Grape Seed Procyanidin B2 Inhibits Human Aortic Smooth Muscle Cell Proliferation and Migration Induced by Advanced Glycation End Products

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Advanced glycation end product (AGE)-induced vascular smooth muscle cell (VSMC) proliferation is vital to the progression of diabetic vasculopathy. A grape seed procyanidin extract has been reported to possess anti-oxidative and anti-inflammatory properties and to display a significant cardiovascular protective effect, but little is known about the underlying mechanism. The objective of this present study was to determine whether GSPB2 (grape seed procyanidin B2), which is a dimeric procyanidin and more biologically active, could inhibit AGE-induced VSMC proliferation by affecting the production of ubiquitin COOH-terminal hydrolase 1 (UCH-L1), the degradation of IκB-α and nuclear translocation of NF-κB in human aortic smooth muscle cells (HASMCs). Our data show that GSPB2 preincubation markedly inhibited AGE-induced proliferation and migration of HASMCs in a dose-dependent manner and upregulated the protein level of UCH-L1. Further studies revealed that the GSPB2 pretreatment markedly attenuated the degradation of IκB-α and nuclear translocation of NF-κB by modulating ubiquitination of IκB-α in AGE-exposed HASMCs. These results collectively suggest that AGE-induced HASMC proliferation and migration was suppressed by GSPB2 through regulating UCH-L1 and ubiquitination of IκB-α. GSPB2 may therefore have therapeutic potential in preventing and treating vascular complications of diabetes mellitus.

Key words: grape seed procyanidin B2; advanced glycation end product; ubiquitin COOH-terminal hydrolase 1; IκB-α; human aortic smooth muscle cell

It is well known that hyperglycemia in diabetic patients can increase the risk of developing such cardiovascular complications as atherosclerosis and hypertension.1,2 It accelerates the progression of cardiovascular disease by effecting wall thickening in large arteries.3 This effect is particularly marked by the proliferation of vascular smooth muscle cells (VSMCs). However, the mechanism linking hyperglycemia and the pathogenesis of macrovascular disease remains to be further defined.

There is growing evidence that the production and accumulation of advanced glycation end products (AGEs) is involved in the pathogenesis of diabetic vascular complications.4 Binding of AGEs to their receptor RAGE on VSMCs leads to an intracellular oxidative stress response through the increased activation of transcription factors such as NF-κB.5,6 Many studies have shown that the NF-κB signal pathway is associated with VSMC migration, invasion and proliferation.7 IκB proteins are known to regulate NF-κB activity in the cytoplasm. IκB-α is forwarded to be ubiquitinated by the relevant enzyme and degraded by the 26S proteasome in response to extracellular stimuli. NF-κB is subsequently translocated to the nucleus and activates gene transcription.8

The ubiquitin-proteasome system is involved in the regulation of such cellular processes as the degradation of intracellular proteins, proliferation and signal transduction. Recent studies have shown that the increased ubiquitin-proteasome activity induced by oxidative stress may play a crucial role in the progression of atherosclerosis in diabetic patients.9 In contrast, the de-ubiquitinating enzymes are negative regulators of ubiquitin-dependent proteolysis. UCHs (ubiquitin COOH-terminal hydrolases)10 are known as a member of the family of de-ubiquitinating enzymes. As one of the UCH isozymes, UCH-L1 was first found to be predominantly expressed in central and peripheral neurons and to be involved in the pathogenesis of such neurodegenerative disorders as Parkinson’s disease and Alzheimer’s disease.11 It has been reported that UCH-L1 was also expressed in endothelial cells and smooth muscle cells in atherosclerotic lesions from human carotid arteries.12

Grape seed procyanidin extracts (GSPEs) derived from grape seeds have been shown to exert radical-scavenging, anti-oxidative, anti-inflammatory and anti-tumoral effects.13-15 Dimeric procyanidin B2 is one of

