Journal of Ethnopharmacology 148 (2013) 27–36

Contents lists available at SciVerse ScienceDirect

Journal of Ethnopharmacology

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

Protection of glycyrrhizic acid against AGEs-induced endothelial dysfunction through inhibiting RAGE/NF-κB pathway activation in human umbilical vein endothelial cells

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ARTICLE INFO

Article history:
Received 21 November 2012
Received in revised form
7 March 2013
Accepted 8 March 2013
Available online 22 March 2013

Keywords:
AGEs
Endothelial dysfunction
Apoptosis
Inflammation
Oxidant stress
RAGE

ABSTRACT

Ethnopharmacological relevance: Licorice (*Glycyrrhiza uralensis* roots) is used as a traditional medicine for the treatment of diabetes mellitus and its vascular complications. Glycyrrhizic acid (GA, also known as Glycyrrhizin), a triterpenoid saponin glycoside, is considered to be a bioactive component in Licorice and is beneficial to diabetic vascular complications.

Aim of study: The present study was conducted to evaluate the potential protective activities on AGEs-induced endothelial dysfunction, including anti-apoptosis, antioxidant stress and anti-proinflammatory responses, and explore the underlying mechanism.

Materials and methods: Human umbilical vein endothelial cells (HUVECs) were incubated and pre-treated with GA (10^{-9}–10^{-6} M) or RAGE-Ab (5 μg/ml) in the presence or absence of 200 μg/ml AGEs. AO/EB fluorescence staining assay was performed to evaluate anti-apoptosis activity. The superoxide dismutase (SOD) activity and malondialdehyde (MDA) level in cell supernatant were detected by kits while the intracellular reactive oxygen species (ROS) generation was determined by 2,7-dichlorodihydrofluorescin diacetate (DCFH-DA) kit. Immunocytochemistry analysis was designed to determine transforming growth factor beta1(TGF-β1) protein expression while immunofluorescence analysis for RAGE and NF-kB. The protein expressions of TGF-β1, RAGE and NF-kB were analyzed by Western blot analysis.

Results: Pretreatment with GA at a concentration of 10^{-8}–10^{-6} M significantly reduced the AGEs-induced apoptosis in HUVECs. GA significantly increased antioxidant enzyme SOD activity and decreased peroxide degradation product MDA level in a dose-dependent manner. Furthermore, GA also remarkably inhibited the overgeneration of AGEs-induced ROS. Both immunocytochemistry analysis and western blot analysis showed that GA significantly decreased the protein expression of poinflammatory cytokine TGF-β1 in a similar manner which RAGE-Ab did. Additionally, AGEs-induced RAGE and NF-kB protein expressions were down-regulated significantly by the pretreatment with GA or RAGE-Ab.

Conclusion: These findings provide evidences that GA possesses protective activity on AGEs-induced endothelial dysfunction, including anti-apoptosis, anti-inflammation and antioxidant stress, *via* inhibiting RAGE/NF-kB pathway. GA might be an alternative for the prevention and treatment of diabetic vascular complications in an appropriate dosage.

1. Introduction

Endothelial dysfunction has been regarded as one of the essential causes involved in the pathogenesis of diabetes mellitus and its vascular complication diseases (Naka et al., 2012). Many factors have been found to be associated with endothelial dysfunction and contribute to the onset and development of diabetic vascular complications. Advanced glycation end products (AGEs), the non-enzymatic glycation of reducing sugar and the amino groups of lipids, nucleic acids and proteins, have been found to

Abbreviations: AGEs advanced glycation end products; ROS, reactive oxygen species; GA, glycyrrhizic acid; HUVECs, human umbilical vein endothelial cells; AO/EB, acridine orange/ethidium bromide; BSA, non-glycated bovine serum albumin; AG, aminoguanidine; VE, Vitamin E; SOD, superoxide dismutase; MDA, malondialdehyde; TGF-β1, transforming growth factor beta1; DMEM, Dulbecco's modified Eagle's medium; DCF, 2,7-dichlorofluorescein; DCFH-DA, 2,7-dichlorodihydrofluorescin diacetate.

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http://dx.doi.org/10.1016/j.jep.2013.03.035

trigger and aggravate the endothelium damage in diabetic vascular complications (Alkhalaf et al., 2012; Morita et al., 2012). Many experimental studies have identified that the interaction between AGEs and their receptor RAGE elicits intracellular oxidative stress and then leads to cascade signaling, such as inflammation and apoptosis responses (Pazdro and Burgess, 2012; Schaffer et al., 2012). The over-generation of AGEs-induced reactive oxygen species (ROS) is partially caused by sustained NF-kB activation in endothelial cells (Morita et al., 2012). Moreover, AGEs also upregulate the expression of transforming growth factor-β1 (TGF-β1) which is associated with the pro-inflammation response in endothelial dysfunction (Feng et al., 2012).

Glycyrrhizic acid (GA, also known as Glycyrrhizin, 3-*O*-(2-*O*-beta-D-glucopyranuronosyl-alpha-D-glucopyranuronosyl)-18beta-glycyrrhetinic acid, Chemical formula: $C_{42}H_{62}O_{16}$, molecular weight=822.94), a triterpenoid saponin glycoside, is a conjugated compound of glycyrrhetinic acid and glucuronic acid (Fig. 1) (Asl and Hosseinzadeh, 2008). Phytochemical study shows that GA is one of the major compounds in the root of *Glycyrrhiza uralensis* Fish. (Fabaceae). It has been well shown that GA has various potential bioactivities, such as antioxidant, anti-virus, cardiovascular protection (Tripathi et al., 2009), immunomodulatory, hepatoprotective (Haleagrahara et al., 2011), as well as resistance to endothelium damage (Quaschning et al., 2001), etc. There is a growing body of evidence indicating that GA has an hypoglycemic effect in noninsulin-dependent diabetes model mice (Takii et al., 2001). Additionally, GA prevents venous thrombosis in infusion rat model by suppressing the adherence of neutrophils to the venous endothelium (Nakata et al., 2008). However, the protection of GA on AGEs-induced endothelial dysfunction and its underlying mechanisms are not fully elucidated.

