Experimental Gerontology xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

Genipin ameliorates age-related insulin resistance through inhibiting hepatic oxidative stress and mitochondrial dysfunction

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ARTICLE INFO

Article history:
Received 10 May 2013
Received in revised form 6 September 2013
Accepted 9 September 2013
Available online xxxx

Section Editor: Dr. Andrzej Bartke

Keywords:
Genipin
Oxidative stress
Insulin resistance
Mitochondria
Aging

ABSTRACT

Insulin resistance (IR) increases with age and plays a key role in the pathogenesis of type 2 diabetes mellitus. Oxidative stress and mitochondrial dysfunction are supposed to be major factors leading to age-related IR. Genipin, an extract from *Gardenia jasminoides* Ellis fruit, has been reported to stimulate insulin secretion in pancreatic islet cells by regulating mitochondrial function. In this study, we first investigated the effects of genipin on insulin sensitivity and the potential mitochondrial mechanisms in the liver of aging rats. The rats were randomly assigned to receive intraperitoneal injections of either 25 mg/kg genipin or vehicle once daily for 12 days. The aging rats showed hyperinsulinemia and hyperlipidemia, and insulin resistance as examined by the decreased glucose decay constant rate during insulin tolerance test (kITT). The hepatic tissues showed steatosis and reduced glycogen content. Hepatic malondialdehyde level and mitochondrial reactive oxygen species (ROS) were higher, and levels of mitochondrial membrane potential (MMP) and ATP were lower as compared with the normal control rats. Administration of genipin ameliorated systemic and hepatic insulin resistance, alleviated hyperinsulinemia, hyperglyceridemia and hepatic steatosis, relieved hepatic oxidative stress and mitochondrial dysfunction in aging rats. Furthermore, genipin not only improved insulin sensitivity by promoting insulin-stimulated glucose consumption and glycogen synthesis, inhibited cellular ROS overproduction and alleviated the reduction of levels of MMP and ATP, but also reversed oxidative-stress associated with JNK hyperactivation and reduced Akt phosphorylation in palmitate-treated L02 hepatocytes. In conclusion, genipin ameliorates age-related insulin resistance through inhibiting hepatic oxidative stress and mitochondrial dysfunction.

1. Introduction

Type 2 diabetes mellitus (T2DM) is the most common chronic metabolic disease in the elderly, which seriously impairs their life quality and lifespan due to specific complications. The prevalence of diabetes in the elderly is higher and increasing covering at least 50 million individuals 65 years of age or older in 2000 and estimated 130 million in 2030 in the world (Wild et al., 2004). Insulin resistance plays a key role in the pathogenesis of T2DM in the elderly (Krentz et al., 2013; Viljoen and Sinclair, 2011). Evidence showed that insulin sensitivity gradually decreased during the aging process in humans and animals (Carvalho et al., 1996; DeFronzo, 1981; Goodman et al., 1983). However, the exact mechanisms involved in age-related insulin resistance have not been adequately defined.

There is growing evidence that oxidative stress and mitochondrial dysfunction are the important factors leading to insulin resistance during the aging process (Petersen et al., 2003; Phielix et al., 2011; Rains and Jain, 2011). Oxidative stress increases with age and exacerbates stress signals contributing to the development of insulin resistance. Mitochondria are the primary origin of reactive oxygen species (ROS) as inevitable by-products of oxidative phosphorylation. Excessive metabolic substrates usually produced during aging flux into the mitochondria resulting in the spin-down of electrons and overproduction of ROS, which can induce oxidative stress and then impair mitochondrial function to form a vicious cycle. Indeed, mitochondria-derived ROS has been considered as an initiating agent of palmitate-induced insulin resistance (Nakamura et al., 2009). Lee et al. further confirmed that overexpression of catalase in mitochondria of protected mice from age-associated mitochondrial damage and insulin resistance (Lee et al., 2010). Therefore, targeting antioxidants to mitochondria seems to be a promising strategy for preventing and ameliorating insulin resistance in elderly population.

