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Small molecule adiponectin receptor agonist GTDF protects against skeletal muscle atrophy

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ABSTRACT

Skeletal muscle atrophy is a debilitating response to several major diseases, muscle disuse and chronic steroid treatment for which currently no therapy is available. Since adiponectin signaling plays key roles in muscle energetics, we assessed if globular adiponectin (gAd) or the small molecule adiponectin mimetic 6-C-β-D-glucopyranosyl-(2S,3S)-(+)-5,7,3′,4′-tetrahydroxydihydroflavonol (GTDF) could ameliorate muscle atrophy. Both GTDF and gAd induced C2C12 myoblast differentiation. GTDF and gAd effectively prevented reduction in myotube area and suppressed the expressions of atrophy markers; atrogin-1 and muscle ring finger protein-1 (MuRF1) in models of steroid, cytokine and starvation −induced muscle atrophy. The protective effects of GTDF and gAd were routed through AMPK and AKT activation and thereby stimulation of PPAR gamma coactivator 1α and inhibition of forkhead box O transcription factors. Finally, GTDF and gAd mitigated dexamethasone-induced muscle atrophy *in vivo*. Together, our results demonstrate that activating adiponectin signaling may be an effective therapeutic strategy against skeletal muscle atrophy.

1. Introduction

In addition to being a major structural and mechanical organ, skeletal muscle is the primary protein reservoir in vertebrates. Skeletal muscle is highly plastic in nature in relation to various physical and chemical stimuli and can convert proteins into free amino acids for hepatic gluconeogenesis and energy production during starvation and pathological conditions (Gomes et al., 2001). Excessive protein breakdown and lack of new protein synthesis in skeletal muscle can result in muscle atrophy. Muscle atrophy is characterized by loss of muscle mass leading to partial or complete loss of its function. Major diseases like AIDS, cancer, chronic obstructive pulmonary disorder, diabetes, renal failure, cardiac failure and septicemia lead to muscle atrophy (Sacheck et al., 2007). Muscle atrophy also may occur as a debilitating response to muscle disuse, malnutrition, fasting, steroid administration and denervation due to loss of motor neurons, and it is a consequence of biological aging as well (Bonaldo and Sandri, 2013). Skeletal muscle mass depends on number of muscle fibers, their type, size and balance between myofibrillar protein synthesis and degradation (Siriett et al., 2007). The two important proteolytic systems controlling myofibrillar protein turnover are ubiquitin proteasome machinery and the autophagy-lysosome machinery (Sacheck et al., 2007,Bonaldo and Sandri, 2013). Proteasome machinery inhibitors

Abbreviations: GTDF, 6-C-β-D-glucopyranosyl-(2S,3S)-(+)-5,7,3′,4'-tetrahydroxydihydroflavonol; gAd, globular adiponectin; AdipoR, Adiponectin receptor; AMPK, AMP-activated protein kinase; MuRF1, Muscle ring finger protein-1; FoxO, Forkhead box O; PGC-1α, PPAR gamma coactivator 1α; Dex, Dexamethasone; LPS, Lipopolysaccharide.

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like bortezomib (Velcade) and MG132 have been shown to effectively block myofibrillar proteolysis *in vitro* as well as in muscle atrophy induced by denervation *in vivo* (Caron et al., 2011).

A comparative cDNA microarray analysis of mRNA samples from muscles of different atrophy models including fasting, cancer cachexia, streptozotocin induced diabetes mellitus, chronic renal failure and denervation revealed that a common subset of genes were up or down-regulated in all the atrophy models (Gomes et al., 2001,Sacheck et al., 2007,Lecker et al., 2004,Jagoe et al., 2002,Bodine et al., 2001a,b). These common set of genes which were coordinately regulated in atrophy were named as "atrogenes" (Sacheck et al., 2007,Sandri et al., 2004, 2006). The most highly upregulated atrogenes in muscle atrophy are the two "muscle-specific" E3 ubiquitin ligases Atrogin-1 (also called Muscle Atrophy F-box protein or MAFbx) and Muscle-specific Ring Finger protein1 (MuRF1). While MuRF1 is involved in degradation of myocyte structural proteins such as myosin heavy chain 1,4,8, MyBPc, alpha actin, filamin c etc (Cohen et al., 2009), atrogin-1 majorly regulates the stability of myocyte functional components such as the key myogenic transcription factor MyoD (Lagirand-Cantaloube et al., 2009). MyoD is involved in skeletal muscle differentiation and maintenance of differentiated myotubes (Lagirand-Cantaloube et al., 2009). The protein level of MyoD gets heavily depleted in various models of muscle atrophy and overexpression of a mutant MyoD that does not get ubiquitinylated protects against muscle atrophy *in vivo* (Lagirand-Cantaloube et al., 2009). Ablation or depletion of MuRF1 and atrogin-1 alone or in combination have been shown to protect against skeletal muscle atrophy against various atrogenic stimuli (Bonaldo and Sandri, 2013). Forkhead box O (FoxO) family of transcription factors are the major positive regulators of both MuRF1 and atrogin-1 expression either via direct transcriptional regulation of atrogin-1 (Sandri et al., 2004) or via an indirect pathway involving the upregulation of the myokine myostatin that inhibits myogenesis (Lokireddy et al., 2011). FoxO proteins, in particular FoxO1 transcription is enhanced in different atrophy models (Lecker et al., 2004). On the other hand, the FoxO group of transcription factors are deactivated by PI3K/AKT signaling and activation of AKT prevents muscular atrophy (Sandri et al., 2004,Stitt et al., 2004). PGC-1α, a transcriptional coactivator expressed highly in tissues with high energy demand has been shown to undergo robust downregulation in various forms of muscle atrophy and its overexpression counters the effect of FoxO on muscle mass, via downregulation of atrogenes (Sandri et al., 2006). In addition to Atrogin-1 and MuRF1, lysosomal protease cathepsin L has also been implicated in various forms of muscle atrophy including fasting and glucocorticoid-induced muscle atrophy where its expression is upregulated (Sacheck et al., 2007,Lecker et al., 2004,Deval et al., 2001). FoxO1 has been reported as a direct activator of cathepsin L transcription (Yamazaki et al., 2010).

