas in myotubes upregulation of DTNA, VDR, myosin skeletal fast (MSF) and Myosin Slow (MSLOW) protein markers was verified (2C).

F2: RT-qPCR analysis



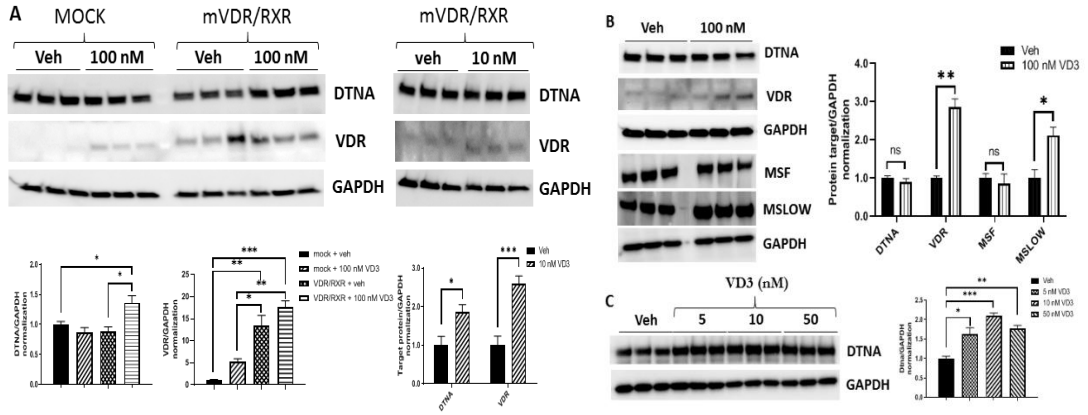
F3: Western blot analysis



(3) Dtna response to VD3 is dependent on VDR expression levels in H2K-mdx52 myogenic cells

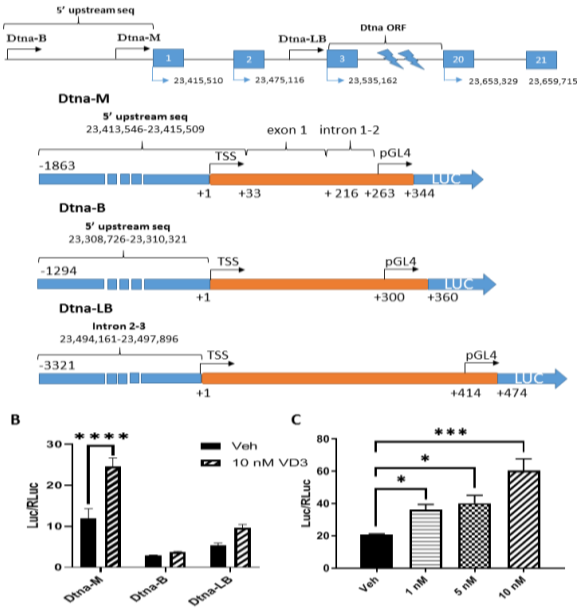
In H2K-mdx52 early myotubes, low levels of VDR expression do not permit elevation of DTNA upon 100 nM of VD3 administration, however co-transfection of VDR/RXR expression plasmids raised DTNA levels at 10 nM and 100 nM of VD3 administration (4A). In H2K-mdx52 myotubes, administration of 100 nM VD3 raised VDR and MSLOW expression levels but not DTNA and MSF levels (4B).

F4: Dtna response to VD3 is dependent on VDR expression levels in H2K-mdx52 myogenic cells

