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Novel mechanisms for botanical effect

Bioactives of *Artemisia dracunculus* L. enhance insulin sensitivity by modulation of ceramide metabolism in rat skeletal muscle cells

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ABSTRACT

Objective: An increase in ectopic lipids in peripheral tissues has been implicated in attenuating insulin action. The botanical extract of *Artemisia dracunculus L.* (PMI 5011) improves insulin action, yet the precise mechanism is unknown. The aim of this study was to determine whether the mechanism by which the bioactive compounds in PMI 5011 improve insulin signaling is through regulation of ceramide metabolism.

Methods: L6 Myotubes were separately preincubated with 250 μM palmitic acid with or without PMI 5011 or four bioactive compounds isolated from PMI 5011 and postulated to be responsible for the effect. The effects on insulin signaling, ceramide, and glucosylceramide profiles were determined.

Results: Treatment of L6 myotubes with palmitic acid resulted in increased levels of total ceramides and glucosylceramides, and cell surface expression of gangliosides. Palmitic acid also inhibited insulin-stimulated phosphorylation of protein kinase B/Akt and reduced glycogen accumulation. Bioactives from PMI 5011 had no effect on ceramide formation but one active compound (DMC-2) and its synthetic analog significantly reduced glucosylceramide accumulation and increased insulin sensitivity via restoration of Akt phosphorylation.

Conclusions: The observations suggest that insulin sensitization by PMI 5011 is partly mediated through moderation of glycosphingolipid accumulation.

Introduction

Skeletal muscle insulin resistance (IR) is a major contributor to reduced whole-body glucose disposal in obesity and type 2 diabetes mellitus (T2DM). A strong correlation between IR and lipid accumulation in tissues is observed with weight gain, prolonged physical inactivity, and/or systemic hyperlipidemia [1]. Exposure of muscle to excessive lipids leads to accumulation of triglycerides (TGs), diglycerides (DGs), and ceramides that initiate pathways leading to the inactivation of various insulin-signaling intermediates [1–3]. A previous study using L6 myotubes exposed to high levels of free fatty acids (FFAs) confirmed the role of ceramides but not DGs and TGs in attenuating insulin signaling by inhibiting protein kinase B (Akt-1 and Akt-2) phosphorylation [3]. In this regard, interventions that improve insulin sensitivity such as pharmaceutical agents i.e., thiazolidinediones and lifestyle interventions i.e., exercise, have been shown to lower muscle ceramide levels in humans and rodents [4,5].

Plants have traditionally been a rich source of medicinal compounds considered safe as complementary therapies for many indications, including diabetes. The ethanolic extract of the

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perennial herb *Artemisia dracunculus L.* or Russian tarragon (termed PMI 5011) has been shown to significantly decrease blood glucose levels in genetic and chemically induced murine models of diabetes and improve insulin sensitivity [3,6]. The previous study [3] reported that this botanical modulated the role of ceramides on insulin signaling but the mechanism involved was not secondary to an effect on the formation or accumulation of ceramides. With PMI 5011 intervention, we observed increased insulin signaling via preservation of insulin-stimulated Akt-1 and Akt-2 phosphorylation despite the presence of high ceramide levels in cells.

Sphingolipid metabolism is complex and involves hundreds of molecular species and metabolic pathways. Ceramide provides the platform for the synthesis of more complex sphingolipids and leads to the formation of sphingosine, sphingosine 1-phosphate, ceramide 1-phosphate, sphingomyelin, and the glycosphingolipids [glucosylceramides, galactosylceramides, lactosylceramides, sulfatides, and gangliosides] [7,8]. In this study we focused on the glycosphingolipid pathway and how perturbations in this pathway contribute to aberrant IR and the role of PMI 5011 bioactives in preventing accumulation of lipid intermediates. To explore the role PMI 5011 compounds on the glycosphingolipid pathway (Fig. 1) in relation to insulin signaling, we used tandem mass spectrometry and flow cytometry with monoclonal antibodies to quantify the intracellular level of glucosylceramides and cell surface expression of ganglioside monosialosyllactosylceramide (GM3), respectively, in rat skeletal muscle cells in the presence of excess FFAs and PMI 5011 bioactives. Our data demonstrates that despite significant ceramide accumulation, in the presence of excess FFAs, the bioactive compounds in PMI 5011 preserve insulin sensitivity. Although ceramide levels remained unchanged, one specific bioactive compound i.e., 2′,4-dihydroxy-4′- methoxydihydrochalcone (DMC 2) and its synthetic analog (Fig. 2) down-regulated the expression of glucosylceramide synthase (GCS) the enzyme that catalyses the transfer of the carbohydrate moiety from a sugar-nucleotide, uridine 5-diphosphate (UDP)-glucose to the ceramide resulting in lower glucosylceramide levels in the cells. Using an inhibitor of GCS (D–*threo*-1-phenyl-2 decanoylamino-3-morpholino-1-propanol hydrochloride [D-*threo*-PDMP]) and interference RNA (RNAi) we show that lowering of glycosphingolipids, without significant reduction of ceramides, reverses IR. Based on these observations, we postulated that PMI 5011 inhibition of glycosphingolipid synthesis is one of the mechanisms by which this botanical in increases insulin sensitivity in vitro and in vivo.

