Grape Seed Proanthocyanidin Extracts Enhance Endothelial Nitric Oxide Synthase Expression through 5′-AMP Activated Protein Kinase/Surtuin 1–Krüpple Like Factor 2 Pathway and Modulate Blood Pressure in Ouabain Induced Hypertensive Rats

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Grape seed proanthocyanidin extracts (GSPE) belonging to polyphenols, possess various biological effects including anti-inflammation, anti-oxidant, anti-aging, anti-therosclerosis, etc. GSPE is potential in regulating endothelial function. However, the underlying mechanism is not clear yet. In this study, by small interfering RNA (siRNA) knocking down, we proved that GSPE increase endothelial nitric oxide synthase (eNOS) expression in human umbilical vessel cells (HUVECs) in vitro, which was attributed to its transcription factor Krüpple like factor 2 (KLF2) induction. Furthermore, GSPE activate 5′-AMP activated protein kinase (AMPK) and increase surtun 1 (SIRT1) protein level, critical for KLF2 induction. We also illuminated the role of GSPE in hypertension treatment. By chronic administration of GSPE in ouabain induced hypertensive rats model, we access the effect of GSPE on blood pressure regulation and the possible mechanisms involved. After 5 weeks feeding, GSPE significantly block the ouabain induced blood pressure increase. The aortic NO production impaired by ouabain was improved. In conclusion, GSPE increase eNOS expression and NO production in an AMPK/SIRT1 dependent manner through KLF2 induction, and attenuate ouabain induced hypertension.

Key words grape seed proanthocyanidin; endothelial nitric oxide synthase; hypertension; Krüpple like factor 2

Grape seed proanthocyanidin extracts (GSPE), belonging to polyphenols, is a potent antioxidant extracted from grape seeds and skins. The superoxide anion scavenging activity of GSPE was stronger than vitamin C, vitamin E or any other antioxidant.1) It has been reported to have protective effects on various cardiac disorders, correct dyslipidemia associated with high-fat diet,2) decrease arterial pressure in estrogen-depleted female spontaneously hypertensive rats,3) prevent diabetic nephropathy and neuropathy from progressing,4,5) and most importantly, ameliorate atherosclerosis,6) possibly attributed to its endothelial protective potential.

Endothelial dysfunction is a hallmark of various cardiovascular diseases. In the vascular endothelium, nitric oxide (NO) is a principal mediator of normal endothelial function, inhibiting platelet adhesion and aggregation, preventing leukocyte adhesion and migration, and most importantly, relaxing smooth muscle cells to regulate the vessel tone.7) The main source of NO is from the endothelial nitric oxide synthase (eNOS). Although eNOS was originally thought to be constitutively expressed, its expression can be modulated by a variety of stimuli. Regulation of eNOS expression occurs at both transcriptional and post-transcriptional levels.8–10) Previously, our colleagues proved that GSPE up-regulate eNOS expression through AKT pathway and induce NO production in H2O2 treated human umbilical vessel cells (HUVECs).11) However, the detailed mechanism is not clear. It is demonstrated that in HUVECs, the eNOS promoter is regulated by transcription factor Krüpple like factor 2 (KLF2),12,13) the key factor in shear stress induced eNOS expression. Whether this molecular is involved in GSPE enhanced eNOS expression is unknown.

In this study, we mainly address the role of KLF2 in GSPE induced eNOS expression in vitro. Considering the key effect of endothelium-mediated vessel dilation in hypertension, we also tested the hypothesis that GSPE may attenuate ouabain induced blood pressure increase in vivo.

MATERIALS AND METHODS

Animals and Treatment Thirty male Sprague-Dawley (SD) rats (5–6 weeks old, supplied by Experimental Animal Center of Shandong University, China) weighted 180–220 g were housed in a 12:12-h light-dark cycle at 24°C, and had free access to tap water and standard rat chow ad libitum for 7 d to acclimatize before entering the study. All protocols were approved by the Institutional Animal Care and Use Committee of the Qilu Hospital, Shandong University.

