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Research Paper

Insulin-sensitizing and insulin-mimetic activities of *Sarcopoterium spinosum* extract

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ABSTRACT

Ethnopharmacological relevance: *Sarcopoterium spinosum* is an abundant plant in Israel, used by Bedouin medicinal practitioners for the treatment of diabetes. In our previous study we validated the anti-diabetic activity of *Sarcopoterium spinosum*. The aim of this study was to further clarify its mechanism of action.

Materials and methods: In-vivo studies were performed on KK-a/y mice given the extract for 6 weeks. Insulin tolerance test was performed, and relative pancreatic islets area was measured. Mechanisms of action were investigated in L6 myotubes using protein array, Western blot analysis and confocal microscopy. Glucose uptake assays were performed in 3T3-L1 adipocytes.

Results: *Sarcopoterium spinosum* extract reduced fasting blood glucose and improved insulin sensitivity in treated mice. Hypertrophic islets were detected in diabetic, but not in *Sarcopoterium spinosum*-treated mice. *Sarcopoterium spinosum* phosphorylated PTEN on ser380 and thr382/383, which are known inhibitory sites. PKB was not phosphorylated by *Sarcopoterium spinosum*, however, translocation of PKB from cytoplasm to the membrane and nucleus was detected. Target proteins of PKB were regulated by *Sarcopoterium spinosum*; GSK3β was phosphorylated and cytosolic localization of FoxO was increased. Glucose uptake was increased in a PI3K and AMPK-independent mechanism.

Conclusions: We suggest that *Sarcopoterium spinosum* inhibited PTEN and activated PKB by a mechanism which is independent of ser473 and thr308 phosphorylation. Other post translation modifications might be involved and should be analyzed further in order to understand this unique PKB activation. Identifying the active molecules in the extract, may lead to the development of new agents for the treatment of insulin resistance.

1. Introduction

Type 2 Diabetes mellitus (T2DM) is a common chronic metabolic disease with a prevalence of over 300 million people over the world, and a prediction of doubling this number by 2030 (Danaei et al., 2011). The disease is characterized by insulin resistance of target tissues, caused by reduced transmission of insulin signaling, combined with progressive functional deterioration and increased death of insulin secreting pancreatic β-cells. These two pathological processes are manifested by impaired glucose tolerance of affected individuals, leading to hyperglycemia, as well as other metabolic abnormalities, mainly impaired lipid profile. Several pharmacological agents are currently available for the treatment of T2DM, acting via different mechanisms of action, (including insulin secretagogues (sulfonylureas), insulin sensitizers (metformin), thiazolidinediones, α-glucosidase inhibitors, amylin analogs,

Abbreviations: AMPK, AMP-activated protein kinase; BAD, Bcl 2-antagonist of cell death; 2-DG, 2-deoxy-D-glucose; FoxO, forkhead transcription factor; GSK3β, glycogen synthase kinase 3β; IBMX, isobutylmethylxanthine; IKK, inhibitor of nuclear factor kappa-B kinase; IR, insulin receptor; ITT, insulin tolerance test; MAPK, mitogen-activated protein kinases; MEK, mitogen-activated protein kinase kinase; mTor, mammalian target of rapamycin; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; PIP3, phosphatidylinositol (3,4,5)-triphosphate; PI3K, phosphatidylinositide 3-kinase; PKA, protein kinase A; PKB, protein kinase B; PRAS40, proline-rich Akt substrate of 40 kDa; P70S6K, protein 70 kDa ribosomal subunit 6 kinase; PTEN, phosphatase and tensin homolog; *Sarcopoterium spinosum*, *Sarcopoterium spinosum*; T2DM, type 2 diabetes mellitus; RAS-GRF1, rat sarcoma related protein, G-protein regulated factor.

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incretin mimetics, as well as insulin) (Nelson, 2011). However, although the number of approved medications is growing, the goal of treatment, which is maintaining HbA1C of $<7\%$ is still difficult to be achieved; There is a significant fraction of patients that are not accurately responding to the medications, and failed to meet the desired glycemic goal (Khunti and Davies, 2010). In addition, it was reported that in those patients that are initially responding to the therapy, the hypoglycemic agents lose their effectiveness in a significant percentage of patients 3–5 years after the beginning of the treatment, as indicated by elevated HbA1C (Turner et al., 1999; Kahn et al., 2006). Lastly, there are some safety considerations with some of the agents (mainly Thiazolidinediones, but also metformin and others) (Nelson, 2011). These data emphasize the need for developing new drugs based on new molecules and mechanisms of action that might improve the glycemic control of the patients.