Adiponectin is an adipokine whose circulating level is depleted in a number of metabolic diseases including insulin resistance, diabetes and cancer (Khan et al., 2015,Singh et al., 2014a,b,Yamauchi et al., 2014). Despite its importance in health and pathophysiology, adiponectin replenishment therapy is not possible owing to the large size of this peptide and its complex multimerization properties (Singh et al., 2014a,b,Yamauchi et al., 2014). Recently, we identified a small molecule orally active adiponectin receptor (AdipoR) agonist called GTDF that shows a binding preference for AdipoR1 (Singh et al., 2014a,b). Our results in osteoblasts revealed that MuRF1 and atrogin-1 were also expressed in bone cells and their expressions were increased during early-onset diabetes-induced osteopenia (Khan et al., 2015). In the same study GTDF was found to downregulate MuRF1 and atrogin-1 and enhance the expression and activity of PGC-1α via AdipoR1 (Khan et al., 2015). Adiponectin, in particular globular adiponectin that has a strong affinity for AdipoR1 also has been shown to induce myogenic differentiation (Fiaschi et al., 2009). In this study we thus investigated if GTDF also could induce myogenesis and if activation of adiponectin signaling events by GTDF or gAd could protect from skeletal muscle atrophy.

2. Material and methods

2.1. Reagents

All cell culture reagents were from Life Technologies (Thermo Fisher Scientific, Carlsbad, CA). Fine chemicals were from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Globular adiponectin (gAd) used for *in vitro* experiments was from ATGen global (Gyeonggi-do, South Korea). 6-C-β-D-glucopyranosyl-(2S,3S)-(+)-5,7,39,49-tetrahydroxydihydroflavonol (GTDF) (purity 98%) was purified as previously reported (Sharan et al., 2011). ON-TARGETplus SMARTpool siRNAs were from Dharmacon (GE healthcare, Lafayette, CO). Vectashield was from Vector Laboratories (Burlingame, CA).

2.2. Cell cultures

C2C12 myoblast cells were acquired from ATCC (Manassas, VA) and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in 5% CO2 humidified atmosphere. For differentiation, 80% confluent C2C12 cultures were washed with PBS and shifted from growth medium to differentiation medium (DM) composed of DMEM containing 2% horse serum (HS). When differentiation inducing capabilities of gAd or GTDF were assessed, serum starved C2C12 cells were incubated with medium containing GTDF (0.1 μM) or gAd (1 μg/mL) (without HS). To induce Dexamethasone (Dex) or inflammation -induced muscle atrophy, fully differentiated myotubes were treated with 1 μM Dex or LPS (1 μg/mL) + TNF-α (15 ng/mL) (LT) for 48 h in differentiation media. For starvation-induced muscle atrophy, fully differentiated myotubes were incubated in phosphate buffer saline (PBS) for 6 h.

2.3. siRNA transfection and treatment of myotubes

C2C12 myotubes in 6 well plates were transfected with 0.1 μM Control siRNA (siC) or 0.1 μM of AdipoR1 siRNA (siAdipoR1) using DharmaFECT 1 transfection reagent (GE healthcare) according to manufacturer's protocol. 48 h after transfection, myotubes were treated with vehicle and or GTDF (0.1 μM) or gAd (1 μg/mL) for 24 h. Cells were then treated with LT (LPS (1 μg/mL) +TNF-α (15 ng/mL) for 48 h to induce myotube atrophy (GTDF or gAd and stressor (LT) was replenished every 24 h) following which cells were harvested for subsequent analysis.

2.4. Measurement of the area of immunofluorescence-labeled myotubes

Following different treatments, C2C12 myotubes were fixed with 4% formaldehyde for 20 min at room temperature. Fixed cells were then washed three times with PBS and then permeabilized and blocked with 0.1% Triton X-100 in PBS with 2% horse serum (HS) and 1% BSA for 60 min at room temperature. Anti-myosin heavy chain antibody (MHC) (Sigma-Aldrich) was added (1:100 in PBS supplemented with 1% BSA) and incubated for overnight at 4 °C. Samples were then washed three time with PBS, and then incubated with Alexa Fluor tagged goat anti-mouse (1:500) secondary antibody (Life Technologies) for 1 h at room temperature in PBS + 1% BSA. After three washes in PBS, cells were stained with