Thirty rats were randomized into three groups (n=10, each), treated with nitric sodium (NS group), ouabain (O group), and ouabain plus GSPE (GO group, the proanthocyanidin content of all animals was measured by tail-cuff plethysmographic Systolic Blood Pressure (SBP) Measurement. The SBP of all animals was measured by tail-cuff plethysmographic (TCP) method with a rat tail BP monitor (RBP-I, Clinical Medicine Institute, Beijing Sino-Japan Friendship Hospital, China) at baseline and the end of the experiment. The rats

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were kept calm and conscious till pulsatory signals from the arteria caudalis were displayed steadily. At least 10 determinations were made on each rat and the mean of 6 readings within a 5–10 mmHg range was taken as the SBP of rat.14) The systolic and diastolic blood pressures were measured during 30 min with a pressure transducer (model 1050BP, UFI, Inc., Morro Bay, CA, U.S.A.) and then recorded using an interface and software for computer data acquisition (model MP100A, BIOPAC System, Inc., Santa Barbara, CA, U.S.A.).

**Tissue Collection** After five weeks of treatment, the rats were sacrificed by decapitation. Thoracic aortas were rapidly excised and dissected.

**Cell Culture, Antibodies, and Reagents** HUVECs were cultured in M199 medium in Dulbecco’s modified Eagle’s medium (DMEM), both supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 1% antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) at 37°C under 5% CO₂. Anti-eNOS, anti-phospho-5′-AMP activated protein kinase alpha (AMPKα) (Thr-172), anti-sirtuin 1 (SIRT1), anti-β-actin, horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies were from Cell Signaling Technology. The NO production of cultured HUVECs were measured by Nitrate/Nitrite Colorimetric Assay Kit (Nanjing Jiancheng Bioengineering Institute), following the protocol.

**Small Interfering RNA (siRNA) Knockdown** HUVECs were seeded on a 6-well plate and grown to 50–70% confluence before transfection. For each well, 2 µg of total DNA construct was transfected in Lipofectamine 2000 (Invitrogen, 2 µL/µg DNA). Twenty-four hours after transfection, cells were treated with GSPE for certain time length and then washed with phosphate buffered saline (PBS) and lysed on ice in lysis buffer.

Total RNA was isolated with the use of Trizol reagent (Invitrogen). Reverse transcription was carried out with 3 µg of total RNA by the Superscript II reverse transcriptase (Invitrogen). The synthesized cDNA was used to perform real-time quantitative polymerase chain reaction (qPCR) with the iQ SYBR Green supermix (Bio-Rad, Hercules, CA, U.S.A.) on the iCycler real-time PCR detection system (Bio-Rad). The sequences of primer sets were: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ATG ACA TCA AGA AGG TGG TG and CAT ACC AGG AAA TGA GCT TG. KLF2, AGA CCT ACA CCA AGA GTT GTC CAT GC and CAT GTG CCG TTT CAT GTG CAG C eNOS, TCT CCG CCT CGCT CTA TG and AGC CAT ACAGG ATT GTC CG CC.

**Protein Isolation and Immunoblotting** HUVECs were lysed with radio immunoprecipitation assay (RIPA) buffer (1% Igepal CA-630, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS) in 1X PBS) containing protease and phosphatase inhibitors. Proteins for in vivo experiments were isolated from the aorta harvested from each group. Equal amounts of protein were separated on SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane (Bio-Rad). After 1-h blocking with 5% bovine serum albumin (BSA), the membrane was probed with various primary antibodies and the appropriate secondary antibodies conjugated with horseradish peroxidase (HRP), followed by ECL detection (GE Healthcare). The protein bands were quantified by using the ImageJ software (National Institutes of Health).

**Statistics** All data were presented as mean±standard deviation. Statistical significance of the differences was
calculated by unpaired Student’s t-test or one way analysis of variance (ANOVA) by SPSS® version 11.5 (SPSS® Inc., Chicago, IL, U.S.A.) for Windows®. A p value less than 0.05 was considered statistically significant.

RESULTS

GSPE Induce eNOS Expression in Both mRNA and Protein Level and Increase Its Bioavailability In order to address whether GSPE regulate eNOS expression at transcription level, the HUVECs were incubated with GSPE (10 µg/mL) for 24 h, and the mRNA level of eNOS was detected by real-time PCR. Cells treated with GSPE displayed three times increase of eNOS mRNA compared to the dimethyl sulfoxide (DMSO) control, as shown in Fig. 1A. The protein level of eNOS detected by Western Blot also increase parallel to the mRNA level after 24 h treatment, and last for 48 h (Fig. 1B). The eNOS bioavailability was enhanced at the same time, as the NO production increased significantly after 24 h GSPE treatment measured by nitrate/nitrite colorimetric assay (Fig. 1C).

