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An ethanolic extract of *Artemisia dracunculus* L. regulates gene expression of ubiquitin-proteasome system enzymes in skeletal muscle: Potential role in the treatment of sarcopenic obesity

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Abstract

Objective—Obesity is linked to insulin resistance, a primary component of metabolic syndrome and type 2 diabetes. The problem of obesity-related insulin resistance is compounded when age-related skeletal muscle loss, called sarcopenia, occurs with obesity. Skeletal muscle loss results from elevated levels of protein degradation and prevention of obesity-related sarcopenic muscle loss will depend on strategies that target pathways involved in protein degradation. An extract from *Artemisia dracunculus*, termed PMI5011 improves insulin signaling and increases skeletal muscle myofiber size in a rodent model of obesity-related insulin resistance. This study examines the effect of PMI5011 on the ubiquitin-proteasome system, a central regulator of muscle protein degradation.

Materials and Methods—Gastrocnemius and vastus lateralis skeletal muscle was obtained from KK-A^y obese diabetic mice fed a control or 1% (w/w) PMI5011-supplemented diet. Regulation of genes encoding enzymes of the ubiquitin-proteasome system was determined using realtime qRT-PCR.

Results—While *MuRF-1* ubiquitin ligase gene expression is consistently down-regulated in skeletal muscle, *atrogen-1*, *Fbxo40* and *Traf6* expression is differentially regulated by PMI5011. Genes encoding other enzymes of the ubiquitin-proteasome system ranging from ubiquitin to ubiquitin-specific proteases are also regulated by PMI5011. In addition, expression of the gene

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The authors have no conflicts of interest to disclose.

Author Contributions:

HKB, ZQW, WTC and ZEF designed the experiments; HKB, GK, BCD, ZEF processed and analyzed data; DR provided the PMI5011 botanical extract; HKB and ZEF wrote the manuscript and all authors contributed to preparation of the final manuscript.

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encoding the microtubule-associated protein-1 light chain 3 (*LC3*), a ubiquitin-like protein pivotal to autophagy-mediated protein degradation, is down-regulated by PMI5011 in the vastus lateralis.

Conclusion—PMI5011 alters the gene expression of ubiquitin-proteasome system enzymes that are essential regulators of skeletal muscle mass. This suggests PMI5011 has therapeutic potential in the treatment of obesity-linked sarcopenia by regulating ubiquitin-proteasome-mediated protein degradation.

Keywords

botanical; ubiquitin; proteasome; atrophy

Introduction

When combined with obesity, the age-related loss of muscle mass that characterizes sarcopenia is strongly associated with developing insulin resistance and metabolic syndrome. The National Health and Nutrition Examination Survey III (NHANESIII) found that sarcopenic obese individuals have increased insulin resistance and elevated HbA1C levels compared to obese individuals without sarcopenia [1]. The higher incidence of insulin resistance in sarcopenic obesity is consistent with the role of skeletal muscle as the primary site of insulin-mediated glucose disposal. The link between sarcopenia and insulin resistance is also evident in NHANESII data for adults under 60 years of age [1]. In this age group, sarcopenia is significantly associated with insulin resistance in lean or obese individuals, indicating reduced skeletal muscle mass may be an early predictor of metabolic syndrome or type 2 diabetes.

Skeletal muscle loss involves an imbalance between protein synthesis and degradation that favors degradation. While protein synthesis and degradation are regulated by insulin, a number of studies indicate that skeletal muscle protein degradation is particularly sensitive to insulin's action [2–5] and protein turnover is increased in animal models of sarcopenic obesity [6]. Protein degradation in skeletal muscle is carried out primarily by the ubiquitin-proteasome system, which catalyzes the degradation of most cellular proteins and acts in connection with the autophagy-lysosomal proteolytic system that degrades organelles and protein aggregates [7]. Multiple ubiquitin polypeptides are shuttled to the substrate protein via a series of enzymatic reactions that are initiated by the ubiquitin activating enzyme (E1) followed by transfer of the E1-bound ubiquitin to a ubiquitin conjugating enzyme (E2) and the targeted protein. Transfer of ubiquitin to the target protein is catalyzed by the ubiquitin (E3) ligases, the enzymes responsible for substrate recognition and selectivity. Once modified by multiple ubiquitin molecules, the substrate is recognized by the 26S proteasome and degraded, releasing amino acids and recycling ubiquitin (Figure 1) [8]. The ubiquitin-proteasome system is regulated by insulin and various models of skeletal muscle loss associated with insulin resistance show striking increases in components of the ubiquitin proteasome system [9–11], particularly the muscle-specific ubiquitin ligases Muscle Ring Finger-1 (MuRF-1) and Muscle Atrophy F-box protein (MAFBx, also called Atrogin-1) [9]. The essential role of the ubiquitin-proteasome system in skeletal muscle protein degradation makes this pathway an important target in developing therapeutic options for treating sarcopenia.