Genipin is an aglycone derived from geniposide, an iridoid glucoside extracted from *Gardenia jasminoides* Ellis fruit which has long been used

Abbreviations: Akt, also called protein kinase B (PKB); ATP, adenosine tri-phosphate; BCA, bicinchoninic acid; H2DCFDA, 2,7-dichlorofluorescein diacetate; IR, insulin resistance; ivITT, intravenous insulin tolerance tests; JNK/SAPK, c-Jun kinases/stress-activated protein kinase; kITT, the glucose decay constant rate during insulin tolerance test; MDA, malondialdehyde; MMP, mitochondrial membrane potential; ROS, reactive oxygen species; T2DM, type 2 diabetes mellitus; TC, total cholesterol; TG, triacylglycerols.

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0531-5565/$ – see front matter © 2013 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.exger.2013.09.001

in Asian countries as an herbal medicine with choleretic, hepatoprotective effect and hypoglycemic effect (Kimura et al., 1982; Miyasita, 1976; Tseng et al., 1995). Genipin has been reported to possess various pharmacological actions such as anti-oxidation, anti-inflammation and anti-apoptosis (Kim et al., 2010; Koo et al., 2004). Zhang et al. proposed that genipin could reverse obesity- and high glucose-induced β cell dysfunction and stimulate insulin secretion by modulating mitochondrial function (Zhang et al., 2006). However, the effect of genipin on insulin resistance and the potential mechanisms remain unknown.

It is well known that the liver plays a central role in maintaining glucose and lipid metabolism. As the primary site of insulin action, it is inherently linked to the development of systemic insulin resistance. In fact, hepatic insulin resistance has been reported to be the primary event leading to diabetes and the subsequent development of peripheral tissue insulin resistance (Michael et al., 2000). Though genipin has been used as a hepatoprotective agent (Kim et al., 2010; Shang et al., 2009), its effect on hepatic insulin resistance is short of reports.

In this study, the effect of genipin on age-related insulin resistance was explored and the mitochondrial mechanisms were investigated in 18-month-old SD rats and palmitate-treated L02 hepatocytes.

2. Materials and methods

2.1. Reagents

Genipin was purchased from WaKo Pure Chemical Industries (Osaka, Japan). Palmitate was purchased from Sigma-Aldrich (St Louis, MO, USA). Antibodies against phospho-JNK/SAPK (Thr183/Tyr185), JNK/SAPK, phospho-Akt (ser473) and Akt (pan) were all purchased from Cell Signaling Technology (Danvers, MA). Anti-rabbit secondary antibodies conjugated to horseradish peroxidase were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). RPMI 1640 medium, fetal bovine serum and other cell culture products were from Gibco-Invitrogen (Scotland, UK).

2.2. Animals and treatments

All animal studies were consistent with the guidelines for the care and use of medical laboratory animals (Ministry of Health, China) and were approved by Animal Ethics Committee of Dalian Medical University. Male Sprague–Dawley rats (3 and 18 months old) used here were housed in climate-controlled quarters with 12 h light cycle and were fed *ad libitum* throughout their lifespan. The 18-month-old rats were randomly assigned to receive intraperitoneal injections of either genipin of 25 mg/kg (aging genipin-treated group, AG) or vehicle (aging control group, AC) once daily for 12 days (Okada et al., 2007; S.Y. Wu et al., 2009). The 3-month-old rats received vehicle alone (normal control group, NC). After administration, at least six rats from each group randomly were used to perform intravenous insulin tolerance tests. The other rats were sacrificed after 12 h fasting. Blood samples were collected for measurement of fasting plasma glucose, triacylglycerols (TG) and total cholesterol (TC) by using a biochemical analyzer (HITACHI 7600-020, Tokyo, Japan) and fasting plasma insulin by ELISA kit (Mercodia AB, Sylveniusgatan, Uppsala, Sweden). Fresh liver tissues were used for measurements of levels of mitochondrial ROS, mitochondrial membrane potential (MMP) and ATP. Some of the liver remnants were removed, fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (HE) and examined blindly. The rest of the liver remnants were collected and snap-frozen in liquid nitrogen and stored at −80 °C for malondialdehyde (MDA) determination and glycogen analysis.