GSPE Induce KLF2 Expression, Critical for eNOS Induction KLF2 is a well proved eNOS transcription factor, to delineate the role of KLF2, we first detected KLF2 mRNA expression under GSPE treatment by real-time PCR. Cells treated with GSPE displayed three times increase of eNOS mRNA compared to the dimethyl sulfoxide (DMSO) control, as shown in Fig. 1A. The protein level of eNOS detected by Western Blot also increase parallel to the mRNA level after 24 h treatment, and last for 48 h (Fig. 1B). The eNOS bioavailability was enhanced at the same time, as the NO production increased significantly after 24 h GSPE treatment measured by nitrate/nitrite colorimetric assay (Fig. 1C).

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GSPE Activate AMPK and Induce SIRT1 Expression AMPK is a cellular energy sensor and plays important roles in vascular biology and SIRT1, a nicotinamide adenine dinucleotide-dependent deacetylase modulates metabolic homeostasis and longevity, both of which are proved to be associate with KLF2 and eNOS expression in endothelial cells (ECs). To know whether AMPK and SIRT1 is involved in GSPE induced ECs protection, HUVECs were treated with GSPE and the AMPK phosphorylation and SIRT1 expression were detected at 30 min, 1 h and 2 h by Western blot. As seen in Fig. 3A, GSPE induce both AMPK phosphorylation and SIRT1 protein level in a time-dependent manner. To know whether AMPK or SIRT1 is critical for GSPE induced KLF2 expression, AMPK and SIRT1 was knockdown by siRNA in HUVECs and treated with GSPE. As shown in Figs. 3B and C, either AMPK or SIRT1 knockdown blocked the induction of KLF2 mRNA level by GSPE, indicating that the effect of GSPE on KLF2 is both AMPK and SIRT1 dependent.

GSPE Alleviate Ouabain Induced Hypertension and Increase Endothelium NO Production in Rats Here we established ouabain induced hypertensive rat model, studied chronic administration of GSPE on blood pressure. There is no difference on baseline systolic blood pressure at the start of the experiment as shown in Fig. 4A. After five weeks treatment with ouabain, the mean systolic blood pressure of the ouabain group was about 23 mmHg higher than that in the N.S. control group, while the ouabain induced blood pressure increase was significantly blocked by GSPE administration,
almost 13 mmHg lower than that in the ouabain group (Fig. 4A). Oubain treatment impaired aortic NO production \((p < 0.05)\) and when fed with GSPE, the decreased NO production was revised \((p < 0.05)\), as shown in Fig. 4B.

**DISCUSSION**

Grape seed proanthocyanidin extracts (GSPE) is a kind of polyphenol extracted from grape seeds and skins. It has been reported to have multiple cardioprotective effects, including anti-dyslipidemia, anti-hypertension and atheroprone, possibly attributed to its endothelial protective potential.\(^2,3,6\)

Endothelial dysfunction is a hallmark of various cardiovascular diseases including hypertension. In the vascular endothelium, nitric oxide (NO) produced by the endothelial nitric oxide synthase (eNOS), is a principal mediator of normal endothelial function.\(^7\) Impaired endothelial function is closely related with decreased NO production. Previously, our colleagues already proved that GSPE up-regulate eNOS expression and induce NO production through AKT pathway in H\(_2\)O\(_2\) treated HUVECs,\(^11\) however, the detailed mechanism is not clear.

Regulation of eNOS expression occurs at both transcriptional and post-transcriptional levels.\(^8-10\) In this study, we proved that eNOS is regulated by GSPE at transcription level since eNOS mRNA increase in HUVECs after GSPE treatment. There are many factors involving in eNOS transcription including. In HUVECs, the eNOS promoter is regulated by transcription factor KLF2,\(^12,13\) the key factor in shear stress induced eNOS expression. Moreover, KLF2 exhibit multiple bioavailabilities including anti-inflammation, anti-thrombosis and anti-angiogenesis, leading us to link GSPE with KLF2, since GSPE also display multiple endothelial protective effects.\(^18,19\) In this study, we proved GSPE induce eNOS expression dependent on KLF2, which partly explained its pleiotropic effects.