An botanical extract of *Artemisia dracuncululus* L. (termed PMI5011) decreases blood glucose and insulin levels and improves insulin action in skeletal muscle by increasing insulin signaling in animal models of diabetes [12, 13]. The effect of PMI5011 extends to regulation of the ubiquitin-proteasome system in skeletal muscle. In the KK-A^y murine model of obesity-related type 2 diabetes, dietary intake of PMI5011 inhibits proteasome and non-proteasome proteolytic activity, alters the overall pattern of substrate ubiquitylation, and decreases MuRF-1 and Atrogin-1 gene and protein expression. PMI5011-mediated changes in proteasome activity and ubiquitylation levels *in vivo* correlate with larger myofiber size, suggesting PMI5011-mediated regulation of the ubiquitin-proteasome system leads to preservation of skeletal muscle mass in the presence of obesity and insulin resistance. In the current study, we show that PMI5011 differentially regulates the expression of genes encoding a range of ubiquitin-proteasome enzymes, providing further evidence that the ubiquitin-proteasome system is a target of PMI5011.

Materials and Methods

Animal Studies

All animal studies were conducted as approved by the Pennington Biomedical Research Center Institutional Animal Care and Use Committee. Briefly, six-week-old male KK-A^y mice (n=16) (Jackson Laboratory; Bar Harbor, ME) were single housed in animal rooms maintained at 23°C with a 12-h light–dark cycle. The mice were fed a low-fat diet containing 16.4 kcal% protein, 10.5 kcal% fat, and 71.3 kcal% carbohydrate (D12329; Research Diets, Inc.; New Brunswick, NJ). At 10 weeks of age, the mice were randomly divided into a control group (n=8) and a PMI5011-treated group (n=8). The control group was fed the low fat diet *ad libitum* and the PMI5011 treatment group was fed *ad libitum* the low-fat diet containing 1% (w/w) PMI5011. Measurements of food intake, body weight, blood glucose and insulin were carried out and previously reported [14]. After twelve weeks on the diets, the mice were euthanized after a four hour fast and skeletal muscle (gastrocnemius and vastus lateralis) was harvested.

Analysis of gene expression

Total RNA was purified from the skeletal muscle tissue using an RNeasy Fibrous Tissue Minikit (Qiagen, Valencia, CA). In each case, RNA (200 ng) was reverse transcribed using Multiscribe Reverse Transcriptase (Applied Biosystems, Carlsbad, CA) with random primers at 37°C for 2 hour. Real-time PCR was performed with TaqMan chemistry using the 7900 Real-Time PCR system and universal cycling conditions (50°C for 2 minutes; 95°C for 10 minutes; 40 cycles of 95°C for 15 seconds and 60°C for 1 minute; followed by 95°C for 15 seconds, 60°C for 15 seconds and 95°C for 15 seconds). The results were normalized to Cyclophilin B mRNA or 18S rRNA levels and analyzed using the 2^{-CT} method with the control values used as the calibrator.

Statistical Analysis

Statistical significance for the gene expression data was determined using a two-tailed t test. All statistical analysis was carried out using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). Variability is expressed as the mean $\pm$ standard error of the mean.