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Abbreviations: AGE, advanced glycation end product; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; GSPB2, grape seed procyanidin B2; GSPE, grape seed procyanidin extract; HASMC, human aortic smooth muscle cell; MTT, 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide; RAGE, receptor for advanced glycation end products; ROS, reactive oxygen species; SMC, smooth muscle cell medium; UCH, ubiquitin COOH-terminal hydrolase; VSMC, vascular smooth muscle cell
the components of GSPE which may be more active than other water-soluble polyphenols in its biological activities, i.e., inhibiting the formation of AGEs in the BSA-glucose model and nNOS activity in vitro.[16–18] The objective of the present study is to determine whether grape seed procyanidin B2 (GSB2) has the capability to inhibit the AGE-induced proliferation and migration of human aortic smooth muscle cells (HASMCs) and to clarify the underlying molecular mechanism for the effect of GSB2 on NF-κB translocation by affecting the production of the de-ubiquitinating enzyme, UCH-L1.

Materials and Methods

Materials. GSB2 (more than 95% pure, lot no. 20080915) was provided by Jianfeng (Tianjin, China). Bovine serum albumin (BSA), d-glucose, collagenase, a trypsin/EDTA solution, dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2)-5-diphenyl tetrazolium bro- mide (MTT) were purchased from Sigma (St. Louis, USA). Fetal bovine serum (FBS) was obtained from Gibco (Grand Island, USA), and HASMCs and the smooth muscle cell medium (SMCM) were obtained from ScienCell Research Laboratories (California, USA). The antibodies of total UCH-L1, IκB-α, and NF-κB were purchased from Cell Signaling Technology (Beverly, USA). Tissue culture flasks and plates were supplied by Costar (Cambridge, USA), and all other reagents were of standard commercial high-purity grade.

AGE-BSA preparation. AGEs were prepared by incubating 50 mg/mL of BSA with 0.5 mol/L of glucose in 0.2 mol/L of phosphate-buffered saline (PBS, pH 7.4) in the dark at 37 °C for 12 weeks. The solution was sterilized by passing it through a 0.2-μm filter before incubation. Unmodified BSA was incubated under the same conditions in the absence of glucose as a control. The unincorporated sugars were removed by extensive dialysis after incubation. The concentration of AGEs was determined by V-2001 fluorescence spectrophotometry (Hitachi, Japan; 390 nm excitation wavelength, 450 nm emission wavelength). The fluorescence values for the test substances were measured at a protein concentration of 1 mg/mL, the results being expressed as AGE units (1 U = 1 mg/mL of the AGE-BSA standard).

The AGE concentration in AGE-modified BSA was 94.5 U/mg of proteins, whereas that in the control group was 0.5 U/mg of proteins. The endotoxin concentration was measured by the limulus amebocyte lysate assay (Sigma, St. Louis, USA) which revealed negligible values (<0.2 μg/L).

Cell cultures. SMCM consisted of 500 mL of the basal medium, 10 mL of fetal bovine serum (FBS, cat. no. 0010), 5 mL of a smooth muscle cell growth supplement (0.5 ng/mL of EGF, 5 mg/mL of BFGF, 50 mg/mL of Amphotericin B and 50 μg/mL of gentamicin), and 5 mL of a penicillin (100 U/mL)/streptomycin (100 μg/mL) solution (P/S, cat. no. 0503). HASMCs were incubated in SMCM and maintained at 37 °C in 5% CO2/95% mixed ambient air. HASMC experiments were performed on 5 to 8 passages. HASMCs were grown to 80–90% confluence and made quiescent by serum dye exclusion, and cell survival was estimated with an MTT viability assay. The fluorescence values for the test substances were measured by microscopic examination after trypan blue dye exclusion, and cell survival was estimated with an MTT assay. The objective of the present study is to determine whether grape seed procyanidin B2 (GSB2) has the capability to inhibit the AGE-induced proliferation and migration of human aortic smooth muscle cells (HASMCs) and to clarify the underlying molecular mechanism for the effect of GSB2 on NF-κB translocation by affecting the production of the de-ubiquitinating enzyme, UCH-L1.

