Grape seed procyanidin B2 ameliorates mitochondrial dysfunction and inhibits apoptosis via the AMP-activated protein kinase–silent mating type information regulation 2 homologue 1–PPARγ co-activator-1α axis in rat mesangial cells under high-dose glucosamine

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Abstract
Grape seed procyanidin B2 (GSPB2), an antioxidative and anti-inflammatory polyphenol in grape seed, has been found to have protective effects on diabetic nephropathy. Based on its favourable biological activities, in the present study, we aimed to investigate whether GSPB2 could inhibit apoptosis in rat mesangial cells treated with glucosamine (GlcN) under high-dose conditions. The results showed that the administration of GSPB2 (10 μg/ml) significantly increased the viability of mesangial cells treated with GlcN at a dose of 15 mM. We found that GSPB2 inhibited apoptosis in mesangial cells using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphates (dUTP) nick-end labelling staining and flow cytometry technique (P<0.05 for both). GSPB2 treatment also suppressed oxidative stress by elevating the activity of glutathione peroxidase (P<0.05) and superoxide dismutase (P<0.01), as well as prevented cellular damage. GSPB2 enhanced the mRNA expression of nuclear respiratory factor 1, mitochondrial transcription factor A and mitochondrial DNA copy number in mesangial cells as determined by real-time PCR (P<0.05 for each). Finally, GSPB2 treatment activated the protein expression of PPARγ co-activator-1α (PGC-1α), silent mating type information regulation 2 homologue 1 (SIRT1) and AMP-activated protein kinase (AMPK) in mesangial cells. These findings suggest that GSPB2 markedly ameliorates mitochondrial dysfunction and inhibits apoptosis in rat mesangial cells treated with high-dose GlcN. This protective effect could be, at least in part, due to the activation of the AMPK–SIRT1–PGC-1α axis.

Key words: Apoptosis; Diabetic nephropathy; Grape seed procyanidin B2; Mesangial cells; Mitochondrial dysfunction

Diabetic nephropathy (DN) is one of the most serious and common microvascular complications of diabetes mellitus, and is a major cause of end-stage renal disease[1]. Mesangial cells play a vital role in the pathogenesis of DN. Mesangial cell proliferation and hypertrophy have been identified to be early events in the progress of diabetic kidney disease[2]. However, mesangial cell apoptosis, which has been observed in both experimental and human glomerular diseases including DN, becomes an important event as the disease progresses[3–5]. It has been demonstrated that mesangial cell apoptosis is related to an increase in albuminuria[6] and may play a pathological role in progressive glomerulosclerosis by reducing the number of mesangial cells[4]. Therefore, seeking effective methods for inhibiting mesangial cell apoptosis may be of great clinical importance in the treatment of DN.

Oxidative stress is considered to be a trigger of mesangial cell apoptosis[7]. Reactive oxygen species (ROS), such as O$_2^-$, H$_2$O$_2$, HO$_2$ and OH, are generated when stimulated by pro-inflammatory factors. Excess ROS generation leads to increased metabolism accumulation in diabetes[8,9]. There is mounting evidence that ROS initiate mesangial cell apoptosis in vitro[10,11]. Mitochondria are the major source of ROS. Mitochondrial dysfunction could lead to more ROS generation and result in further oxidative stress and increased apoptosis.

Abbreviations: AMPK, AMP-activated protein kinase; DN, diabetic nephropathy; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GlcN, glucosamine; GSH-Px, glutathione peroxidase; GSPB2, grape seed procyanidin B2; MDA, malondialdehyde; MEM, minimum essential medium; mtDNA, mitochondrial DNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NRF-1, nuclear respiratory factor 1; PARP, poly(ADP-ribose) polymerase; PGC-1α, PPARγ co-activator 1α; RAGE, receptor for advanced glycation end products; ROS, reactive oxygen species; SIRT1, silent mating type information regulation 2 homologue 1; SOD, superoxide dismutase; TFAM, mitochondrial transcription factor A; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling.

