European Review for Medical and Pharmacological Sciences 2012; 16: 299-304

Grape seed proanthocyanidins extracts promote apolipoprotein A-I mRNA expression in HepG2 cells under experimental sugar and high-sugar conditions

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Abstract. – *Objectives:* **In this study, we investigated the effect of grape seed proanthocyanidins extracts (GSPE), which have been proved to have anti-oxidative and anti-aging functions, on the expression of apoA-I at mRNA level of HepG2 cells *in vitro* under the experimental conditions of high-sugar and sugar.**

Materials and Methods: **Cell viability was measured by sulforhodamine B (SRB). The apoA-I mRNA expression was assayed by real-time fluorescence quantitative polymerase chain reaction. Firstly, HepG2 cells were incubated in 10% inactivated newborn calf serum in Dulbecco's Modified Eagle Medium (DMEM). Next, cells were incubated with high-sugar and sugar serum-free medium, and added different concentration of GSPE (2.5, 5 and 10 μg/ml) for more than 24 hours, and thereafter, investigated whether GSPE can promote more apoA-I expression in HepG2 cells under the experimental conditions of high-sugar and sugar.**

Results: **In this experiment, HepG2 cells were incubated with high-sugar and sugar serum-free medium, and HepG2 cells incubated with high-sugar medium produced less apoA-I at mRNA level. The difference was significant ($p < 0.05$). When HepG2 cells were incubated with GSPE at concentration of 20 μg/ml or above for about 4 hours, cell viability measured by SRB was lower than 50%. However, cell viability of HepG2 cells incubated with GSPE at concentration of 10 μg/ml or below was higher than 70%. Therefore, we chose the HepG2 cells incubated with GSPE concentration of 2.5, 5, 10 μg/ml to observe the effect of GSPE on the mRNA expression of apoA-I. After incubated with GSPE, the apoA-I expression of HepG2 cells were significantly elevated at mRNA level compared to that of high sugar control ($p < 0.05$). Moreover, this action of GSPE showed dose dependent, and the dose of 2.5 μg/ml was optimal.**

Conclusions: **GSPE (concentration of higher than 20 μg/ml) could inhibit HepG2 cell survival, and in HepG2 cells, endogenous apoA-I was significantly suppressed following 24h of exposure to high concentrations of glucose. Meanwhile GSPE could promote expression of apoA-I dose dependently at mRNA level when its concentration was lower than 10 μg/ml.**

Key Words:

Grape seed proanthocyanidins extracts, Apolipoprotein A-I, HepG2 cell, mRNA.

Introduction

It is well known that macro-angiopathy artery, atherosclerosis, is one of the vascular complications of both type 1 and 2 diabetes mellitus. The pathologic changes of diabetic macro-angiopathy can be from endothelial dysfunction to formation of atherosclerotic plaque[1], but its main remains unknown, in which oxidative stress induced by chronic hyperglycemia may play a key role[2-3].

Proanthocyanidins are naturally occurring polyphenolic compounds widely available in fruits, vegetables, nuts, seeds, flowers and bark. Grape seed proanthocyanidins extracts (GSPE), which are derived from grape seeds, have been reported to possess a variety of potent properties, including antioxidant, antiinflammation, radical-scavenging, and cardiovascular protecting activity, anti-tumor, and so on[4-14]. Our previous experiments showed that GSPE had a protective effect on aortic tissue in the diabetic rats. And in the

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proteomic study of aortic tissue of diabetic rats, a total of 25 protein spots included 17 up-regulated and 8 down-regulated spots after GSPE treatment were identified. One of the 17 up-regulated spots was identified by LTQ type electrospray ionization-tandem mass spectrometry (LTQ-ESI-MS/MS, Thermo Finnigan Scientific, CA, USA) as apolipoprotein A-I (apoA-I)[14].

Previous studies have proved that apoA-I, the major apolipoprotein of high density lipoprotein (HDL), by removing cholesterol and cholesteryl ester hydroperoxides from oxidized low density lipoprotein and foam cells, is a protective factor of cardiovascular disease. And there is a negative relationship between the level of apoA-I and cardiovascular diseases[15-16]. The aim of this study was to explore the effect of GSPE on the expression of apoA-I mRNA in HepG2 cells under experimental sugar and high-sugar conditions and find the role of target of GSPE.