Materials and methods

Source and characterization of PMI 5011 and active compounds

The study used a well-characterized ethanolic extract of *Artemisia dracunculus* L., called PMI 5011, as well as four bioactive compounds previously identified and isolated from PMI 5011 using bioactivity-guided fractionation: davidigenin, sakuranetin, DMC-2 and 2′,4-dihydroxy-4′-methoxydihydrochalcone (DMC-1) as previously reported [6,9–11]. A chemically synthesized version of DMC-2 was also tested.

Standards and reagents

Ceramide and glucosylceramide standards were purchased from Avanti Polar Lipids (Alabaster, AL, USA). D-*threo*-PDMP and palmitic acid were obtained from Sigma-Aldrich (St. Louis , MO, USA). The predesigned RNAi for UDP-glucose ceramide glucosyltransferase (GCS) and its forward and backward primers were obtained from Ambion Life Technologies (Carlsbad, CA, USA).

Cell culture and treatments

L6 myoblasts were obtained from the American Type Culture Collection and maintained at 37°C, 95% air, and 5% CO_2 in low-glucose Dulbecco's modified Eagle's medium supplemented with 10% calf blood serum and antibiotics. For individual experiments, myoblasts were subcultured onto 6- or 12-well plates, grown to 80% to 90% confluence, and differentiated into fused myotubes for 5 d by switching to media with 2% horse serum. All cells used were within five passages.

Cultures were exposed to 250 μM palmitic acid conjugated with 1% bovine serum albumin (BSA). PMI 5011 extract and each of the five test compounds were

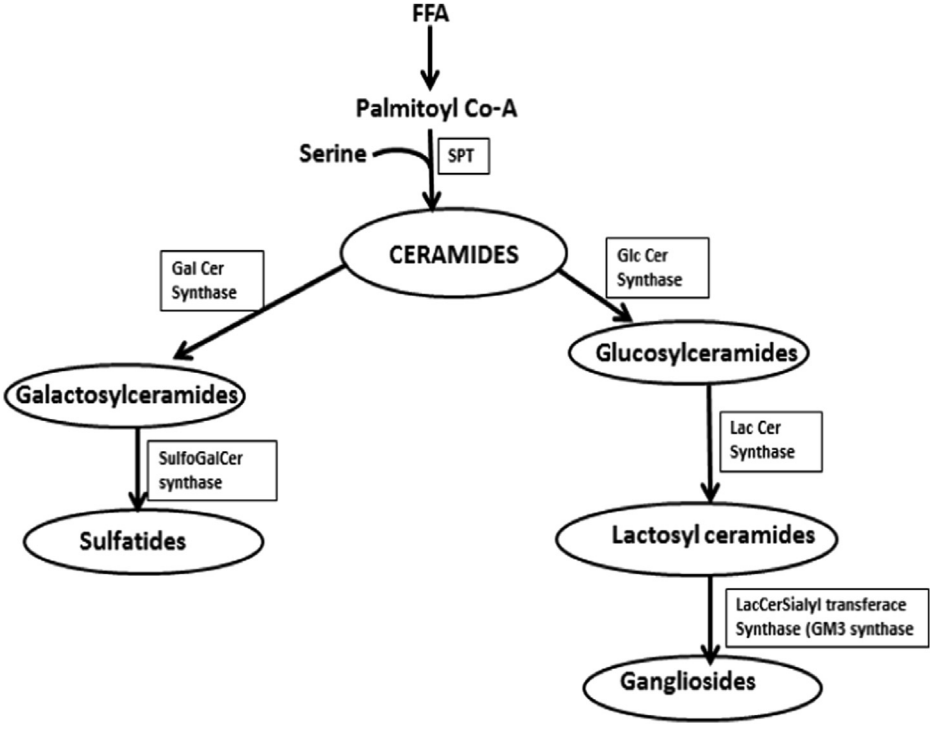

Fig. 1. Demonstrates ceramide metabolism pathway and cellular targets for PMI 50111 compounds. CO-A, coenzyme A; FFA, free fatty acid; GM3, ganglioside monosialosyllactosylceramide.