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It has been reported that mitochondrial dysfunction resulting from the decrease in mitochondrial DNA (mtDNA) is associated with the pathogenesis of diabetes mellitus and its complications including DN\(^{(12)}\). Therefore, the inhibition of mitochondrial dysfunction may be an effective way to suppress apoptosis and slow down the progression of DN.

Proanthocyanidin extracts, derived from grape seed, have been reported to possess a wild range of potent biological activities including free radical scavenging, antioxidant, anti-inflammatory and anti-tumour activities\(^{(19,20)}\). The overexpression of the transcriptional co-activator PPAR (PGC-1\(\alpha\)) can prevent aldosterone-induced mitochondrial dysfunction in podocytes and regulate mitochondrial oxidative stress\(^{(15,16)}\). AMP-activated protein kinase (AMPK) and silent information regulator T1 (SIRT1) have been found to be capable of regulating PGC-1\(\alpha\) activity and energy metabolism of mitochondria\(^{(17)}\). It has been suggested that high glucose-induced mesangial cell damage lowered the activity of the AMPK–SIRT1–PGC-1\(\alpha\) axis may offer new approaches for the treatment of mesangial cell apoptosis and DN.

Materials and methods

**Chemicals and reagents**

GSPB2 was purchased from Jianfeng Inc. (>95% pure, batch no. 20080915). Minimum essential medium (MEM) and fetal bovine serum (FBS) were obtained from Gibco Invitrogen Corporation. Trypsin, dimethyl sulfoxide (DMSO), GlcN and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyterazolium bromide (MTT) were all purchased from Sigma. The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphates (dUTP) nick-end labelling (TUNEL) detection kit was obtained from KeyGen Biotech. The Annexin-V-fluorescein (FLUOS) Staining Kit was obtained from Roche Diagnostics.

**Materials and methods**

Rat mesangial cells (HBZY-1) were obtained from the Center of Type Culture Collection, Chinese Academy of Medical Sciences. The cells were cultured in MEM supplemented with 10% FBS and incubated at 37°C under a humidified atmosphere of 5% CO\(_2\). The cells were used at the fifth passage. All experiments were performed in the logarithmic phase of cell growth. The cells were incubated for 24 h in serum-deprived (0-2% FBS) MEM. Then, mesangial cells were cultured in MEM and 10% FBS with or without 15 mM GlcN and two concentrations of GSPB2 (2.5 and 10 \(\mu\)g/ml) for 24 h. The highest concentration of GSPB2 in plasma was 14 \(\mu\)g/ml after being taken for 2 h; so these two concentrations of GSPB2 can be reached in vivo. Cells treated with 15 mM mannitol instead of GlcN were used as osmolarity control.

**Cell culture**

Rat mesangial cells (HBZY-1) were obtained from the Center of Type Culture Collection, Chinese Academy of Medical Sciences. The cells were cultured in MEM supplemented with 10% FBS and incubated at 37°C under a humidified atmosphere of 5% CO\(_2\). The cells were used at the fifth passage. All experiments were performed in the logarithmic phase of cell growth. The cells were incubated for 24 h in serum-deprived (0-2% FBS) MEM. Then, mesangial cells were cultured in MEM and 10% FBS with or without 15 mM GlcN and two concentrations of GSPB2 (2.5 and 10 \(\mu\)g/ml) for 24 h. The highest concentration of GSPB2 in plasma was 14 \(\mu\)g/ml after being taken for 2 h; so these two concentrations of GSPB2 can be reached in vivo. Cells treated with 15 mM mannitol instead of GlcN were used as osmolarity control.

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propidium iodide mixture, and incubated for 15 min at 25°C in the dark. The samples were analysed by flow cytometry (BD FACSCalibur).

**Colorimetric method**

The colorimetric method was applied to detect the levels of glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and malondialdehyde (MDA), which were used to reflect the severity of oxidative stress. Mesangial cells (1 × 10^6 in 15 ml medium) were seeded in 75 cm^2 tissue culture flasks. The results were calculated with the standard curve, according to the manufacturer's instructions (Beyotime Biotechnology).

