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Hepatocyte growth factor has a role in the amelioration of diabetic vascular complications via autophagic clearance of advanced glycation end products: Dispo85E, an HGF inducer, as a potential botanical drug

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Abstract

The aim of this study was to evaluate the effect and elucidate the potential mechanism of the extract of rhizomes from *Dioscorea alata* L. cv. Phyto, Dispo85E, on accelerating the elimination of advanced glycation end products (AGEs) in vitro and in vivo. Primary mouse nonparenchymal cells (NPCs) were used to evaluate the drug effect on AGEs clearance and autophagic-lysosomal activity. In an animal study, we used AGEs-induced diabetic mice to evaluate the drug effect on AGEs-induced vascular complications. Our results indicated that Dispo85E enhanced the endocytosis and degradation activity of AGEs in hepatic NPCs. Furthermore, the hepatocyte growth factor (HGF) expression level was positively correlated with the clearance capacity of the AGEs in NPCs after Dispo85E treatment. In addition, the effects of Dispo85E on the degradation and uptake capability of ^{14}C-AGEs were abolished in the presence of an anti-HGF neutralizing antibody. We further demonstrated that recombinant mouse HGF could enhance the endocytosis and autophagic clearance of AGEs in NPCs. The in vivo data indicated that Dispo85E increased hepatic HGF messenger RNA expression levels and decreased serum AGEs level in diabetic mice. Moreover, the function of retina and kidneys was improved by Dispo85E treatment in AGEs-induced diabetic mice. These results suggest that HGF may have an important role in the elimination of AGEs. This study suggests that Dispo85E is a botanical drug with a novel mechanism that enhances the clearance of AGEs through HGF-induced autophagic-lysosomal pathway and is a candidate drug for the treatment of diabetic vascular complications.

1. Introduction

Advanced glycation end products (AGEs) have been proposed as having a crucial role in the pathogenesis of diabetic vascular complications [1]. At present, it has been suggested that liver is the main organ for metabolism of circulating AGEs under normal physiologic conditions [2]. Such clearance is largely achieved by active endocytic uptake of hepatic nonparenchymal cells (NPCs) including sinusoidal endothelial cells and Kupffer cells [3]. However, in diabetes, the overloaded AGEs may disturb the normal clearance mechanisms [4,5]. Therefore, the approach to promote uptake and degradation of AGEs may be an effective way for ameliorating diabetic complications.

Hepatocyte growth factor (HGF) has originally been recognized and cloned as a potent mitogen of primary cultured hepatocytes [6]. In the liver, HGF is synthesized and secreted by NPCs [7]. It has been demonstrated that HGF functions as a therapeutic agent for a variety of disorders in different cells under diabetic conditions [8,9]. Besides, the serum HGF concentration has been reported to have a significant negative correlation with hemoglobin A_{1c} levels in human and presented with lower level in KKAy diabetic mice [10]. Therefore, a decreased local concentration of HGF may be a trigger for organ dysfunction in diabetes.

The Chinese yam (*Dioscorea alata*) has been widely used as an antidiabetes herbal medicine in China for more than 2000 years. Besides, according to the recent human experience on Chinese yam as folk remedy for treating diabetic patients with nephropathy or vascular

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complications, we tried to evaluate the effect and to elucidate the potential mechanism of the extract of rhizomes from *D alata* L. cv. Phyto, Dispo85E, based on our rationale for human experienced medicine-derived drug discovery.

2. Materials and methods

2.1. Preparation of Dispo85E

Dried and peeled tubers of *D alata* L. cv. Phyto were immersed in 85% (vol/vol) ethanol to extract small molecules. The extract was condensed with a rotary vacuum evaporator, lyophilized, and stored at −20°C before use.

2.2. Animals

Eight- to 10-week-old specific pathogen-free male C57BL/6 mice were obtained from the National Laboratory Animal Center (Taipei, Taiwan). The protocol of the experiments was approved by the Animal Research Committee of National Yang-Ming University (Guide for Animal Experiments, National Yang-Ming University).