Materials and Methods

Materials

The HepG2, human hepatoma cells chosen for the studies were purchased from Shanghai Institute of Cell Resource Center for Health Science Academy (Shanghai, China). GSPE (proanthocyanidin content exceeds 96%, Lot No: G050412) were provided by Jianfeng, Inc. (Tianjin, China). Bovine serum albumin (BSA), trypsin/EDTA solution, dimethyl sulfoxide (DMSO) and sulforhodamine B (SRB) were purchased from Sigma (St Louis, MO, USA). Fetal bovine serum, high-sugar (Cat#11995) and sugar (Cat#11885) serum-free medium were obtained from GIBCO (Invitrogen Biotech, Carlsbad, CA, USA). ApoA-I and β-actin primer sets were synthesized by TaKaRa Biotech Co, Ltd (Dalian, China).

Cell Culture

HepG2 cells were incubated in Dulbecco Modified Eagle Medium (DMEM) containing 10% fetal bovine serum at 37°C, under humidified 5% CO_2 in room air. All experiments were performed with cells at passages of 2-3. At cell confluence, DMEM medium containing 10% fetal bovine serum was replaced by high-sugar and sugar serum-free medium and GSPE of concentrations of 2.5, 5, 10, 20, 40 and 80 μg /ml were added for co-incubation. GSPE stock solutions for cell treatment were prepared fresh in dimethyl sulfoxide (DMSO) at concentrations such that the final concentration of the solvent in cell suspension never exceeded 0.1% (v/v). Respective controls were treated with equal volume of DMSO. Under all conditions cell viability was judged by Sulforhodamine B (SRB).

RT-PCR Procedures

HepG2 cells were incubated in high-sugar and sugar serum-free medium and GSPE of concentrations of 0, 2.5, 5, 10 μg /ml were added for co-incubation. According to the manufacturer's instructions, total RNA was extracted from HepG2 cells using the UNIQ-10 Trizol total RNA extract kit (Invitrogen Biotech Co. Ltd., Carlsbad, California**,** USA) following stimulation. The concentration of total RNA was determined by spectrophotometry (Eppendorf Ltd, Hamburg, Germany) at 260-nm wavelength. The integrity of RNA samples was checked by gel electrophoresis (Bio-Rad, Hercules, California, USA) in 1% agarose gel stained with ethidium bromide. To synthesis the first strand cDNA, realtime reverse transcription was performed using 1 μl of total RNA in a 10 μl final volume of reaction mixture 10× reaction buffer, 10 mM dNTP mixure (the mixture of dATP, dCTP, dGTP and dTTP), rNase inhibitor, avian myeloblastic virus (AMV) Reverse Transcriptase, Random 9 mers. By incubation at 30°C for 10 min, 42°C for 30 min and 99°C for 5 min, The reaction was stopped by incubation at and 5°C for 5 min. Thereafter, polymerase chain reaction (PCR) was carried out using 5 μl of cDNA obtained as templates in a 20 μl/sample reaction volume containing 0.125 μl of TaKaRa Ex Taq® HS (hot start), 0.25 μl of upstream primers, 0.25 μl of downstream primers, and 14.375 μl of double-distilled water (PCR kit from TaKaRa Biotech Co, Ltd, China). The primers designed were as follows:

apoA-I (sense) 5'-ATCGAGTGAAGGACCTGGC-3'
(antisense) 5'-AGCTTGCTGAAGGTGGAGGT-3'

β-actin (sense) 5'-GGCATCGTGATGGACTCCG-3'
(antisense) 5'-GCTGGAAGGTGGACAGCGA-3'

The amplification profile consisted of an initial denaturation at 94°C for 3 minutes followed by denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 1 min with a number of 35 cycles. Of the PCR

products obtained (apoA-I 154 bp, β-actin 594bp), 10 ml were visualized by ethidium bromide staining and photographed under UV radiation after 1.5% agarose gel electrophoresis. Identified signal intensity of apoA-I PCR product band was normalized with that of the β-actin PCR product and expressed as a ratio. The experiment was repeated 3 times with 3 different batches of cells.

Statistical Analysis

Results were expressed as means±SD on the basis of at least 3 separate experiments. Statistical significance of the differences among experimental groups was calculated by unpaired Student *t* test and one-way ANOVA. $p < 0.05$ was considered statistically significant.

