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Maslinic acid modulates glycogen metabolism by enhancing the insulin signaling pathway and inhibiting glycogen phosphorylase

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[ABSTRACT]

AIM: To investigate the molecular signaling mechanism by which the plant-derived, pentacyclic triterpene maslinic acid (MA) exerts anti-diabetic effects.

METHOD: HepG2 cells were stimulated with various concentrations of MA. The effects of MA on glycogen phosphorylase a (GPa) activity and the cellular glycogen content were measured. Western blot analyses were performed with anti-insulin receptor *β* (IR*β*), protein kinase B (also known as Akt), and glycogen synthase kinase-3*β* (GSK3*β*) antibodies. Activation status of the insulin pathway was investigated using phospho-IR*β*, as well as phospho-Akt, and phospho-GSK3*β* antibodies. The specific PI3-kinase inhibitor wortmannin was added to the cells to analyze the Akt expression. Enzyme-linked immunosorbent assay (ELISA) was used to measure the effect of MA on IR*β* auto-phosphorylation. Furthermore, the effect of MA on glycogen metabolism was investigated in C57BL/6J mice fed with a high-fat diet (HFD).

RESULTS: The results showed that MA exerts anti-diabetic effects by increasing glycogen content and inhibiting glycogen phosphorylase activity in HepG2 cells. Furthermore, MA was shown to induce the phosphorylation level of IR*β*-subunit, Akt, and GSK3β. The MA-induced activation of Akt appeared to be specific, since it could be blocked by wortmannin. Finally, MA treatment of mice fed with a high-fat diet reduced the model-associated adiposity and insulin resistance, and increased the accumulated hepatic glycogen content.

CONCLUSION: The results suggested that maslinic acid modulates glycogen metabolism by enhancing the insulin signaling pathway and inhibiting glycogen phosphorylase.

[KEY WORDS] Maslinic acid; Insulin signal transduction; Glycogen phosphorylation a; Glycogen metabolism

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Introduction

Abnormalities in glucose metabolism have been implicated in the etiology of several of the most common diseases affecting modern-day society, including diabetes [1], ischemic heart disease [2], stroke [3], and cancer [4]. An important component of the glucose metabolism process is glycogen metabolism, which itself plays a key role in several physiologic and pathologic processes. For example, under normal physiologic conditions, glycogen serves as an energy storage molecule, while dysfunctional glycogen metabolism can manifest as hyperglycemia [1], ischemic heart disease [2], and ischemic brain disease [3], much like dysfunctional glucose metabolism. Type 2 diabetes, in particular, is strongly associated with dysfunctional hepatic and peripheral glucose metabolism. The link between perturbed hepatic glycogen metabolism and diabetes involves its effects on insulin, which maintains blood glucose homeostasis [5].

The pentacyclic triterpenes have recently been recognized as bioactive compounds with therapeutic potential for a wide range of human diseases. Studies have demonstrated a variety of biological properties for these plant-derived compounds, including anti-inflammatory, hepatoprotective, gastroprotective, anti-ulcer, anti-viral (human immunodeficiency virus, HIV),

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anti-cancer, anti-diabetic, hypolipidemic, anti- atherosclerotic, and immunoregulatory effects [6]. Maslinic acid (MA) is a pentacyclic triterpene acid that is abundant in olive fruit skin, and has recently attracted much research attention due to its promising anti-tumor [7], anti-HIV [8], and anti-oxidation activities [9].

This laboratory recently reported that MA and other related pentacyclic triterpenes represent a new class of inhibitors of glycogen phosphorylase, and demonstrated that their glucose-lowering activity in adrenaline-induced diabetic mice might be due, at least in part, to modulation of hepatic glycogen metabolism [10-12]. In another study, several naturally-occurring pentacyclic triterpenes were characterized as potential new multi-target drugs for diabetes [13-18]. In that survey of more than 700 pentacyclic triterpene compounds, it was shown by our group that some were effective in lowering blood glucose levels in various diabetic animal models, including a rodent model of diabetes [19-21].

The aim of the present study was to gain insight into the cellular and molecular effects of MA on hepatic glycogen metabolism and the related signaling pathways using an *in vitro* cell system (HepG2 cells) and an *in vivo* mouse model fed with a high-fat diet.

Materials and Methods

Derivation of maslinic acid

Maslinic acid (Fig. 1) was derived from commercially available oleanolic acid (purity > 95%; Guangxi Changzhou Natural Products Development Co., Ltd.) using a semi-synthetic process previously described [16].