2.3. Isolation and treatment of hepatic NPCs

Isolation of hepatic NPCs was performed according to the method previously described with minor modifications [11,12]. The primary NPCs were seeded in 96-well microplate at 6×10^4 cells per well or in 24-well microplate at 4×10^5 cells per well and cultured in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. After 48 hours, the medium was replaced with Dulbecco modified Eagle medium supplemented with 2% fetal calf serum containing Dispo85E or recombinant mouse (rm) HGF at indicated concentrations for 24 hours. The drug effect on lysosomal activity was evaluated by using LysoTracker Red (LTR) stain (Invitrogen, Carlsbad, CA) as previously described [13]. Endocytosis and degradation activity were evaluated as previously described with minor modifications [5]. Western blot analysis was performed with anti-HGF monoclonal antibody (Ab) (R&D Systems, Minneapolis, MN) or anti-LC3B polyclonal Ab (Novus Biological, Littleton, CO) followed by a secondary peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G Ab (Jackson ImmunoResearch, West Grove, PA).

2.4. Animal models

Ten- to 12-week-old male C57B/6 mice were randomly divided into 6 groups as follows: the normal group given tail vein injections with sterile phosphate-buffered saline (twice a week for 4 weeks); the mice serum albumin (MSA) group given tail vein injections with MSA (4 mg per mice twice a week for 4 weeks); and 4 AGEs groups given tail vein injections with AGEs-MSA (4 mg per mice twice a week for 4 weeks). The normal group and MSA group were fed with a normal diet. Four AGEs groups were fed with a normal diet containing different concentrations (0, 40, 200, and 1000 mg/[kg d]) of Dispo85E ad libitum from day 7 to day 62. At week 8t, urine and serum samples were collected; proteinuria and serum levels of AGEs were analyzed. Electroretinographic examination was performed at 8 weeks after Dispo85E treatment. At the end of the experiment, all mice were killed by cervical dislocation. The drug effect on hepatic HGF messenger RNA (mRNA) expression was analyzed by real-time polymerase chain reaction. The blood glucose concentrations were measured by an Accu-Check Advantage glucometer (Roche, Basel, Switzerland).

2.5. Statistical analysis

All results are expressed as means and standard deviations. The statistical significance was evaluated by 1-way analysis of variance followed by least significance difference post hoc test. A level of $P < .05$ was considered statistically significant.

3. Results

3.1. Dispo85E enhances the clearance of AGEs through HGF-induced autophagic-lysosomal activity

The results indicated that endocytic uptake and degradation of ^{14}C-AGEs-MSA by NPCs were significantly increased by the presence of Dispo85E (Fig. 1A, B). Besides, the Western blot demonstrated that Dispo85E increased HGF expression in hepatic NPCs (Fig. 1C). Therefore, these data suggest that Dispo85E, an HGF inducer, enhances the hepatic clearance of the formed AGEs.

Next, we tested the potential involvement of HGF in the Dispo85E-induced clearance of AGEs. The data showed that the endocytic uptake of ^{14}C-AGEs-MSA by the NPCs was significantly enhanced by the presence of 10 and 30 ng/mL rmHGF, and the amount of degraded ^{14}C-AGEs-MSA was similarly increased with rmHGF treatment (Fig. 1D, E). Moreover, the effects of Dispo85E were completely inhibited by cotreatment with an anti-HGF neutralizing Ab (Fig. 1A, B). These results indicate that HGF has a crucial role in Dispo85E-enhanced clearance of AGEs in NPCs.

We also examined whether HGF had a direct association with autophagic-lysosomal activity by measuring LTR staining and levels of LC3 by Western blotting. Quantification data of LTR staining showed that the lysosomal activity in NPCs was significantly enhanced by the presence of rmHGF (Fig. 1F). In addition, Western blot analysis showed that with treatment with rmHGF, the LC3-II/LC3-I ratio was significantly increased compared with the untreated control (Fig. 1G). These results demonstrated an unexpected role for HGF in increasing autophagic-lysosomal activity. Besides, our data further demonstrated that Dispo85E accelerated autophagic-lysosomal activity in NPCs (Supplementary Figure S1).