**Quantitative real-time PCR and analyses of mitochondrial DNA content**

Total RNA and DNA from 10^6 mesangial cells were isolated by TRIzol reagent (Invitrogen) and the DNeasy Tissue Kit (Qiagen Sciences), respectively. The sequences are shown in Table 1. The expression levels of target genes, nuclear respiratory factor 1 (NRF-1) and mitochondrial transcription factor A (TFAM), were determined by real-time RT-PCR, and the relative mtDNA content was detected using quantitative real-time PCR. Reverse transcription was performed using a reaction kit according to the manufacturer's protocol (Promega Reverse Transcriptase System). The ABI 7500 Real-time PCR Detection System was used to perform real-time PCR. mtDNA and β-actin were used for the amplification of nuclear and mtDNA, respectively. Cycling conditions were as follows: denaturation programme (95°C for 10 min), and amplification and quantification by forty cycles of 95°C for 15 s and 60°C for 30 s. The values were determined relative to the control sample after normalising to the values of β-actin as the control gene, and calculated by the comparative cycle threshold (ΔΔCt) method.

**Western blot analysis**

Cells from each group were collected and lysed for Western blotting. The whole process of operation was completed on ice. Protein concentrations were determined using a BCA protein assay kit (Beyotime Biotechnology), following the manufacturer's instructions. Equal amounts of protein were separated by 10% SDS–PAGE and transferred to polyvinylidene fluoride membranes. After being blocked with 5% non-fat milk or 5% bovine serum albumin for 1 h, the membranes were incubated overnight at 4°C with the following primary antibodies: phospho-AMPK Thr172 (1:200; Santa Cruz Biotechnology), SIRT1 (1:200; Santa Cruz Biotechnology), NRF-1 (1:200; Cell Signaling Technology), poly(ADP-ribose) polymerase (PARP, 1:1000; Cell Signaling Technology), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:500; ZSGB-BIO), receptor for advanced glycation end products (RAGE, 1:200; Santa Cruz Biotechnology) and β-actin (1:1000; Cell Signaling Technology). Goat anti-rabbit or anti-mouse IgG (1:4000; ZSGB-BIO) was used as the secondary antibody. Then, immune complexes were detected by enhanced chemiluminescence with Super ECL Plus Detection Reagent (Applygen) and analysed using Image Pro Plus 6.0 Software (Media Cybernetics). Values were corrected with those of β-actin used as the control.

**Statistical analysis**

Data were assessed by SPSS 13.0 (SPSS Inc.). Results are presented as means and standard deviations. Homogeneity of the data was first tested by selecting the option ‘Homogeneity of variance’ in SPSS software. If variances were equal, data were evaluated by means of a one-way ANOVA with Bonferroni correction; otherwise, Tamhane’s T2 test was performed. P < 0.05 was considered as statistically significant.

**Results**

**Effects of grape seed procyanidin B2 on the viability of mesangial cells treated with high-dose glucosamine**

The number of mesangial cells cultured under normal glucose concentrations was not altered by GSPB2 treatment (Fig. 1(a)). In contrast, GlcN significantly suppressed cell viability compared with the control group (P < 0.05). However, cell viability was significantly improved in the GSPB2 (10 μg/ml)-treated group (P < 0.05; Fig. 1(b)).

**Effects of grape seed procyanidin B2 on the apoptosis of mesangial cells treated with high-dose glucosamine**

Similar results were demonstrated using the TUNEL staining (Fig. 2(a)) and flow cytometry (Fig. 2(b)) methods. There were no differences observed between the control and mannitol-treated groups. GlcN significantly increased the percentage of apoptotic cells compared with the control group (P < 0.05). Interestingly, the treatment with GSPB2 protected against GlcN-induced cell apoptosis (P < 0.05).