Results

Effect of GSPE of Different Concentrations on HepG2 Cell Viability

When HepG2 cells were incubated with GSPE of concentrations of higher than 20 μg /ml, cell viabilities were significantly lower than other concentrations. After 4 hours of co-incubation with GSPE of concentrations of higher than 20 μg/ml, HepG2 cells showed apoptosis, cell shrinking, nuclear fragmentation and cell foaming (showed in Figure 1). While HepG2 cell viabilities were higher than 70% until 24 hours after co-incubation with GSPE of concentrations of lower than 10 μg/ml. The viabilities of HepG2 cells co-incubation with GSPE of different concentrations under high-sugar conditions were showed in Table I and Figure 2.

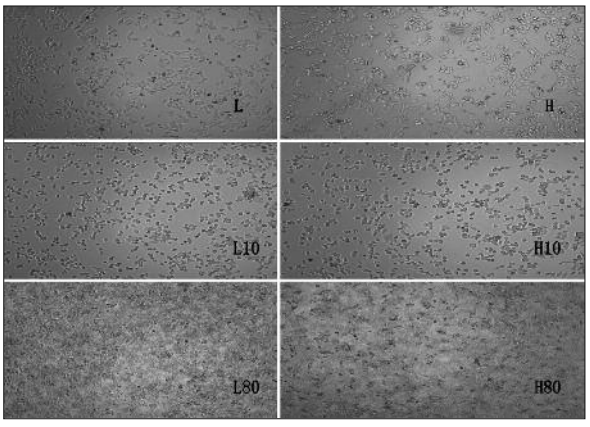

Figure 1. Rappresentative light micrographs of HepG2 cells (×100) co-incubated with different concentrations of GSPE. (L: sugar condition; H: high sugar condition; L10: sugar condition with 10 μg/ml GSPE; L80: sugar condition with 80 μg/ml GSPE; H10: high sugar condition with 10 μg/ml GSPE; H80: high sugar condition with 80 μg/ml GSPE.

Table I. Viabilities of HepG2 cells co-incubation with GSPE of different concentrations under high-sugar conditions.

GSPE of different concentrations	Viabilities of HepG2 cells
2.5 μg/ml	92.00% ± 8.80%
5 μg/ml	81.00% ± 7.80%
10 μg/ml	72.00% ± 12.60%
20 μg/ml	56.00% ± 8.20%
40 μg/ml	21.00% ± 4.35%

Effect of GSPE on apoA-I mRNA Levels in HepG2 Cell in High-sugar and Sugar Conditions

After pre-experiment, we chose concentrations of 0, 2.5, 5 and 10 μg/ml of GSPE to co-incubate with HepG2 cells under sugar and high-sugar conditions. The mRNA expression of apoA-I was evaluated by reverse transcriptase-PCR (RT-PCR). Under sugar condition of HepG2 cells, apoA-I PCR products had a marked increase compared to that under high-sugar condition when concentration of GSPE was 0 μg/ml (1.838±0.240, $p < 0.01$). Co-incubation with different concentrations of GSPE promoted the levels of apoA-I mRNA expression in the HepG2 cells in a dose-dependent manner. Promotion of apoA-I mRNA levels differed ranging from 1.838±0.240 to 5.198±0.896 for 2.5 μg/ml, to 4.705±0.514 for 5 μg/ml, to 3.004±0.509 for 10 μg/ml ($p < 0.01$, *vs.* sugar alone) under sugar condition respectively.

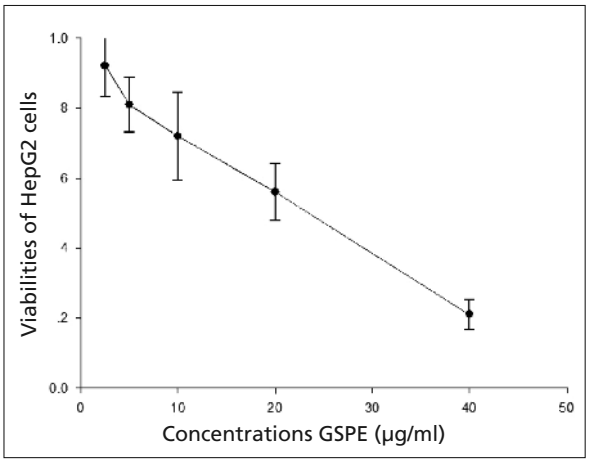

Figure 2. Viabilities of HepG2 cells co-incubation with GSPE under high-sugar conditions.

Table II. Effect of GSPE of different concentrations on endogeneous apoA-I mRNA in HepG2 cells.