Cell viability analysis. The cells were harvested by trypsinization to examine the effects of GSB2 on HASMC proliferation. The cell viability was measured by microscopic examination after trypan blue dye exclusion, and cell survival was estimated with an MTT assay. The objective of the present study is to determine whether grape seed procyanidin B2 (GSB2) has the capability to inhibit the AGE-induced proliferation and migration of human aortic smooth muscle cells (HASMCs) and to clarify the underlying molecular mechanism for the effect of GSB2 on NF-κB translocation by affecting the production of the de-ubiquitinating enzyme, UCH-L1.

Statistical analysis. Data are expressed as the mean ± standard deviation. A statistical analysis between groups was conducted by a one-way analysis of variance (ANOVA) followed by Tukey’s HSD test for multiple comparisons. A p value <0.05 is considered statistically significant. All analyses were performed with SPSS for Windows software version 10.0 (SPSS, Chicago, USA).

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Results

Effect of GSPB2 on the proliferation of AGE-exposed HASMCs

We performed an MTT proliferation assay to investigate the effect of GSPB2 on HASMC proliferation. Neither unmodified BSA nor DMSO had any effect on cell viability. The cell viability of AGEs was significantly higher by approximately 60% for 48 h ($p < 0.01$). Pretreating HASMCs with different concentrations of GSPB2 significantly improved the AGE-stimulated cell viability in a dose-dependent manner ($p < 0.01$) (Fig. 1).

Effect of GSPB2 on the migration of AGE-exposed HASMCs

A Transwell migration chamber with a collagen-coated polycarbonate filter was used to determine the effect of GSPB2 on HASMC migration. AGE-stimulated HASMCs significantly increased the number of migrating cells for 48 h ($p < 0.01$). However, pretreating HASMCs with different concentrations of GSPB2 significantly improved the AGE-stimulated cell migration in a dose-dependent manner ($p < 0.01$) (Fig. 2).

Effect of GSPB2 on the expression of UCH-L1 in AGE-exposed HASMCs

We determined the effect of GSPB2 on the expression of UCH-L1 by western blotting. Stimulation of HASMCs with AGEs (200 mg/L) for 48h significantly reduced the UCH-L1 protein expression. Pretreating with GSPB2 significantly improved the AGE-stimulated expression of UCH-L1 for 48h ($p < 0.05$) (Fig. 3A and B).

Effect of GSPB2 on the ubiquitination of IkB-α in AGE-exposed HASMCs

An immunoprecipitation analysis demonstrated the ubiquitination of IkB-α in HASMCs. Stimulation of HASMCs with AGEs resulted in an increased ubiquitination of IkB-α and lower level of IkB-α, whereas pretreating with GSPB2 significantly attenuated the degradation of IkB-α by affecting the ubiquitin-mediated protein degradation in AGE-exposed HASMCs ($p < 0.05$) (Fig. 4).

Discussion

Macrovascular disease is the major cause of mortality in a diabetic patient population. 19,20) The pathogenesis of macrovascular disease is characterized by the proliferation and migration of HASMCs within the intima and media. 21) AGEs have been associated with VSMC proliferation and migration in diabetic vasculopathy. 5) Many studies have demonstrated the impairment of AGEs on human umbilical-vein endothelial cells 22–24)
which cannot mimic the typical state in the aorta of diabetic patients. We used AGEs in this present study to stimulate HASMCs and approach the real pathological changes in the human macrovascular wall so that the results may provide more clinical sense.

