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Research Paper

Interventions Targeting Glucocorticoid-Krüppel-like Factor 15-Branched-Chain Amino Acid Signaling Improve Disease Phenotypes in Spinal Muscular Atrophy Mice

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ABSTRACT

The circadian glucocorticoid-Krüppel-like factor 15-branched-chain amino acid (GC-KLF15-BCAA) signaling pathway is a key regulatory axis in muscle, whose imbalance has wide-reaching effects on metabolic homeostasis. Spinal muscular atrophy (SMA) is a neuromuscular disorder also characterized by intrinsic muscle pathologies, metabolic abnormalities and disrupted sleep patterns, which can influence or be influenced by circadian regulatory networks that control behavioral and metabolic rhythms. We therefore set out to investigate the contribution of the GC-KLF15-BCAA pathway in SMA pathophysiology of Taiwanese *Smn*$^{-/-}$;*SMN2* and *Smn*$^{2B/-}$ mouse models. We thus uncover substantial dysregulation of GC-KLF15-BCAA diurnal rhythmicity in serum, skeletal muscle and metabolic tissues of SMA mice. Importantly, modulating the components of the GC-KLF15-BCAA pathway via pharmacological (prednisolone), genetic (muscle-specific Klf15 overexpression) and dietary (BCAA supplementation) interventions significantly improves disease phenotypes in SMA mice. Our study highlights the GC-KLF15-BCAA pathway as a contributor to SMA pathogenesis and provides several treatment avenues to alleviate peripheral manifestations of the disease. The therapeutic potential of targeting metabolic perturbations by diet and commercially available drugs could have a broader implementation across other neuromuscular and metabolic disorders characterized by altered GC-KLF15-BCAA signaling.

1. Introduction

Transcriptional regulation is one of the main control mechanisms of metabolic processes [1]. Krüppel-like factor 15 (KLF15) is a transcription factor expressed in a multitude of metabolic tissues including skeletal muscle [2] where it is involved in regulation of lipid [3], glucose [4], and amino acid metabolism [5]. Specifically, *KLF15* displays a diurnal pattern of expression, and regulates branched-chain amino acids (BCAA) metabolism and utilization in a circadian fashion [5]. BCAAs (isoleucine, leucine and valine) are a major source of essential amino acids in muscle (35%) [6]. Accumulating evidence in various species suggest that BCAAs promote survival, longevity [7,8] and repair of exercise- and sarcopenia-induced muscle damage [9,10]. Both KLF15 and BCAAs are modulated by circadian secretion of glucocorticoids (GCs) and activity of the glucocorticoid receptor (GR) [11,12]. GCs are

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also used surreptitiously by endurance athletes for their ergogenic properties [13] and as treatment for genetic muscle pathologies [14].

The neuromuscular disease spinal muscular atrophy (SMA) is the most common autosomal recessive disorder leading to infant mortality [15]. It is characterized by degeneration of α-motoneurons in the ventral horn of the spinal cord as well as progressive muscle weakness and atrophy [16,17]. SMA is a monogenic disease caused by homozygous deletions or mutations within the *survival motor neuron 1* (*SMN1*) gene [18,19]. Complete loss of SMN is embryonic lethal in mice [20]. However, humans have at least one copy of the highly homologous *SMN2* gene, which generates a low amount of functional protein that allows for embryonic development, while not being sufficient for complete rescue in the event of *SMN1* loss. This is due to a nucleotide transition in *SMN2* that favors alternative splicing of exon 7 and production of a non-functional truncated protein [19,21,22]. Whilst several cellular functions for SMN have been defined [23–25], it remains elusive why a lack of the ubiquitously expressed SMN results in the canonical SMA phenotype.

Although motoneurons are the primary cellular targets in SMA, a number of tissues outside the central nervous system (CNS) also contribute to disease pathophysiology [26], with skeletal muscle being the most prominently afflicted [27]. As muscle plays an important role in maintaining systemic energy homeostasis [28], intrinsic muscle defects can have severe consequences on whole-body metabolism. Various studies in SMA animal models and patients report metabolic abnormalities such as abnormal fatty acid metabolism [29–31], defects in glucose metabolism and pancreatic development [32,33] and the coexistence of diabetes mellitus and diabetic ketoacidosis in SMA patients [34,35]. The observation that dietary supplementation improves lifespan of SMA mice [36–38] further supports the hypothesis that metabolic perturbations contribute to SMA pathology. We thus postulate that intrinsic metabolic defects in skeletal muscle play a contributory role in whole-body metabolic perturbations in SMA.

Here, we identify dysregulation of the GC-KLF15-BCAA pathway in skeletal muscle as a key pathological event in SMA. Notably, we demonstrate that pharmacological and dietary interventions that modulate this pathway lead to significant phenotypic improvements in SMA mice. Our results reveal the importance of the GC-KLF15-BCAA axis in SMA pathogenesis and highlight its potential as a therapeutic target to attenuate muscle and metabolic disturbances in SMA. The accessibility and ease of administration of the dietary and drug treatments identified in our study make them exciting clinical avenues to investigate not only in SMA patients but also in individuals with other neuromuscular and neurodegenerative diseases where GC-KLF15-BCAA signaling may be altered.