There are multiple factors regulating KLF2 expression, involving phosphoinositide-3-kinase (PI3K)-dependent/Akt-independent pathway\(^20\) and MEK5/ERK5/MEF2 signaling pathway,\(^21\) which is AMPK dependent.\(^15\) AMPK is a highly conserved sensor of cellular energy status that is activated under conditions of low intracellular ATP. Recently, it is reported that AMPK activation regulate blood pressure and vascular tone in spontaneously hypertensive rats.\(^22\) Many stimuli such as hypoxia, estrogen, shear stress, adiponectin, and statins can act on the vascular EC to activate AMPK. Besides KLF2 induction to provoke eNOS expression, AMPK also increase eNOS activity by phosphorylates Ser1177 and Ser633 to enhance the NO bioavailability in ECs.\(^23\)

Recently, AMPK are reported to co-ordinate with SIRT1 in...
7) Taddei S, Ghiadoni L, Virdis A, Versari D, Salvetti A. Mechanisms of the substrate activity. SIRT1, also known as Sirtuin 2 (silent mating type information regulation 2 homolog), is a NAD⁺-dependent class III histone deacetylase, which acts primarily by removing acetyl groups from lysine residues within proteins in the presence of NAD⁺. Canto and Zhen Chen proved that PGC-1α and eNOS phosphorylation by AMPK primes their deacetylation by SIRT1, therefore enhance their activity. We then studied the role of AMPK and SIRT1 in GSPE induced KLF2 expression. Our results show that GSPE induced KLF2 expression in ECs is both AMPK and SIRT1 dependent. Whether these two molecules act synergistically in KLF2 expression needs further investigation.

One intriguing phenomenon in our study is the fast induction of SIRT1 expression by GSPE. The SIRT1 protein level increased 30 min after GSPE treatment. By real-time PCR detection, we prove that SIRT1 mRNA level did not change (data not shown), indicating a post-transcription regulation of SIRT1 by GSPE. A possible explanation is increased protein stability, since Ford et al. found that phosphorylation at serine 27 by JNK2 increase SIRT1 protein stability. However, the effect of GSPE on JNK2 is still unknown.

Blunted endothelium-mediated vasodilation is common in various cardiovascular diseases, including hypertension. Ouabain, a digitalis compound, works as an endogenous regulator of blood pressure and Na⁺, K⁺-ATPase activity. In rats and human hypertension, the endogenous ouabain or a closely related isomer increase and is associated with other cardiovascular complications, such as cardiac hypertrophy, heart failure, and myocardial infarction. Endogenous ouabain is a prehypertrophic hormone and may affect cardiovascular function and structure, being responsible for cardiac remodeling which contributes to an increased risk of morbidity events. Chronic administration of exogenous ouabain induces hypertension in normotensive rats and hypertensive vascular remodeling, which is a ideal model to study hypertension. In recent years, nutraceuticals and functional foods have attracted considerable interest as potential alternative therapies for treatment of hypertension, especially for prehypertensive patients. Nowadays, researchers are seeking a new, safe reagent for blood pressure control and natural remedies catch much attention, esp. garlic, folic acid and Crataegus. Considering the excellent endothelial protective effect of GSPE, we fed the ouabain induced hypertensive rats with GSPE and got lowered blood pressure, making GSPE a potential natural reagent in hypertension prevention.

REFERENCES


Fig. 4. GSPE Alleviate Ouabain Induced Hypertension and Increase Aortic NO Production in Rats

(A) GSPE alleviated ouabain induced blood pressure increase. Thirty rats were randomized into three groups (n=10, each), treated with nitric sodium (1 mL·kg⁻¹·d⁻¹ 0.9% saline intra-peritoneally and 1 mL·0.9% N.S. orally), ouabain (27.8 µg·kg⁻¹·d⁻¹ intra-peritoneally), and ouabain (27.8 µg·kg⁻¹·d⁻¹ intra-peritoneally) plus GSPE (250 mg/kg·d) intragastrically. The SBP of all animals was measured by tail-cuff plethysmographic (TCP) method with a rat tail BP monitor at baseline and the end of the experiment. There is no difference on baseline systolic blood pressure at the start of the experiment, after five weeks treatment with ouabain, the mean systolic blood pressure of the ouabain group was about 23 mmHg higher than that in the NS control group, while the ouabain induced blood pressure increase was significantly blocked by GSPE administration, almost 13 mmHg lower than that in the ouabain group. (B) GSPE increase aortic NO production in ouabain induced hypertensive rats. Ouabain treatment impaired aortic NO production (p<0.05) and when fed with GSPE, the decreased NO production was revised (p<0.05).