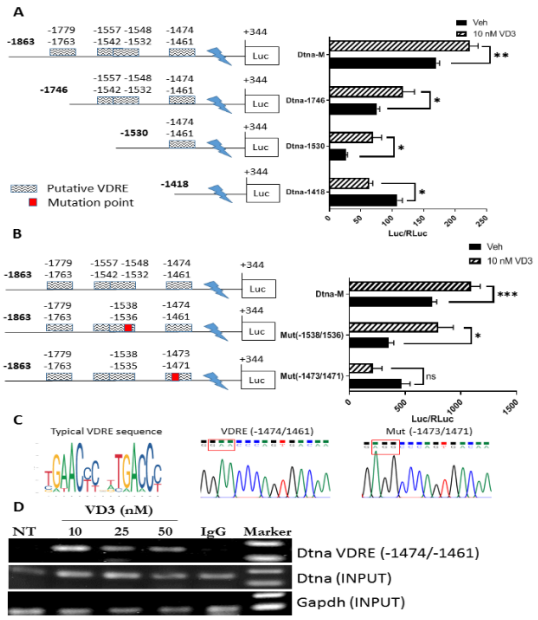


(4) Dtna response to VD3 is dependent on VDR expression levels in H2K-mdx52 myogenic cells. We have cloned three Dtna promoters, named Dtna-M, Dtna-B and Dtna-LB, located in chromosome 18 into the pGL4 vector as shown in F5A (Exons are depicted as blue squares. TSS: transcription start site, ORF: open reading frame) and ascertained that Dtna-M only is induced by VD3 expression in C2C12 cells (F5B) and this induction is dose dependent (F5C). Truncations of Dtna-M promoter indicates loss of Dtna-M VD3-dependent induction for the shortest Dtna-1418 construct only (F6A). abolishment of Dtna-M –VD3 induction in Mut (-1473/-1471) only, confirms the functionality of (-1474/1461) VDRE (6B) whereas sequencing analysis confirmed disruption of the hexameric pattern in Mut (-1473/-1471) (6C). CHIP-PCR amplification demonstrates binding of VDR to the VDRE region (6D)

F5 Generation of muscle specific Dtna promoter



F6 Identification of a functional VDRE in Dtna-M promoter



（5）まとめ

Herein, we have identified Dtna, a homologue of dystrophin and a key component of DAPC, as a gene induced explicitly by VD3 and confirmed that this induction is VDR-mediated and specific. Administration of low doses of VD3 during individual phases of C2C12 myogenic differentiation upregulated Dtna and Vdr gene and protein expression. Experiments in H2K-mdx52 cells indicated that DTNA upregulation by VD3 can occur in the absence of dystrophin but only in elevated VDR/RXR levels. Through site-directed mutagenesis and ChIP assays we have validated a VDRE site in muscle-specific Dtna promoter in myogenic cells. Furthermore, we have demonstrated that at least in vitro, the fast fiber marker (MSF) upregulation by VD3 in myotubes was dependent on higher levels of VDR, whereas the slow fiber marker upregulation (MSLOW) occurred in lower VDR expression levels. The current study reveals a novel mechanism of VDR-mediated regulation for Dtna, which may be positively explored in treatments aiming to stabilize the DAPC in musculoskeletal diseases.

５．主な発表論文等

〔雑誌論文〕　計2件（うち査読付論文　2件／うち国際共著　2件／うちオープンアクセス　2件）

１．著者名 Shouta Miyatake, Yoshitaka Mizobe, Maria K. Tsoumpra, Kenji Rowel Q. Lim, Yuko Hara, Fazel Shabanpoor, Toshifumi Yokota, Shin'ichi Takeda, Yoshitsugu Aoki	４．巻 14
２．論文標題 Scavenger Receptor Class A1 Mediates Uptake of Morpholino Antisense Oligonucleotide into Dystrophic Skeletal Muscle	５．発行年 2019年
３．雑誌名 Molecular Therapy Nucleic Acids	６．最初と最後の頁 520-535
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.omtn.2019.01.008	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

１．著者名 Yuichi Takashi, Hidetaka Kosako, Shun Sawatsubashi, Yuka Kinoshita, Nobuaki Ito, Maria K. Tsoumpra, Masaomi Nangaku, Masahiro Abe, Munehide Matsuhisa, Shigeaki Kato, Toshio Matsumoto, Seiji Fukumoto	４．巻 May 2019-online
２．論文標題 Activation of unliganded FGF receptor by extracellular phosphate potentiates proteolytic protection of FGF23 by its O-glycosylation	５．発行年 2019年
３．雑誌名 Proceedings of the National Academy of Sciences of the United States of America	６．最初と最後の頁 PNAS online
掲載論文のDOI（デジタルオブジェクト識別子） 10.1073/pnas.1815166116	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

〔学会発表〕　計3件（うち招待講演　0件／うち国際学会　0件）

１．発表者名 Maria K. Tsoumpra
２．発表標題 The vitamin D/VDR axis regulates the expression of selected components of dystrophin associated protein complex
３．学会等名 Vitamin D Workshop, Tokyo, Japan
４．発表年 2018年

１．発表者名 Maria K. Tsoumpra
２．発表標題 The vitamin D/VDR axis regulates expression of selected DAPC genes
３．学会等名 The 41st Annual Meeting of the Molecular Biology Society of Japan
４．発表年 2018年

１．発表者名 Maria K. Tsoumpra
２．発表標題 The Vitamin D/VDR axis regulates selected components of dystrophin associated protein complex in healthy and atrophying myotubes
３．学会等名 Sarcopenia Meeting, Japan
４．発表年 2018年

〔図書〕　計1件

１．著者名 Nakamura A, Aoki Y, Tsoumpra M, Yokota T, Takeda S	４．発行年 2018年
２．出版社 Humana Press, New York, NY	５．総ページ数 12
３．書名 Exon Skipping and Inclusion Therapies. Methods in Molecular Biology	

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考