2. Materials and Methods

2.1. Animals

The Taiwanese $Smn^{-/-}$;*SMN2* (FVB/N background, FVB·Cg-Smn1tm1HungTg(SMN2)2Hung/J, RRID: J:59313), $Smn^{2B/-}$ (C57BL/6 background, RRID: not available) and *KLF15 MTg* (C57BL/6 background, RRID: not available) mice were housed either in individual ventilated cages in the typical holding rooms of the animal facility or in circadian isolation cages (12 h light:12 h dark cycle, LD12:12). Experiments with the $Smn^{-/-}$;*SMN2* and *Klf15 MTg* mice were carried out in the Biomedical Sciences Unit, University of Oxford, according to procedures authorized by the UK Home Office (Animal Scientific Procedures Act 1986). Experiments with the $Smn^{2B/-}$ mice were carried out at the University of Ottawa Animal Facility according to procedures authorized by the Canadian Council on Animal Care. Prednisolone (5 mg tablets, Almus) was dissolved in water (1 tablet in 5 mL) and administered by gavage on every second day starting at P0 until death in the severe Taiwanese $Smn^{-/-}$;*SMN2* SMA mouse model. In the $Smn^{2B/-}$ mouse model, prednisolone or saline was administered by gavage every two days from P0 to P20. For the $Smn^{2B/-}$ mouse model treatment, weaned mice were given daily wet chow at the bottom of the cage to ensure proper access to food. BCAA peptides (Myprotein) were diluted in water (300 mg in 2 mL) and administered to the severe Taiwanese $Smn^{-/-}$;*SMN2* SMA mice by gavage starting at P5. Pip6a-PMO and Pip6a-scrambled compounds were delivered by facial vein injections at P0 and P2 (10 μg/g diluted in 0.9% saline) to WT and severe Taiwanese $Smn^{-/-}$;*SMN2* SMA mice. Prednisolone and BCAAs were administered to the animals around the same time each day. Litters were randomly assigned to treatment prior to birth. For survival studies, animals were weighed daily and culled upon reaching their defined humane endpoint. To reduce total number of animals used, the fast-twitch *tibialis anterior* and *triceps* muscles from the same animal were used interchangeably for respective molecular and histological analyses. Sample sizes were determined based on similar studies with SMA mice.

2.2. Peptide-PMO Synthesis

Pip6a Ac-(RXRRBRRXRYQFLIRXRBRXRB)-COOH was synthesized and conjugated to a PMO chemistry as previously described [39]. The full length *SMN2* enhancing PMO (5′-ATTCACTTTCATAATGCTGG-3′) and scrambled PMO (5′- TACGTTATATCTCGTGATAC-3′) sequences were purchased from Gene Tools LLC.

2.3. qPCR

Skeletal muscles were harvested at several time-points during disease progression and immediately flash frozen. For circadian experiments, liver, heart, white and brown adipose tissue (WAT and BAT), spinal cord and *tibialis anterior* muscles were harvested from P2 and P7 pups every 4 h over a 24 h period (ZT1 = 9 am, ZT5 = 1 pm, ZT9 = 5 pm, ZT13 = 9 pm, ZT17 = 1 am, ZT21 = 5 am). RNA was extracted with the RNeasy MiniKit (Qiagen) except for WAT and BAT where the RNeasy Lipid Tissue MiniKit (Qiagen) was used. Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). qPCR was performed either using TaqMan Gene Expression Mastermix (ThermoFisher Scientific) or SYBR green Mastermix (ThermoFisher Scientific) and primers were from Integrated DNA Technologies (see Supplementary Experimental Procedures). For SYBR green qPCRs, *RNA polymerase II polypeptide J* (*PolJ*), was used as a validated housekeeping gene. *PolJ* has previously been demonstrated as being stably expressed between tissues and in different pharmacological conditions [40]. For circadian experiments and TaqMan qPCRs, housekeeping genes for each tissue were determined using the Mouse geNorm Kit and qbase+ software (Primerdesign).

2.4. PCR Arrays

RNA from skeletal muscle was extracted using the RNeasy Microarray Tissue Mini Kit (Qiagen). cDNA was made using RT^2 First Strand Kit (Qiagen). qPCRs were performed using Mouse Amino Acid Metabolism I & II PCR arrays (PAMM-129Z and PAMM-130Z, SABiosciences). Data was analyzed with the RT Profiler PCR Array Data Analysis version 3.5 and mRNA expression was normalized to the geometric average of the two most stably expressed housekeeping genes between all samples.

2.5. Immunoblots With Mouse Tissues

Triceps were isolated from P7 $Smn^{-/-}$;*SMN2* mice and healthy control littermates and snap frozen in liquid nitrogen. The tissue was lysed in 200 μL RIPA buffer (150 mM NaCl, 50 mM Tris, 0.5% sodium deoxycholate, 0.1% TX-100, 5 mM sodium pyrophosphate, 2 mM β-glycerophosphate, 1 × EDTA-free protease inhibitor (Roche), 1 × PhosSTOP phosphatase inhibitor (Roche), pH 7.5) using Precellys 24 homogenizer (Stretton Scientific). Total protein (20 μg per lane)

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