2.3. Intravenous insulin tolerance test

Intravenous insulin tolerance tests (ivITT) were performed on 6 h fasted rats injected with 0.75 U/kg human regular insulin (Novo Nordisk, North Carolina, USA). Blood glucose was measured at 0, 4, 8, 12, and 16 min after injection using a glucometer (Accu-check Advantage, Roche, Mannheim, Germany). Glucose values were then converted into natural logarithm (Ln); the slope was calculated using linear regression [time × Ln (glucose)] and multiplied by 100 to obtain the glucose decay constant rate during insulin tolerance test (kITT) per minute (Lehnen et al., 2010).

2.4. Analysis of glycogen contents of hepatic tissues

Glycogen contents of hepatic tissues were assayed according to the method described before (Capozza et al., 2005) with slight modifications. In brief, the hepatic tissues were weighed and treated with 1 N NaOH (3:1, w/v) at 100 °C for 20 min. Glycogen was precipitated from the supernatant with ethanol (2:1, v/v). The glycogen pellet was dissolved in water and glycogen content was determined by anthrone-reagent method.

2.5. Determination of MDA content in hepatic tissues

The stored liver remnants were homogenized in ice-cold phosphate buffered saline and then centrifuged at 800 *g* for 15 min. The supernatants were used to detect MDA contents by the recommended steps using a commercial kit (Jiancheng Bioeng, Nanjing, China). Protein concentration was determined by the bicinchoninic acid (BCA) protein assay according to the manufacturer's instructions (Beyotime Biotech, Shanghai, China).

2.6. Hepatic mitochondria isolation and analysis of MMP and ROS

Hepatic mitochondria were isolated from freshly harvested livers by differential centrifugation as we have described before (Q. Wu et al., 2009). MMP was determined using rhodamine 123, a fluorescent cationic dye labeling mitochondria as described previously (Emaus et al., 1986). ROS generation was assessed using the probe 2,7-dichlorofluorescein diacetate (H2DCFDA) which can be cleaved by esterases and then oxidized by ROS in cells and mitochondria to yield the fluorescent product DCF (Duranteau et al., 1998). The fluorescence was assayed using a fluorescence spectrophotometer (HITACHI 650-60).

2.7. Determination of ATP levels in hepatic tissues

ATP levels in the hepatic tissues were assayed as described previously (Q. Wu et al., 2009). Freshly harvested liver remnants were rapidly homogenized in ice cold buffer. The homogenate solutions were boiled for 3 min and then centrifuged at 800 *g* for 10 min. ATP levels in the supernatants were detected by ATP bioluminescent assay kit (Roche, Mannheim, Germany).

2.8. L02 cell culture and assignment

The L02 hepatocytes (China Center for Type Culture Collection) were exposed to 0.25 mM palmitate for 24 h in the presence or absence of genipin at 50 μM or 100 μM respectively. The doses of palmitate and genipin were selected according to cell viability assays (Supplemental Figure 1) and previous studies (Gao et al., 2010; Kojima et al., 2011). Control cells were treated with the solvent of palmitate only.

For glycogen determination, cells were incubated for 3 h in serum-free RPMI 1640 medium containing 100 nM insulin. Glycogen was precipitated and detected as mentioned in Section 2.4. Glucose consumption assays were carried out as previously described (Wan et al., 2009). Cells were cultivated in RPMI 1640 medium containing 11.1 mM glucose and 100 nM insulin for 12 h. Glucose concentration in culture medium was determined with a glucose assay kit (Sigma). Glucose consumption levels were the differences of glucose levels in culture medium of parallel well without cells and test sample well. The changes of MMP and ROS

Please cite this article as: Guan, L., et al., Genipin ameliorates age-related insulin resistance through inhibiting hepatic oxidative stress and mitochondrial dysfunction, Exp. Gerontol. (2013), http://dx.doi.org/10.1016/j.exger.2013.09.001