Results

Although there are at least 600 genes encoding ubiquitin ligases [15], very few ubiquitin ligases are currently identified as regulating skeletal muscle mass. Genes encoding the muscle-specific Atrogin-1 and MuRF-1 ligases are the most strongly induced in muscle atrophy [9, 16]. Figure 2 shows *atrogin-1* gene expression is reduced by PMI5011 in gastrocnemius muscle, but strongly up-regulated by PMI5011 in the vastus lateralis. In contrast, *MuRF-1* gene expression is inhibited in both the gastrocnemius and vastus lateralis. Other ligases that are reported to play a role in muscle loss are the muscle-specific F-box domain protein 40 (Fbxo40), the substrate recognition component of an SCF-type (for Skp1, cullin-1, F-box) ubiquitin ligase [17] and the TNF α receptor associated factor-6 (TRAF6) [18]. Although *Fbxo40* gene expression trends upward in gastrocnemius muscle with dietary intake of PMI5011, it is inhibited in the vastus lateralis. Gene expression of *TRAF6* is not regulated by PMI5011 in the gastrocnemius, but is significantly up-regulated in the vastus lateralis, in parallel with *atrogin-1* (Figure 2)

We also observed differential regulation by PMI5011 of other ubiquitin-proteasome system components in the skeletal muscle. In mammals, ubiquitin is transcribed from four genes. *UbA52* and *UbA80* encode ubiquitin fused to ribosomal protein L40 or S27a [19]. *Ubb* and *Ubc* are polyubiquitin genes consisting of four or nine tandem ubiquitin coding units, respectively [20]. As shown in Figure 3A, while *UbA52* expression is inhibited by PMI5011 in the gastrocnemius, all of the ubiquitin genes are up-regulated in the vastus lateralis.

Ubiquitin-specific proteases (USP) are a class of deubiquitylating enzymes that reverse the ubiquitin modification of proteins, antagonizing the effect of ubiquitylation and recycling free ubiquitin (reviewed in [21]). Two of the ubiquitin-specific proteases, *Usp14* and *Usp19*, are up-regulated with muscle atrophy [9, 22]. PMI5011 consistently decreases the gene expression of *Usp14*, but *Usp19* gene expression is elevated in the gastrocnemius and decreased in the vastus lateralis (Figure 3B). The gene encoding the ubiquitin conjugating enzyme *Ube2v1* (ubiquitin conjugating enzyme E2 variant 1) acts in a complex with TRAF6 to modify proteins with lysine 63-linked ubiquitin chains that regulate signaling events non-proteolytically [23, 24]. *Ube2v1* mRNA levels are down-regulated by PMI5011 in gastrocnemius, but like *TRAF6*, up-regulated in the vastus lateralis (Figure 3C).

Genes encoding multiple subunits of the 20S core proteasome and 19S proteasome regulatory particle are up-regulated in atrophying muscle [9]. We assayed the expression of genes that encode two of the 20S proteasome subunits, *PSMA5* and *PSMB3* (Figure 3D). Expression of both genes is inhibited by PMI5011 in gastrocnemius, but unchanged in the vastus lateralis muscle. Finally, we assayed the gene expression of *LC3*, which encodes a ubiquitin-like protein that is essential for forming the autophagosome necessary for lysosomal degradation of organelles in autophagy [25]. Although *LC3* expression is unchanged in gastrocnemius, its expression is significantly down-regulated by dietary intake of PMI5011 in the vastus lateralis (Figure 3E).

Discussion

Sarcopenia is defined as the decline in skeletal muscle mass and strength that occurs with aging [26]. The insulin resistance associated with obesity accelerates sarcopenia by suppressing protein synthesis and stimulating skeletal muscle protein degradation [27], even in the absence of type 2 diabetes [28]. Independent of obesity, sarcopenia may increase the risk of developing insulin resistance and type 2 diabetes due to the loss of skeletal muscle that leads to reduced glucose disposal. When found in combination with obesity, sarcopenic muscle loss is strongly associated with insulin resistance [1], clearly increasing the risk of developing type 2 diabetes. Developing therapeutic agents that prevent or treat sarcopenic muscle loss in obesity will depend on understanding the underlying processes in the skeletal muscle that regulate muscle mass. The current study demonstrates that a botanical extract from *A. dracunculus*, termed PMI5011, alters transcriptional regulation of genes encoding enzymes of the ubiquitin-proteasome system, the central pathway controlling protein degradation in skeletal muscle.