In the present study, we have investigated the molecular mechanism of GA on AGEs-induced endothelial dysfunction in human umbilical vein endothelial cells (HUVECs). The protection effect of GA on AGEs-induced endothelial dysfunction in HUVECs *via* RAGE/NF-kappa B dependent pathway was studied. Our findings in this study may provide a better insight for understanding the potential therapeutic effect of GA on the development of diabetic vascular complications.

2. Material and methods

2.1. Reagents and materials

Glycyrrhizin (purity >99%) was purchased from National Institute For the Control of Pharmaceutical and Biological Products (Beijing, PR China). Acridine orange (AO), ethidium bromide (EB), D-glucose, bovine serum albumin, sodium dodecyl sulfate (SDS), phenylmethylsulphonyl fluoride (PMSF), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and positive drug aminoguanidine (AG) and Vitamin E (VE) were purchased from Sigma (St. Louis, MO, USA). Reactive oxygen species (ROS) DCFH-DA kit was provided by Beyotime Institute of Biotechnology (Nantong, PR China). Superoxide dismutase (SOD) and malondialdehyde (MDA) kits were ordered from Nanjing Jiancheng Bio Co., Ltd.(Nanjing, PR China). SABC kit was offered by Boster (Wuhan, PR China). Immunofluorescence assay rabbit anti-RAGE FITC conjugated kit was purchased from Shanghai Boyao Biotechnology Co., Ltd. (Shanghai, PR China). Transforming growth factor beta1 (TGF-β1), p-NF-kB p65, Bax and RAGE antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Human AGEs ELISA kit was ordered from Shanghai Ximei Biological Technology Co., Ltd. (Shanghai, PR China). In addition, all other materials were from commercial sources.

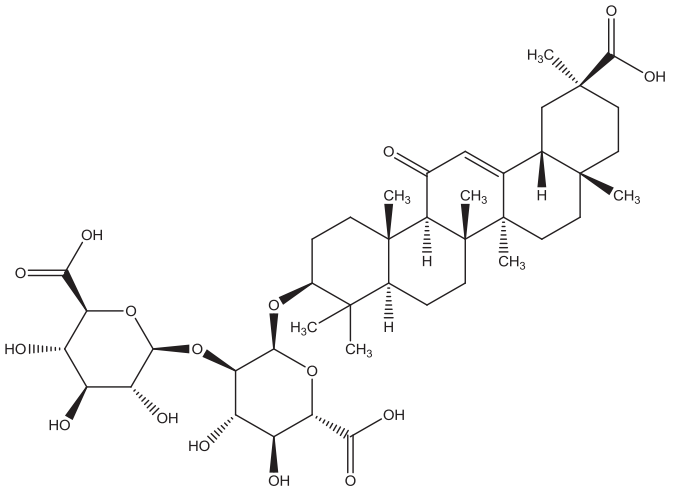

Fig. 1. The chemical structure of Glycyrrhizic acid (GA, chemical formula: $C_{42}H_{62}O_{16}$, molecular weight=822.94).

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) line, obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), was maintained in basal Dulbecco's modified Eagle's medium (DMEM) medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), low-glucose, 80 units/ml of penicillin/streptomycin. Cells were grown on plastic cell culture flasks in a cell incubator at 37 °C and in 95% air/5% CO_2. Medium was changed every 2 days. Cells at 2–4 passages were used for the experiments until they generated a 80–90% confluent layer.

2.3. Preparation of AGEs

The preparation of AGEs proteins, i.e. AGEs-BSA, was performed as described previously (Franke et al., 2009; Xu et al., 2011a). In brief, bovine serum albumin of 5 g and D-glucose of 9 g were dissolved in 0.2 M phosphate buffered saline (PBS, pH=7.2). The mixture was filtrated through 0.22 μm microporous film and incubated at 37 °C under sterile conditions for 12 weeks. At the end of this reaction, unincorporated glucose were then removed by dialysis against PBS (pH=7.2). The final AGEs was determined for its identification in a Gemini EM fluorescence microplate reader (Molecular Devices, USA) at excitation/emission 370/440 nm. The content of AGEs was measured by AGEs ELISA. Moreover, blank control non-glycated BSA (BSA) was incubated in the same conditions with the absence of D-glucose.

2.4. AO/EB fluorescence staining assay for apoptosis

Acridine orange/ethidium bromide (AO/EB) fluorescence staining of HUVECs was performed to evaluate the prevention of GA on AGEs-induced cell apoptosis. HUVECs growed on a coverslip coated with polylysine in 24-well plates. And then cells were incubated at 37 °C and in 5% CO_2 for 48 h. After being washed with PBS for three times, cells were stained with AO/EB (100 mg/ml of AO in PBS; 100 mg/ml EB in PBS) just prior to fluorescence microscopy (Wang et al., 2011b). In this experiment, the normal cells were stained only by AO with bright green while apoptotic cells stained by AO and EB with red–orange. The degree of apoptosis was calculated according to the dense orange areas/bright green areas with Image-Pro Plus software.

2.5. Assays for SOD activity and MDA content

HUVECs were stimulated for 48 h by 200 μg/ml AGEs in the presence or absence of GA. The assays for SOD activity and MDA