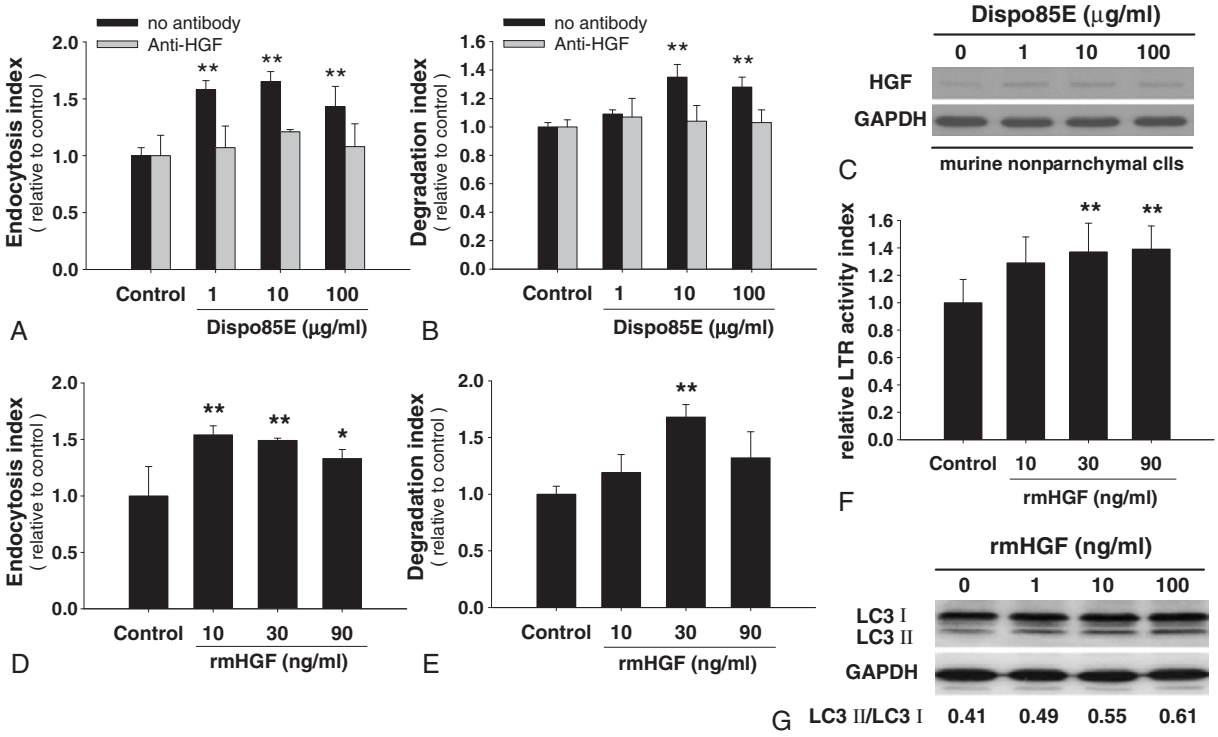

Fig. 1. Dispo85E enhances the clearance of AGEs in NPCs through HGF-induced autophagic-lysosomal activity. The NPCs were treated with indicated concentrations of Dispo85E or rmHGF for 24 hour. A, Effect of Dispo85E on endocytosis of ^{14}C-AGEs (n = 3). B, Effect of Dispo85E on degradation of ^{14}C-AGEs. Anti-HGF neutralizing antibodies block the endocytosis and degradation effect of Dispo85E (n = 3). C, Western blot analysis of HGF expression in NPCs treated with Dispo85E. D. Effect of rmHGF on endocytosis of ^{14}C-AGEs (n = 3). E, Effect of rmHGF on degradation of ^{14}C-AGEs (n = 3). F, Intracellular LTR was measured by fluorescence photometry (n = 6). G, Western blot analysis of LC3. Western blotting of glyceraldehyde-3-phosphate dehydrogenase indicated equal loading of the samples. **Significant difference from control group at $P < .01$; *significant difference from control group at $P < .05$.

