Inhibitory Effects of Hydroxysafflor Yellow A on the Formation of Advanced Glycation End Products in Vitro

Zhenzhen Ni, Zhengbing Zhuge, Wenlu Li, Huimin Xu, Zhongmiao Zhang and Haibin Dai

Department of Pharmacy, Second Affiliated Hospital, Zhejiang University School of Medicine; Hangzhou 310009, China; Department of Pharmacy, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University; Hangzhou 310006, China; and Cancer Institute, Zhejiang University; Hangzhou 310009, China.

Received March 14, 2012; accepted August 21, 2012; advance publication released online August 31, 2012

To investigate the inhibitory effects of hydroxysafflor yellow A (HSYA) on the protein glycation in vitro. Using bovine serum albumin (BSA)-glucose assay, BSA-methylglyoxal (MGO) assay, and N-acetylglucosyl-lysine methyl ester (G.K.) peptide-ribose assay, inhibitory effects of HSYA were investigated. Advanced glycation end products (AGEs) production was assessed by AGEs-specific fluorescence and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In BSA-glucose assay, HSYA concentration dependently decreased AGEs formation, with maximum inhibitory effects at 1 mM by 95%. Further more, HSYA also showed significant inhibitory effects on MGO-mediated protein modification and subsequent cross-linking of proteins. Finally, when co-incubated with G.K. peptide and ribose, HSYA exhibited its antiglycation effects, and the maximum inhibitory effects of HSYA at 1 mM were 84%. Overall, our present study provides the first evidence of the antiglycation effects of HSYA on AGEs formation in vitro.

Key words hydroxysafflor yellow A; glycation; advanced glycation end product; inhibitor of glycation

Advanced glycation end products (AGEs) are a group of complex and heterogeneous compounds. They are the final products of the nonenzymatic reaction between reducing sugars and amino groups in proteins, lipids, and nucleic acids.1,2) AGEs can be divided into brown and fluorescent cross-linking substances such as pentosidine, nonfluorescent cross-linking products such as methylglyoxal-lysine dimers (MOLD), or nonfluorescent, noncross-linking adducts such as carboxymethyllysine (CML) and pyrraline (a pyrrole aldehyde).3,4) Recently, AGEs accumulation in vivo has been implicated as a key pathogenic process in diabetic complications, including neuropathy, nephropathy, retinopathy, cataract and other health disorders such as atherosclerosis, Alzheimer's disease, and normal aging.5–10) Therefore, the discovery and investigation of AGEs inhibitors would offer a potential therapeutic approach for the prevention and treatment of diabetic or other pathogenic complications.

Many compounds were introduced as AGE inhibitors based on their inhibition of AGE formation during incubation of proteins with glucose in vitro. These inhibitors vary widely in structure, the common theme being their nucleophilicity or reactivity with reactive carbonyl intermediates in AGE formation.11) Synthetic products such as aminoguanidine,12) LR compounds13–15) and naturally occurring compounds such as flavonoids,16) cinnamon bark proanthocyanidins17) have been studied for their antiglycation activities in vitro and in vivo. The flower of the safflower plant, Carthamus tinctorius L., has been broadly used in traditional Chinese medicine for treatment of cerebrovascular and cardiovascular diseases.18,19) Hydroxysafflor yellow A (HSYA) (Fig. 1) is a major chemical component of the safflower yellow pigments. It is reported that HSYA has neuroprotective effects through its antioxidative action and can alleviate liver fibrosis, acute lung injury.18–21) Recently, we further showed that HSYA may exert potential therapeutic strategies to improve outcome following traumatic brain injury, which may be through improving mitochondrial activity, antioxidant activity, and antiinflammatory or fibrinolytic effects.22) In theory, the antioxidative action may influence glycation processes as well, yet the effects of HSYA on AGES formation have remained uninvestigated. Here, using bovine serum albumin (BSA)-glucose assay, BSA-methylglyoxal (MGO) assay and N-acetylglucosyl-lysine methyl ester (G.K.) peptide-ribose assay,16) we reported the first evidence of the antiglycation action of HSYA on AGEs formation in vitro.

Materials and Methods

Materials Aminoguanidine (AG), BSA (fraction V, essentially fatty acid free), 1-glucose, ribose and methylglyoxal (MGO, 40%w/v) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). N-Acetylglucosyl-lysine methyl ester (G.K.) peptide was purchased from Bachem AG (Bubendorf, Switzerland). Hydroxysafflor yellow A (HSYA, purity ≥98%) was purchased from Yuanye Biotech. Co. (Shanghai, China).