Previous studies show PMI5011 enhances insulin signaling in skeletal muscle in animal models of obesity-related insulin resistance [13, 14]. Insulin signaling is a potent regulator of the balance between protein synthesis and degradation in skeletal muscle and PMI5011-mediated improvements in insulin sensitivity correlate with inhibition of proteasome and non-proteasome proteolytic activity in the gastrocnemius of obese insulin resistant animals [14]. The dietary intake of PMI5011 also decreases the mRNA and protein levels of atrogin-1 and MuRF-1, two ubiquitin ligases that are up-regulated in muscle atrophy [9, 16], indicating PMI5011 regulates the ubiquitin-proteasome system in skeletal muscle. Moreover, the PMI5011-mediated changes in ubiquitin-proteasome activity are associated with larger myofiber size [14].

In aging, type II muscle fiber size is diminished to a greater extent than type I fibers [26] and the majority of studies focus on the vastus lateralis muscle that contains about 68% type II fibers, a level slightly higher than found in the gastrocnemius (~50% type II fibers) of humans [29]. Although these muscles have similar composition [30], our results indicate the effect of PMI5011 on the ubiquitin-proteasome system in skeletal muscle varies between the two muscles, with the exception of PMI5011-mediated down-regulation of MuRF-1 expression. Given the positive effect of type IIb fiber hypertrophy on insulin sensitivity in obesity [31], muscle-specific effects of PMI5011 on the ubiquitin-proteasome system have important implications for treating sarcopenic obesity.

MuRF-1 ubiquitin ligase is a fiber type II associated protein [32] that targets sarcomeric proteins for degradation [33–35] and accounts for the majority of ubiquitin modification in muscle atrophy [35], making MuRF-1 an attractive target for prevention of muscle loss in sarcopenia. PMI5011-mediated elevation of *atrogin-1* expression in the vastus lateralis and inhibition of *atrogin-1* expression in the gastrocnemius muscle suggest regulation of *atrogin-1* is muscle-specific. Lagirand-Cantaloube *et al* [36] found that atrogin-1 activity links regulation of protein degradation to protein synthesis by targeting the elongation initiation factor 3f (eIF3f), an essential factor in protein synthesis, for degradation. Atrogin-1 dependent inhibition of protein synthesis suggests PMI5011-mediated stimulation of

atrogin-1 expression will increase protein turnover and reduce muscle mass in the vastus lateralis, but not the gastrocnemius. However, recent evidence shows deletion of atrogin-1 accelerates sarcopenic changes in the gastrocnemius of aged mice, possibly by inhibiting the protein turnover of damaged proteins [37]. While it is difficult to envision a beneficial effect of elevated levels of *atrogin-1*, the modest inhibition of *atrogin-1* expression in the gastrocnemius may preserve the physiological function of atrogin-1 in aging muscle.

The PMI5011-mediated increases in *TRAF6* and *Ube2v1* gene expression in vastus lateralis, but not gastrocnemius, are also notable. The TRAF6/Ube2v1 complex is best described in the activation of I κ B kinase, leading to activation of NF κ B in response to proinflammatory signals [38], but recent studies indicate TRAF6 also regulates *MuRF-1*, *atrogin-1* and *LC3* gene expression [39] in starvation-induced muscle atrophy along with AKT phosphorylation, although the evidence is conflicting regarding whether TRAF6 promotes [40] or inhibits AKT phosphorylation [39]. The PMI5011-mediated transcriptional activation of TRAF6/Ube2v1 in vastus lateralis correlates with *atrogin-1* expression, but not *MURF-1* or *LC3*, suggesting PMI5011-mediated changes in *TRAF6* and *Ube2V1* expression are more specific than observed in starvation models of muscle atrophy and possibly more relevant to the chronic muscle loss of sarcopenia.