AGEs have been linked to the neo-intimal formation after vascular injury in diabetes. Numerous studies have revealed that AGEs would induce the production of reactive oxygen species (ROS) which triggers diabetic vascular remodeling and VSMC proliferation and migration. The interaction of AGEs with their receptor, RAGE (receptor for advanced glycation end products), which is expressed on the surface of VSMCs, contributes to the production of numerous pro-inflammatory factors through the activation of a variety of signal pathways, including p21 (ras), mitogen-activated protein kinase (MAPK), nuclear factor-κB (NF-κB), cyclooxygenase-2 and c-jun. The increased oxidative stress contributes to VSMC dysfunctions, resulting in the activation of pro-inflammatory and pro-thrombotic pathways, and increased arterial stiffness. However, the underlying mechanism is unclear.

Although the morbidity and mortality of diabetes has increased year by year around the world, treatment is still restricted to a few classes of drugs. GSPB2, as a natural dietary supplement, displays an anti-oxidative property and protects against diabetic complications. The results of the present study reveal that GSPB2 inhibited the AGE-induced proliferation and migration of HASMCs, and that UCH-L1 may be involved in this process via the inhibition of NF-κB activity.

Several animal and human studies have used antioxidants derived from such plants as green tea, resveratrol and Trigonella foenum graecum to combat diabetic complications, and their reports have demonstrated that antioxidative therapy has decreased the risk of diabetic outcomes. Antioxidative therapy is therefore considered to be a promising strategy to prevent oxidative damage to VSMC in the early stage of diabetic vascular complications. GSPE has been reported by some researchers to have limited bioavail-

Fig. 3. Effects of GSPB2 on the UCH-L1 Expression in AGE-Stimulated HASMCs (A and B).

Data are presented as the expression ratio of UCH-L1/β-actin and given as the mean ± SD from at least three separate experiments. *p < 0.05, **p < 0.01 compared with the control HASMC group; #p < 0.05, ##p < 0.01 compared with the AGE-stimulated HASMC group. GSPB2, grape seed procyanidin B2; HASMCs, human aortic smooth muscle cells.

Fig. 4. Effects of GSPB2 on the Free and Ubiquitinated IκB-α Expression in AGE-Stimulated HASMCs.

Ubiquitinated IκB-α and free IκB-α expression was evaluated in the cytosolic fraction by immunoprecipitating IκB-α and then immunoblotting with anti-ubiquitin or anti-IκB-α antibodies. HASMCs were pretreated for 1 h with GSPB2 (2.5 μmol/L, 5.0 μmol/L and 10.0 μmol/L) and then stimulated for 48 h with AGE-BSA (200 μg/mL). Experiments were performed in triplicate. GSPB2, grape seed procyanidin B2; HASMCs, human aortic smooth muscle cells.

Fig. 5. Effects of GSPB2 on the NF-κB Translocation in AGE-Stimulated HASMCs (A and B).