BSA-GLUCOSE ASSAY The BSA-glucose assay was used to evaluate the ability of HSYA to inhibit the glucose-mediated formation of AGEs. The BSA-glucose assay was used to evaluate the ability of HSYA to inhibit the glucose-mediated formation of AGEs. The BSA-glucose assay was used to evaluate the ability of HSYA to inhibit the glucose-mediated formation of AGEs.
protein glycation using the method described by Rahbar et al.\textsuperscript{14}. Briefly, BSA (50 mg/mL) and glucose (144 mg/mL) in phosphate buffer (pH 7.4) containing 0.2 g/L NaN\textsubscript{3} were incubated under sterile, dark conditions at 37°C for 7 d. In certain experiments, the indicated HSYA was added to the model system in the concentration range of 0.01–1 mM. Fluorescence of samples was measured at the excitation and emission maxima of 330 nm and 410 nm, respectively.

**BSA-MGO Assay** BSA-MGO assay was adopted from Wu and Yen\textsuperscript{16} with some modification, which was used for investigation of inhibitors on middle stage of the glycation of protein. BSA (50 mg/mL) was incubated with 100 mM MGO under sterile, dark conditions in 0.1 M phosphate buffer (pH 7.4) at 37°C for 24 h in the presence or absence of various concentrations of the compounds. In certain experiments, the indicated HSYA was added to the model system in the concentration range of 0.01–1 mM. Fluorescence of samples was measured at the excitation and emission maxima of 330 nm and 410 nm, respectively. AG (50 mM) was used as a positive control. The effects of MGO modification on the cross-linking and aggregation of BSA were investigated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 4% stacking and 10% separating gel. Protein map was visualized by Coomassie blue stain.

**G.K. Peptide-Ribose Assay** G.K. peptide-ribose assay was used to evaluate the ability of HSYA to inhibit the cross-linking of G.K. peptide (last glycation products) in the presence of ribose according to the method of Rahbar et al.\textsuperscript{14}. G.K. peptide (26.7 mg/mL) was incubated with 40 mg/mL ribose under sterile, dark conditions in 0.5 M sodium phosphate buffer (pH 7.4) at 37°C for 24 h. The HSYA was added to the model system in the concentration range of 0.01–1 mM, respectively. At the end of the incubation, samples were analyzed for specific fluorescence (excitation, 340 nm; emission, 420 nm).

**Data Presentation and Statistical Analysis** The % inhibition of AGEs formation = \[\frac{1 - \text{fluorescence of the test group}}{\text{fluorescence of the control group}}\] \times 100%. Results were expressed as the mean±S.D. for the number of separate experiments indicated. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Newman–Keuls test for multiple comparison tests (performed with SPSS software). p < 0.05 was assumed to be statistical different.

**RESULTS**

**Inhibitory Effects of HSYA on Glucose-Mediated Development of Fluorescence of AGEs** When BSA was incubated with glucose for 7 d, the fluorescence intensity of AGEs was increased to about 30000 A.U., which was significantly inhibited by HSYA at concentration 0.1, 0.5 and 1 mM (Fig. 2). The inhibitory effects of 0.1 mM HSYA (35%) were similar to that of 50 mM aminoguanidine (AG) (41%). At the concentration of 0.5 mM and 1 mM, the inhibitory effects of HSYA were 84%, 95%, respectively, which indicated that HSYA was more effective than AG in the prevention of high glucose-mediated protein modification. In this study, the fluorescence intensity of BSA, HSYA (1 mM) and BSA+HSYA (1 mM) were 2263, 887 and 2187 A.U., respectively.

**Inhibitory Effects of HSYA on MGO-Mediated BSA Glycation** The inhibitory effects of MGO-mediated protein glycation by HSYA were determined by fluorescence formation and protein electrophoresis (Fig. 3). HSYA concentration dependently decreased BSA glycation as measured by fluorescence (Fig. 3A). At the concentration of 0.1, 0.5 and 1 mM, HSYA showed significantly inhibitory effects by 11%, 46% and 71%, respectively. AG (50 mM) also exhibited inhibitory effects by 98%. It was shown that MGO can readily react with protein residues such as lysine to produce high molecular weight, cross-linked products.\textsuperscript{4,23} This could be demonstrated...
Diabetes is characterized by high glucose concentrations, which leads to an increased production of ROS. The increased level of ROS leads to damage to various cell components and triggering the activation of specific signaling pathways. It has been reported that HSYA exhibits antioxidative effects, alleviates oxidative stress. In the rat brain, HSYA inhibits the opening of mitochondrial permeability transition pore by a free radical scavenging action. Thus, it would be of great interest to identify the inhibitory effects of HSYA in protein glycation. Our present data clearly revealed that HSYA possesses an antiglycation effects comparable to that of AG in the formation of AGEs in vitro, which may further help to find therapeutic applications of HSYA for the prevention of diabetic complications, as well as other diseases associated with increased glycation of proteins or lipids.

Acknowledgments This project was supported by the Grant from the National Natural Science Foundation of China (81173040), the Zhejiang Province Traditional Chinese Medicine Foundation of China (2012ZZB091), and the Foundation from the Health Bureau of Zhejiang Province (2011KYA065, 2012RCA027).

REFERENCES

13) Figarola JL, Scott S, Loera S, Xi B, Synold T, Weiss L, Rahbar S. Prevention of early renal disease, dyslipidemia and lipid...