PMI5011-mediated transcriptional regulation of *Usp14* and *Usp19*, two deubiquitinases that are up-regulated in muscle atrophy is more pronounced in vastus lateralis. Repression of both deubiquitinases in the vastus lateralis is associated with PMI5011-mediated increases in each of the genes encoding ubiquitin. The role of the deubiquitinases in muscle atrophy is not well understood, but up-regulation of *Usp14* and *Usp19* may function to increase the pool of free ubiquitin available to meet the demands for ubiquitin modification during muscle loss [41]. Consistent with this idea, our results suggest genes encoding ubiquitin are up-regulated in vastus lateralis in response to PMI5011-mediated suppression of *Usp14* and *Usp19*.

Finally, our results showing PMI5011 down-regulates genes encoding 20S proteasome subunits in the gastrocnemius supports our previous finding that PMI5011 inhibits proteasome activity [14]. However, PMI5011 has no effect on expression of the proteasomal subunits in vastus lateralis, raising the possibility that PMI5011 does not inhibit proteasome activity in the vastus lateralis. In contrast, down-regulation of *LC3* expression in vastus lateralis indicates autophagy is inhibited by PMI5011 in the vastus lateralis, but not the gastrocnemius. Given the importance of the ubiquitin-proteasome and autophagy proteolytic systems in disposing of nonfunctional proteins and organelles during aging [37], it will be important to determine if PMI5011-mediated transcriptional regulation of these factors is associated with changes in muscle mass and strength.

PMI5011-mediated transcriptional regulation of components of the ubiquitin-proteasome and autophagy systems is consistent with enhanced insulin-dependent Akt phosphorylation in the skeletal muscle of obese insulin resistant mice treated with PMI5011 [13]. The FoxO1 and FoxO3a members of the FoxO class of forkhead transcription factors are downstream targets of Akt that regulate the gene expression of *atrogin-1* and *MuRF-1* [42, 43] and PMI5011 increases FoxO3a phosphorylation in skeletal muscle [14]. However, our

results predict any effect of PMI5011 on Akt phosphorylation and FoxO protein transcriptional activity will be differentially regulated in skeletal muscle groups. While this is an important consideration in understanding the mechanisms underlying muscle atrophy and the potential effects of PMI5011 in aging muscle, our results show that the ubiquitin-proteasome and autophagy systems are targets of PMI5011 action in skeletal muscle. Given the central role of the ubiquitin-proteasome system in maintaining muscle mass, compounds found in the PMI5011 extract may represent a viable therapeutic option in the prevention and treatment of sarcopenic obesity.

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Figure 1. The ubiquitin-proteasome system

The ubiquitin-proteasome system is an energy dependent multi-enzyme system that targets proteins for degradation by the 26S proteasome. Substrate selectivity of the system is determined by the E3 ubiquitin ligases. There are two major classes of E3 ubiquitin ligases: the homologous to the E6AP carboxyl terminus (HECT) type ligases and the really interesting new gene (RING) type ligases. The RING type ligases can function as monomers or multi-subunit complexes.

Figure 2. PMI5011 regulates the mRNA level of ubiquitin ligases involved in muscle atrophy
Gene expression of *atrogin-1*, *MuRF-1*, *Fbxo40* and *TRAF6* was assayed in the gastrocnemius and vastus lateralis skeletal muscle from KK- A^y obese diabetic mice fed a low fat diet (Control, □) or low fat diet supplemented with 1% w/w PMI5011 (PMI5011, ■) over twelve weeks.

Relative mRNA levels were assayed using realtime qRT-PCR and reported as relative mRNA expression when normalized to 18s rRNA expression and calculated using the 2^{-C_t} method. Statistical significance compared to related control, * $p < 0.05$.

Figure 3. PMI5011 differentially regulates the mRNA level of ubiquitin, ubiquitin system enzymes and LC3, a ubiquitin-like protein required for autophagy

Gene expression was assayed in the gastrocnemius and vastus lateralis skeletal muscle from KK-A^y obese diabetic mice fed a low fat diet (Control, □) or low fat diet supplemented with 1% w/w PMI5011 (PMI5011, ■) over twelve weeks. Relative mRNA levels were assayed using realtime qRT-PCR and reported as relative mRNA expression when normalized to 18s rRNA expression and calculated using the 2^{-Ct} method. Statistical significance compared to related control, **p*<0.05.