Group	Relative expression of apoA-I mRNA
H	1 ± 0.221
H2.5	8.502 ± 1.604*
H5	6.385 ± 0.812*
H10	2.870 ± 0.396*
L	1.838 ± 0.240*
L2.5	5.198 ± 0.896#
L5	4.705 ± 0.514#
L10	3.004 ± 0.509#

*$p < 0.01$: vs. H; #$p < 0.01$: vs. L.

And under high-sugar condition, promotion of apoA-I mRNA levels differed ranging from 1±0.221 to 8.502±1.604 for 2.5 μg/ml, to 6.385±0.812 for 5 μg/ml, to 2.870±0.396 for 10 μg/ml ($p < 0.01$, *vs.* high-sugar alone). The results were showed in Table II, Figures 3 and 4. In contrast, the levels of β-actin mRNA expression remained the same under these conditions.

Discussion

Apolipoprotein A-I (apoA-I), which constitutes 70% of the apolipoprotein content of HDL,

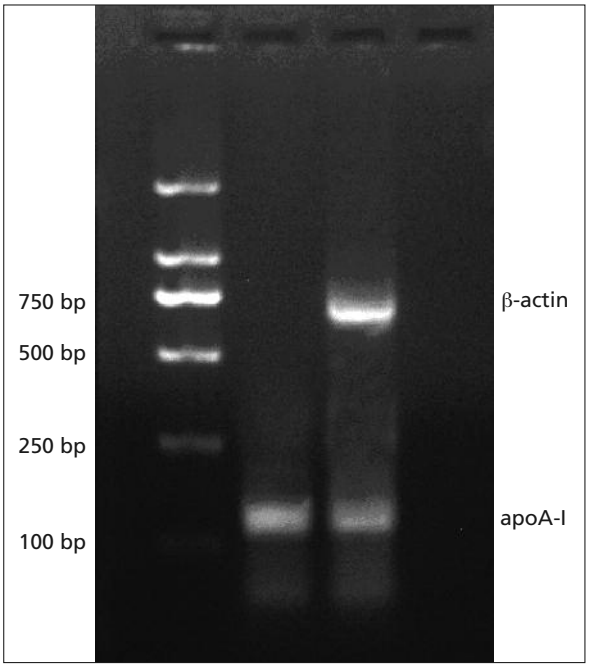

Figure 3. Agarose gel electrophoresis result of apoA-I.

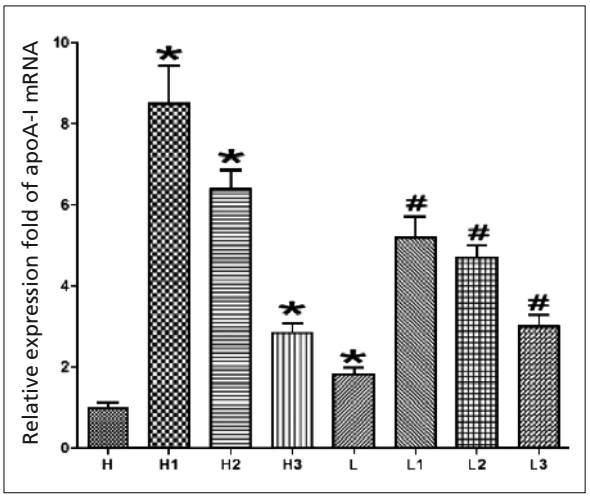

Figure 4. Effect of different concentrations of GSPE on expression of apoA-I mRNA. H: high sugar condition; H1: high sugar condition with 2.5 μg/ml GSPE; H2: high sugar conditions with 5 μg/ml GSPE; H3: high sugar condition with 10 μg/ml GSPE. L: sugar condition; L1: sugar condition with 2.5 μg/ml GSPE; L2: sugar condition with 5 μg/ml GSPE; L3: sugar condition with 10 μg/ml GSPE. *$p < 0.01$: vs. H; #$p < 0.01$: vs. L.