HASMCs were pretreated for 1 h with GSPB2 (2.5 μmol/L, 5.0 μmol/L and 10.0 μmol/L) and then stimulated for 48 h with AGE-BSA (200 μg/mL). Data are presented as the expression ratio of N-NF-κB/C-NF-κB and given as the mean ± SD from at least three separate experiments. *p < 0.05, **p < 0.01 compared with the control HASMC group; #p < 0.05, ##p < 0.01 compared with the AGE-stimulated HASMC group. C-NF-κB, cytoplasmic NF-κB; N-NF-κB, nuclear NF-κB; GSPB2, grape seed procyanidin B2; HASMCs, human aortic smooth muscle cells.
ability; particularly, its absorption in the human body is unclear. However, many studies have shown that the administration of GSPE to diabetic rats effectively improved cardiovascular complications and had an anti-atherosclerotic effect.\(^{37,38}\) A GSPE treatment has decreased the aortic pulse wave velocity and aortic medial thickness by reducing the expression of AGE and RAGE in diabetic aorta.\(^{39}\) GSPE has also demonstrated protective effects against nephropathy,\(^{40}\) retinopathy,\(^{41}\) and damage to peripheral nerves\(^{42}\) and the cerebral cortex\(^{43}\) in STZ-induced diabetic rats. We found in this present study that HASMCs stimulated with AGEs had a higher migration cell number and increased proliferation when compared with unstimulated HASMCs in a dose-dependent manner. Given that AGEs dysregulate VSMC growth and accelerate diabetic complications, GSPB2 may be helpful to prevent and treat vascular injury in diabetes. The mechanism by which GSPB2 blocked the AGE-induced HASMC proliferation and migration involved the regulation of UCH-L1 and ubiquitination of IκB-α. UCH-L1, as a de-ubiquitinating enzyme, can hydrolyze the bonds between Ub and small adducts or unfolded polypeptides.\(^{44}\) Recent studies have reported that UCH-L1 exhibited anti-angiogenesis activity by affecting the development of choroidal new vessels in age-related macular degeneration disease.\(^{45,46}\) Our previous experiments have shown that the expression of UCH-L1 in the retinopathy of diabetic rats was significantly lower than that of control rats by the administration of GSPE to diabetic rats.\(^{31}\) Moreover, recent evidence has shown that UCH-L1 negatively regulated TNF-α-mediated VSMC proliferation by suppressing ERK activation.\(^{47}\) Our data in this present work show that pretreating HASMCs with GSPB2 enhanced the expression of UCH-L1 and reduced the ubiquitination of IκB-α. UCH-L1 with the function of de-ubiquitination may reverse the ubiquitination process of IκB-α and allow ubiquitinated IκB-α to escape from degradation by proteosomes.\(^{12}\) UCH-L1 is therefore most likely to participate in the deterioration of diabetic complications as an important protein and provide novel targets for GSPE treatment.

It is well established that NF-κB is involved in the AGE-mediated effects of RAGE signaling.\(^{5}\) The ubiquitin-proteasome system has been shown to play a crucial role in the activation of NF-κB under conditions of oxidative stress such as atherosclerosis and diabetes.\(^{9,45}\) Activated nuclear NF-κB has been detected in VSMCs after balloon injury to rat carotid arteries and in VSMCs of human atherosclerotic lesions.\(^{6–8}\) Research has confirmed that NF-κB has a close relationship with the proliferation and migration of VSMCs. We observed in this present work that the expression of NF-κB was markedly enhanced in the cytoplasm and reduced in the nucleus of AGE-exposed HASMCs that had been pretreated with GSPB2, suggesting that its translocation to the nucleus was effectively inhibited by GSPB2. IκB-α, the inhibitor of NF-κB in the cytoplasm, prevents translocation of NF-κB into the nucleus. Oxidative stress activates IκB kinase which phosphorylates IκB-α and induces ubiquitination and proteasomal degradation of IκB-α.\(^{8}\) We observed here that pretreating with GSPB2 attenuated the production of ubiquitinated IκB-α, leading to decreased degradation of IκB-α. This suggests that GSPB2 mediated IκB-α protein expression, at least in part, through the ubiquitin-proteasome pathway. The up-regulation of UCH-L1 protein by a GSPB2 treatment also verified its de-ubiquitinating effect on IκB-α,\(^{12}\) indicating that GSPB2 may inhibit nuclear translocation of NF-κB by up-regulating UCH-L1 in AGE-stimulated HASMCs.

We report for the first time in this present study that GSPB2, one of the main components of GSPE, inhibited the AGE-induced proliferation and migration of HASMCs. This was achieved by inhibiting the nuclear translocation of NF-κB and the degradation of its inhibitor, IκB-α, in the cytoplasm of HASMCs. These molecular events may have been the result of the up-regulation of UCH-L1 by GSPB2 which can reverse the ubiquitination of IκB-α. Taken together, the results of our study suggest that the regulation of UCH-L1, IκB-α and NF-κB by GSPB2 contributed to improvement of the VSMC dysfunction resulting from damage by AGEs; this action could exert a protective effect on the vasculopathy in diabetes mellitus.

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