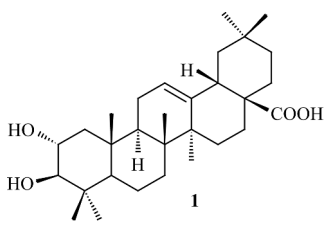

Fig. 1. Structure of maslinic acid (MA).

Cell cluture

HepG2 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in high (4.5 mg·mL^{-1}) glucose content Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (*V*/*V*) fetal bovine serum, 1% (*W*/*V*) glutamine, and 1% (*W*/*V*) penicillin/streptomycin (all reagents from Gibco-BRL, Invitrogen, San Diego, CA, USA) at 37°C in a humidified atmosphere of 5% CO_2/95% air. Cells were passaged by trypsinization (trypsin/EDTA; Sigma Chemical Co., St. Louis, MO, USA) when 90% confluency was reached.

Glycogen Phosphorylase a (GPa) activity assay

HepG2 cells (on 100 mm^2 plates) were used to determine the half maximal inhibitory concentration (IC_{50}) value of MA on GPa activity. First, the HepG2 cells were gently washed three times with ice-cold phosphate-buffered saline (PBS), covered with 50 mmol·L^{-1} Tris-HCl buffer (500 μL), and placed on ice for 30 min to allow hypotonic lysis. Then, the entire sample (lysed cells plus buffer) was harvested by scraping and centrifuged (12 000 *g*, 10 min) to obtain the GPa-containing supernatant and the total protein-containing lysate pellet. The pellet was solubilized by incubating in 1 M KOH, and total protein content was measured by the method of Lowry *et al.* [22].

The supernatant was used to measure GPa activity in the absence of AMP, as previously described [23]. The basic reaction mixture contained 50 mmol·L^{-1} KPi (pH 6.8), 0.8 mmol·L^{-1} EDTA, 0.4 mmol·L^{-1} $NADP^+$ disodium salt, 4 μmol·L^{-1} glucose-1, 6-bisphosphate tetracycloammonium salt, 1.7 U·mL^{-1} G6-PDH, 1 U·mL^{-1} phosphoglucomutase, and cell homogenate. Additionally, various concentrations of MA were present. The reaction was initiated with 2 mg·mL^{-1} glycogen, and the activity of GPa was measured by monitoring increases in the nicotinamide adenine dinucleotide phosphate (NADPH) concentration detected at 340 nm on a spectrophotometer (Tecan, Männedorf, Switzerland).

RT-qPCR

Total RNA was isolated from HepG2 cells using the TRIzol Reagent (Invitrogen) and following the manufacturer's protocol. Complementary DNA was then synthesized by reverse transcription using the Superscript First-Strand Synthesis Kit (Fermentas, Vilnius, Lithuania) and used to measure the gene transcription levels of glycogen phosphorylase by qPCR. The glycogen phosphorylase and GAPDH (internal control) genes were amplified using the SYBR Green Master Mix (Qiagen, Hilden, Germany) and the following primers: glycogen phosphorylase, (forward) 5′- GAT GGT GTA GGA ACC GTG TT -3′ and (reverse) 5′- ATG CGG TCG ATG TCT TTA GG - 3′; GADPH, (forward) 5′-ACC ACA GTC CAT GCC ATC AC-3′ and (reverse) 5′-TCC ACC ACC CTG TTG CTG TA-3′. The thermal cycling reaction was carried out in an iCycler IQ5 instrument (Bio-Rad, Hercules, CA, USA) using the following conditions: initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing for 10 s at 62 °C, and extension at 72 °C for 10 s. The glycogen phosphorylase data was normalized to GAPDH and the relative expression was calculated as $2^{-\Delta ct} \times 100\%$, where Δct was the difference in the ct value between the target gene and GAPDH. All qPCRs were performed in triplicate using three independent samples.

Glycogen content assay

HepG2 cells were seeded in 6-well plates at a density of 5×10^4/mL. When the cells reached 80% confluency, MA was added (to achieve 0.1, 1, and 10 μmol·L^{-1} final concentrations per well) and the cells were incubated for an additional 24 h. The MA affects on glycogen content were measured as previously described [18]. Briefly, the cells were washed three times with ice-cold PBS and lysed by 0.1 mol·L^{-1} NaOH (400 μL). The lysates were then heated at 80°C for 1 h and the glycogen was precipitated by adding 2.5 volumes of ethanol. The samples were placed at 25 °C for 24 h and then centrifuged for 15 min at 12 000 *g*. The pellet was freeze-dried and re-suspended in 50 mmol·L^{-1} sodium acetate buffer (pH 4.8). The glycogen was digested with amyloglucosidase at 37 °C for 90 min, and the resulting glucose was