acts as acceptor for the transfer of phospholipids and free cholesterol from peripheral tissues and transports cholesterol in the liver and other tissues for excretion and steroidogenesis. In particular, apoA-I interacts with the ATP-binding cassette transporter A1 and accepts free cholesterol, and acts as a cofactor of the HDL-associated enzyme lecithin cholesterol acyl transferase (LCAT), thereby mediating the delivery of cholesterol ester to the liver. This process is important in reducing the accumulation of foam cells in the arterial walls[17-19]. Furthermore, apoA-I exerts antioxidant properties by removing lipid hydroperoxides from low-density lipoproteins (LDL), thereby, reducing the oxidation of LDL and consequently the atherogenicity of these lipoproteins. Therefore, apoA-I also shows anti-inflammatory actions through the inhibition of the expression of adhesion molecules in endothelial cells and reducing the recruitment of monocytes in the arterial walls[20]. Beside this, D-4F, the mimetic peptide of apoA-I could inhibit the reactive oxygen species (ROS) in blood circulation, and capacity of anti-oxidant and vascular repair of diabetic rats were increased[21-22]. Recently, research showed apoA-I could stimulate adenosine monophosphate-activated protein kinase to promote glucose absorption[23-25].

Increseased levels of apoA-I could be found in pre-menopausal women, can be induced with

regular exercise, and moderate consumption of alcohol, in particular red wine[26]. Beyond these known factors, there is little in terms of pharmacologic agents that specifically raise the levels of endogenous apoA-I.

Since apoA-I gene was expressed in both small intestine and liver, this study examined the ability of GSPE to affect expression of gene in liver cells. In this experiment, we used sulforhodamine B (SRB) to measure the viabilities of HepG2 cells. SRB is a kind of water-soluble protein dye that can bind with basic amino acids of biological macromolecules. The viabilities are consistent with the quantity of SRB bound with cells[27]. When incubated with GSPE of concentrations higher than 20 μg/ml for about 4 hours, HepG2 cells showed cell shrinkage, nuclear chromatin condensation, nuclear fragmentation and cell foam. After co-incubated with GSPE for 24 hours, the viabilities of HepG2 cells were lower than 50% compared with that of more than 70% when GSPE concentrations were lower than 10 μg/ml. Therefore, we chose GSPE concentration of 2.5, 5, 10 μg/ml as observation of objects.

As we known, apoA-I could be depressed under high-sugar condition. Some researchers found that the rats endogenous apoA-I would be depressed at least an half at transcriptional level under high glucose condition. The target point was at a 50bp fragment (-474 to -7) of apoA-I gene promoter[28]. In our experiment, when HepG2 cells were incubated in high-sugar medium, the expression of apoA-I mRNA was depressed significantly.

Our previous study manifested that GSPE suppressed the hypertrophy and disarray of endothelial cells, decreased proliferation of smooth muscle cells of streptozotocin (STZ)-induced diabetic rats, and led to light microscopic findings similar to those of the control rats[14]. GSPE had been proved that it could markedly down-regulate the receptor for advanced glycation end products (AGEs) and vascular cell adhesion molecule 1 (VCAM-1) gene expression induced by AGEs in endothelial cells, and it could inhibit the formation of ROS[29-30]. Beside this, when HepG2 cells were incubated with GSPE under high-sugar condition, the endogenous apoA-I mRNA was elevated significantly, and this change was dose-dependent. The GSPE concentration of 2.5 μg/ml was optimal. Other more, GSPE caused an increase in the abundance of endogenous apoA-I mRNA following exposure of the agent after 24 hours of treatment under sugar condition and the GSPE concentration of 2.5 μg/ml was also the optimal. GSPE could promote the expression of endogenous apoA-I under both sugar and high-sugar condition, which may provide great clinical advantage.

Various regions related to the apoA-I gene and specifically within the relevant promoter region have been identified that appear to be important for controlling gene activity[31-32]. Resveratrol, mimetic of GSPE, have been discovered to enhance activity of the gene[33]. In this experiment, the primer was within the third and fourth exon region of apoA-I, which might be the activating target point of GSPE.

In summary, GSPE could promote expression of endogenous apoA-I under both sugar and high-sugar conditions. In conjunction with other properties, GSPE may have therapeutic potentials in the treatment and prevention of diabetic vascular complications. However, further studies are needed to elucidate the precise molecular mechanisms underlying the regulation of apoA-I by GSPE.

Acknowledgements

We thank the Center of Medical Biotechnology, Shandong Academy of Medical Sciences, the Department of Experimental Pathology and Physiology, Shandong Academy of Medical Sciences, and the Center for New Drug Evaluation of Shandong University. We also thank Professor Xiang-guo Liu and Professor Yazhou Cui for their assistance. #This study was supported by Shandong Province Natural Science Foundation (No. 2009ZRB02275).

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