The plant kingdom may be described as a huge bank of compounds with different biological activities, which may be used for the treatment of various diseases, either when consumed as food supplements or as a basis for the development of chemically purified drugs. Over 400 plants are suggested by the traditional medicine for the treatment of diabetes (Bailey and Day, 1989; Samad et al., 2009), although only small number of these herbs had been appropriately evaluated. Among these medicinal plants is *Sarcopoterium spinosum* (L.) Sp. (*syn. Poterium spinosum* L.) (Dafni et al., 1984; Yaniv et al., 1987; Ali-Shtayeh et al., 2000; Saad et al., 2005; Al-Qura'n, 2009). *Sarcopoterium spinosum* is a chamaephyte of the Rosaceae family growing throughout the Mediterranean landscape. In the Arab folk medicine *Sarcopoterium spinosum* root cortex extraction is a known treatment for diabetes (Quisenberry and Gjerstad, 1967). Several studies confirmed the anti-diabetic function of *Sarcopoterium spinosum* (Schlutz and Venulet, 1964; Mishkinsky et al., 1966; Shani et al., 1970; Kasabri et al., 2011). In our previous study we showed the glucose lowering effect of the extract and demonstrated that the extract mimics several metabolic functions of insulin, including glucose uptake by myotubes, adipocytes and hepatocytes, glycogen synthesis and inhibition of lipolysis. In addition, *Sarcopoterium spinosum* extract increases viability of pancreatic β-cells and insulin secretion (Smirin et al., 2010). However, the intracellular signaling pathways that are affected by the plant extract and mediate its anti-diabetic activity had not been clarified yet. In this study we investigated the intracellular mechanism of action of *Sarcopoterium spinosum* roots decoction in L6 myotubes and 3T3-L1 adipocytes.

2. Materials and methods

2.1. Chemicals, kits and reagents

Isobutylmethylxanthine (IBMX), dexamethasone, insulin, 2-deoxy-*d*-glucose (2-DG) and inhibitors of proteases and phosphatases were purchased from Sigma. BSA, reagents and media for cell cultures were obtained from Biological Industries (Beit Haemek, Israel). [^{3}H]2-deoxy-*d*-glucose (1 mCi) was purchased from Perkin-Elmer. LY294002 and Compound C were purchased from CalBiochem, Insulin Receptor Phospho-Specific Antibody Microarray was purchased from Full Moon BioSystems. IR and Phospho-IR (tyr 1162/1163) antibodies were obtained from Santa-Cruz Biotechnology. Antibodies against PKB, phospho-PKB (ser 473, thr 308), FoxO, phospho-FoxO (thr 24/32), PTEN and phospho-PTEN (Ser 380, Thr 382/383), AMPK (thr172) and GSK3β (ser-9) were obtained from Cell-Signaling Technology. Secondary antibodies were purchased from Jackson ImmunoResearch.

2.2. Plant material

In order to obtain the roots, *Sarcopoterium spinosum* plants were uprooted from the open area outside the Ariel University, Israel. The plants were identified by the botanical staff of the University as *Sarcopoterium spinosum* (L.) Sp. A voucher specimen (no. HUJ 102531) of the plant has been deposited in the Herbarium of Middle Eastern Flora (Israel National Herbarium) at the Hebrew University of Jerusalem.

2.3. Plant extract preparation

In addition to the data published in ethnobotanical surveys (Dafni et al., 1984; Friedman et al., 1986; Yaniv et al., 1987; Ali-Shtayeh et al., 2000; Hamdan and Afifi, 2004), three local Bedouin medicinal plant practitioners from the Samaria and Negev regions in Israel were interviewed regarding the best method of extraction. The plant was shown to the informants, and its identity was confirmed. The plants were collected and the extracts were prepared according to their instructions. In accordance with the interviews, 100 g fresh *Sarcopoterium spinosum* roots were cut into small pieces on the same day and boiled in 1 L of water for 30 min. The solutions were left for 3 h and the red supernatants were transferred through cloth to a sterile bottle without disturbing the pellet, and kept at 4 °C. Concentrations of 0.01–1 mg/mL of this extract were used in the study.

2.4. Animal experiments

Animal House at the Ariel University operates in compliance with the rules and guidelines set down by the Israel Council for Research in Animals (Israel Ministry of Health), based on the US National Institutes of Health's Guide for the Care and Use of Laboratory Animals, DHEW (NIH, Pub. 78-23). All studies were approved by the institute committee on use and care of Animals. Institutional license number: IL090908.

KK-a/y strain mice, a common model used in the study of potential antidiabetic agents (Frode and Medeiros, 2008; Wang et al., 2013), were purchased from The Jackson Laboratory (Bar Harbor, ME) at age of 4 weeks. The mice were housed in a controlled environment of 20–24 °C, 45–65% humidity, and a 12 h (07:30–19:30) light/dark cycle. All experiments were performed on males, which were housed individually. Mice at age of 6 weeks old were separated into 3 groups: Control mice (KK-a/a), the diabetic, untreated mice (KK-a/y) and *Sarcopoterium Spinosum*-treated KK-a/y mice; $n=6$ in each group). The mice were fed ad-libitum standard rodent chow, and were given ad-libitum drinking water in the control groups, or *Sarcopoterium spinosum* extract instead of their drinking water, daily. Average consumption of water or the extract was measured, and found to be 15 mL/day, which is equal to 600 mg/kg/day powdery lyophilized extract.

At age of 16 weeks insulin tolerance test (ITT) was performed following 6 h of fasting. Glucose was measured at basal, and also 15, 30, 60, 90 and 120 min following intraperitoneal insulin injection (1.25 U/kg). At age of 17 weeks old fasting mice had been sacrificed. Pancreata were isolated, fixed in 4% paraformaldehyde and embedded in paraffin. Consecutive 4 μm sections were cut and stained with hematoxylin and eosin (H&E). The area of the islets and the whole sections were quantified using Image J software, and the percent of islets' area relatively to whole sections' area was calculated.

2.5. Cell culture

L6 myoblasts were grown in MEM-α containing 25 mM glucose, 10% FCS, 2 mM glutamine and 1% ampicillin. Experiments were