Therefore, we suggest that Dispo85E enhances the hepatic clearance of the AGEs through induction of autophagic-lysosomal activity by HGF.

3.2. Dispo85E promotes AGEs clearance and ameliorates renal and retinal functions in AGEs-induced diabetic mice

To evaluate whether Dispo85E directly enhances the elimination of existing AGEs in the circulation, we used the AGEs-induced diabetic model to confirm the effect of Dispo85E on the clearance of AGEs. In this model, our data revealed that Dispo85E increased hepatic HGF mRNA expression (Fig. 2A) and effectively inhibited the increase of serum levels of AGEs (Fig. 2B). In addition, Dispo85E-treated diabetic mice had a statistically significant improvement in renal function by preventing the development of proteinuria (Fig. 2C). Histochemical analysis also indicated that Dispo85E reduced collagen deposition in the kidney compared with the untreated diabetic group (Supplementary Figure S2). Besides, electroretinographic analysis demonstrated that treatment with Dispo85E significantly improved amplitudes of the a-waves and b-waves when compared with the amplitudes in the AGEs-injected control group (Fig. 2D, E). These data suggest that Dispo85E may have the potential to ameliorate AGEs-induced diabetic nephropathy and retinopathy without disturbance of blood glucose levels (Fig. 2F).

4. Discussion

Autophagy is the major channel that delivers cytoplasmic proteins to lysosomes for degradation [14]. Recently, it has been reported that autophagy regulates the clearance of several aggregate prone proteins including polyglutamine-expanded huntingtin in Huntington disease, amyloid β in Alzheimer disease, and oxidative stress–induced cytoplasmic aggregate proteins in diabetes [15-17]. Thus, we are interested whether the effect of Dispo85E-induced AGEs clearance is through activating autophagic-lysosomal pathway.

Based on our previous study, it has been proposed that increased hepatic HGF expression could enhance phagocytosis activity of NPCs [18]. We focused on hepatic HGF expression to explain the clearance effects of Dispo85E on AGEs in this study. To our knowledge, this is the first report to identify HGF affecting endocytosis and autophagic-lysosomal functions in NPCs. These data also suggested that autophagy is a possible route to eliminate the formed AGEs.

