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Proteomic analysis reveals cellular pathways regulating carbohydrate metabolism that are modulated in primary human skeletal muscle culture due to treatment with bioactives from *Artemisia dracunculus* L.

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ABSTRACT

Insulin resistance is a major pathophysiologic abnormality that characterizes metabolic syndrome and type 2 diabetes. A well characterized ethanolic extract of *Artemisia dracunculus* L., termed PMI 5011, has been shown to improve insulin action *in vitro* and *in vivo*, but the cellular mechanisms remain elusive. Using differential proteomics, we have studied mechanisms by which PMI 5011 enhances insulin action in primary human skeletal muscle culture obtained by biopsy from obese, insulin-resistant individuals. Using iTRAQ™ labeling and LC–MS/MS, we have identified over 200 differentially regulated proteins due to treatment with PMI 5011 and insulin stimulation. Bioinformatics analyses determined that several metabolic pathways related to glycolysis, glucose transport and cell signaling were highly represented and differentially regulated in the presence of PMI 5011 indicating that this extract affects several pathways modulating carbohydrate metabolism, including translocation of GLUT4 to the plasma membrane. These findings provide a molecular mechanism by which a botanical extract improves insulin stimulated glucose uptake, transport and metabolism at the cellular level resulting in enhanced whole body insulin sensitivity.

1. Introduction

Insulin resistance is a major pathophysiologic parameter that defines metabolic syndrome and type 2 diabetes. Insulin resistance is typically observed 5–10 years prior to the onset and diagnosis of type 2 diabetes and is accompanied by a compensatory increase in insulin secretion [1]. Insulin resistance has been well described to develop with obesity, result-

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ing from increased food intake, sedentary life style and genetic predisposition [1] and is associated with inflammation, dyslipidemia, carbohydrate dysregulation and cardiovascular diseases [2,3]. Insulin sensitivity can be improved by changes in diet, exercise and use of pharmacological drugs [4]. However, the success rate of maintaining life style changes over prolonged periods of time is low and use of pharmacologic drugs is often accompanied with significant side effects [5]. Thus, nutritional supplementation with naturally occurring products (*i.e.* botanicals) is a desirable alternative to successfully improve and maintain insulin sensitivity.

Botanical extracts have been widely used for centuries in many cultures in efforts to prevent and treat diseases [6]. Metformin, the most commonly used agent for treatment of type 2 diabetes today, has its origins from a plant source [7]. Due to the complex composition of botanicals, very little is known regarding their exact mode of action. Differential global proteomic technologies provide a broad signature of changes in protein levels which allow identification of key pathways and mechanisms responsible for complex biological effects [8,9]. Despite advancements in mass spectrometry based proteomic techniques to understand biological processes at the molecular level, only a limited number of studies have used proteomics to study mechanisms by which botanicals induce biological effects [10–13].

Extracts of *Artemisia* species are widely marketed in over-the-counter dietary supplements. Extracts of *Artemisia* have also been shown to lower blood glucose levels in rats, and rabbits [14,15]. We have recently shown that a well characterized ethanolic extract of *Artemisia dracunculus* L., termed PMI 5011, lowers blood glucose and insulin levels in murine models and improves insulin receptor signaling (*e.g.* Akt phosphorylation and Phosphatidylinositol 3-kinase (PI3K) activity) [16,17]. Our studies have also shown that in primary human skeletal muscle culture (HSMC), PMI 5011 improved insulin receptor signaling (Akt phosphorylation and PI3K activity) and increased glucose uptake and glycogen synthesis [18].

Human skeletal muscle culture can be generated from biopsied skeletal muscle tissue from human subjects and retain the metabolic and biochemical properties of skeletal muscle cells noted in the *in vivo* state [19–25]. Thus, an insulin resistant individual will yield muscle culture that will have diminished insulin signaling and changes in carbohydrate metabolism. Similarly, muscle culture from an insulin sensitive individual will have normal insulin signaling and carbohydrate metabolism. In fact, it has been reported that cultured HSMC from non-diabetic and type 2 diabetic subjects respond to insulin stimulation in a manner consistent with *in vivo* changes in glucose utilization [19–21,24,25]. Thus, HSMC is a good model system to evaluate beneficial effects of botanical extracts under various experimental conditions and to determine molecular mechanisms responsible for improvement in insulin action.

To investigate cellular pathways affected by PMI 5011, we have used two dimensional liquid chromatography–tandem mass spectrometry (2D LC–MS/MS) in conjunction with isobaric tagging for relative and absolute quantification (iTRAQ™) of peptides to measure changes in protein expression levels in primary HSMC from obese insulin resistant subjects due to treatment with PMI 5011. We have further utilized immunohistochemistry and western blot analysis to validate results from proteomics experiments and show that PMI 5011 improves actin filament distribution and enhances translocation of glucose transporter 4 (GLUT4) to the plasma membrane resulting in enhanced glucose uptake, transport and metabolism.

2. Materials and methods

2.1. Extract preparation

Detailed information about the sourcing, growing conditions, quality control, stability, biochemical characterization and specific preparation of the *Artemisia dracunculus* L. extract (PMI 5011) tested in this study has been extensively reported [16,26–29]. Briefly, the *Artemisia dracunculus* L. extract was produced from plants grown hydroponically in greenhouses maintained under uniform and strictly controlled conditions, thereby standardizing the plants for their phytochemical content. Major compounds identified in the extract have included davidigenin, isomer of demethoxydihydrochalcone and sakuranetin [6].

2.2. Primary human skeletal muscle culture (HSMC)

Primary HSMC were prepared as described in detail previously [11,18]. Briefly, freshly removed muscle tissue from biopsies of *vastus lateralis* muscle from five obese diabetic patients was placed in Ham's F-10 media (HyClone Laboratories, Logan, UT) at 4 °C and dissected, minced, washed, dissociated, centrifuged at 600×*g* for 4 min at 37 °C and placed in human skeletal growth medium (SkGM Bullet Kit, Cambrex). Cells were incubated at 37 °C with 95% air and 5% CO_2. Media was changed every 2–3 days. Myoblasts were subcultured and grown to 80–90% confluence. Cells were then differentiated into fused myotubes for 7 days by switching to culture media with 2% horse serum. After starvation, cells were treated with 10 μg/mL of PMI 5011 for 16 h. To evaluate the effects of PMI 5011 on insulin signaling, cultures were treated with 100 nM insulin for 20 min prior to protein extraction. Thus, each experimental set included four HSMC samples: baseline control, PMI 5011 treated, insulin stimulated control and insulin stimulated and PMI 5011 treated. All primary cultured cells used in this study were within five passages.

2.3. Sample preparation

Proteins from all samples were extracted by adding 1 mL of lysis buffer (5 M Urea, 2 M Thiourea, 2% CHAPS, 2% SB3-10, 0.2% Bio-Lyte (pH 3–10), 2% n-dodecyl-b-D-maltoside, 40 mM Tris, 5 mM PMSF, 2 mM TBP and 150U Benzonase) followed by sonication and addition of 50 mM dithiothreitol (DTT) as described previously [11,30,31]. The resulting sample mixture was centrifuged for 30 min at 20,800×*g*, and the supernatant was acetone precipitated and resolubilized in 0.5 M triethylammonium bicarbonate buffer (TEAB; pH 8.5) and 0.8 M urea. The protein concentration was determined using Bradford Protein Assay (Bio-Rad, Hercules, CA).