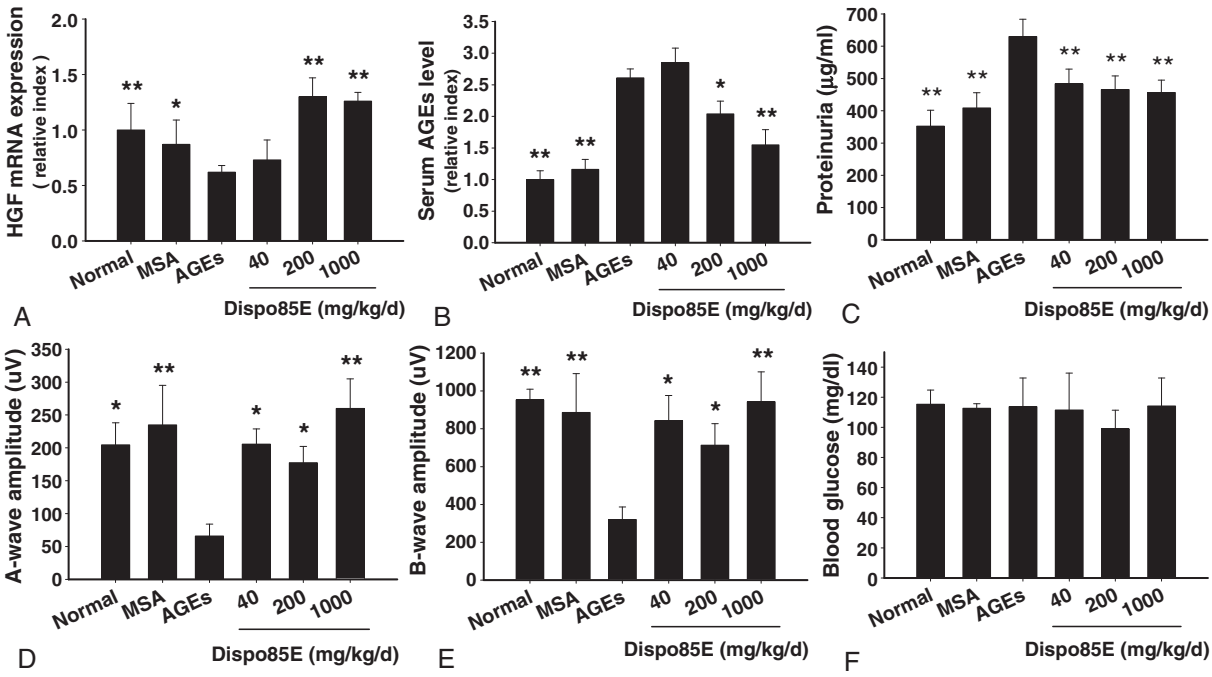

Fig. 2. The effect of Dispo85E on AGEs-induced diabetic mice. A, The HGF mRNA expression in the liver tissue. The mRNA expression level was detected by quantitative real-time polymerase chain reaction. B, Serum levels of AGEs in AGEs-induced diabetic mice. Assay was performed by enzyme-linked immunosorbent assay. C, Proteinuria level of AGEs-induced diabetic mice. Effect of Dispo85E on (D) a-wave and (E) b-wave amplitude of electroretinograms. F, Blood glucose levels in untreated AGEs-induced diabetic mice and Dispo85E-treated diabetic mice. ∗∗Significant difference from control group at $P < .01$; ∗significant difference at $P < .05$. n = 4 to 6 for each group.

Accumulating evidence indicates that AGEs-induced endothelial dysfunction and injury are critical events in the process of diabetic vascular complications [19]. Via RAGE, AGEs generate reactive oxygen species and activate inflammatory signaling cascades that have been implicated in the development of nephropathy and retinopathy [1]. In this study, we demonstrated that Dispo85E can enhance AGEs clearance by increasing hepatic HGF. It was noteworthy that HGF has been demonstrated to prevent AGEs-induced injury and oxidative stress in human endothelial cells [8]. Therefore, we suggested that the enhancement of HGF production by Dispo85E may not only be involved in functional enhancement of AGEs clearance, but also protect against endothelial cells injury from AGEs-induced oxidative stress.

Other than diabetes patients, it is of interest to note that women with polycystic ovary syndrome (PCOS) commonly present with elevated serum AGEs compared with healthy subjects [20]. Previous studies have indicated that insulin resistance may play a critical role in the resulting AGEs elevation in PCOS patients [20,21]. Interestingly, recent study has shown that hepatic autophagy was suppressed in the presence of insulin resistance and hyperinsulinemia [22]. Therefore, we suggested that it was one of the possible reasons to impair AGE elimination from circulation in diabetes or PCOS patients. In this study, we demonstrated that Dispo85E can effectively lower the already formed AGEs in circulation. Therefore, we suggested that Dispo85E might ameliorate AGEs-induced vascular complications in diabetes patients and women with PCOS.

The present study provides evidence that HGF has a pivotal role in promoting autophagic-lysosomal clearance of AGEs. However, when exogenous HGF is administered in vivo, its half-life in the blood is quite short, that is, approximately 5 minutes [23]. Dispo85E, as an HGF inducer, might be a more effective botanical drug for ameliorating diabetic complications. Even if the active components have not yet been identified, the high-performance liquid chromatography fingerprint analysis (Supplementary Figure S3) could help ensure the stability of this botanical drug. At present, Dispo85E already got the permission for a clinical phase II trial from the US Food and Drug Administration (IND no. 106,198).

Based on these findings, we conclude that Dispo85E, an HGF inducer, showed an antidiabetic effect via enhancing AGEs clearance through HGF-induced autophagic-lysosomal pathway, consequently ameliorating diabetic renal and retinal complications. Our data suggest that Dispo85E accelerates elimination of already formed AGEs from circulation and holds a promise as a novel therapeutic drug for the treatment of diabetic vascular complications.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.metabol.2010.08.009.

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