**Effects of grape seed procyanidin B2 on oxidative stress in mesangial cells treated with high-dose glucosamine**

As shown in Table 2, the activities of GSH-Px and SOD and the content of MDA in the mannitol-treated and control groups

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**Table 1. Primer sequences used for real-time PCR**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer (5′–3′)</th>
<th>Reverse primer (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRF-1</td>
<td>TTACTCTGTGCTTGGGTGATGG</td>
<td>CTTGTATGTTTGGTGCTGCTT</td>
</tr>
<tr>
<td>TFAM</td>
<td>GAAAGCACAATCAAGAGGAG</td>
<td>CTGCTTTTTCATGAGACAG</td>
</tr>
<tr>
<td>mtDNA</td>
<td>ATCCCTCCAGATTGTGGAAAT</td>
<td>ACCGAGTATTTGGGATTA</td>
</tr>
<tr>
<td>β-Actin</td>
<td>TAAAGACCTCTTGCAACAC</td>
<td>TAAAGACCTGCAAAATTGTC</td>
</tr>
</tbody>
</table>

NRF-1, nuclear respiratory factor 1; TFAM, mitochondrial transcription factor A; mtDNA, mitochondrial DNA.
were similar. High-dose GlcN-induced oxidative stress in mesangial cells was demonstrated by the decrease in the activities of GSH-Px and SOD in the GlcN-treated group, but an increase in the content of MDA \((P < 0.05\) or \(P < 0.01\)) compared with the control group. On the contrary, the activities of GSH-Px and SOD were improved in the GSPB2 (10 \(\mu g/ml\))-treated group \((P < 0.05\) and \(P < 0.01\), respectively). Although the content of MDA was slightly decreased in the GSPB2-treated group, it did not differ significantly from the control group.

**Effects of grape seed proanthocyanidin extracts on the AMP-activated protein kinase–silent mating type information regulation 2 homologue 1–PPAR\(\gamma\) co-activator 1\(\alpha\) axis in mesangial cells treated with high-dose glucosamine**

The protein expression of the AMPK–SIRT1–PGC-1\(\alpha\) axis in mesangial cells treated with high-dose GlcN was ameliorated by GSPB2 treatment.

**Effects of grape seed proanthocyanidin extracts on the mitochondrial transcription factor A and mitochondrial DNA content in mesangial cells treated with high-dose glucosamine**

Real-time PCR analysis showed that the mRNA expression of \(NRF-1\) and \(TFAM\), two essential mitochondrial biogenesis factors for gene expression in mammals, was significantly decreased in the GlcN-treated group compared with the control and mannitol-treated groups \((P < 0.05\) for each; Fig. 4(a)), and the mtDNA copy number was also significantly reduced \((P < 0.05\) for each; Fig. 4(b)). Interestingly, these changes were markedly reversed after the treatment with GSPB2 \((P < 0.05\) for each). These findings suggest that mitochondrial dysfunction in mesangial cells treated with high-dose GlcN could be ameliorated by GSPB2 treatment.

**Discussion**

To the best of our knowledge, the present study was the first to report that GSPB2 could inhibit high-dose GlcN-induced apoptosis of mesangial cells \textit{in vitro}. This protective effect induced by GSPB2 could be due in part to the suppression of oxidative stress, the amelioration of mitochondrial dysfunction and the activation of the AMPK–SIRT1–PGC-1\(\alpha\) axis in mesangial cells.

Procyanidin absorption depends on the degree of polymerisation. Deprez et al.\textsuperscript{(24)} showed that procyanidin dimers and trimers are absorbed through the human intestinal cell line Caco-2, but procyanidins with an average polymerisation
Fig. 2. Effects of grape seed procyanidin B2 (GSPB2) on the apoptosis of mesangial cells cultured under high-dose glucosamine. (a) Representative terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL)-positive mesangial cells (original magnification ×400) and the quantitative analyses of the results are shown. HPF, high power field. (b) Annexin V-fluorescein isothiocyanate (FITC-A)/propidium iodide (PI-A) staining of mesangial cells in each group is shown. Values are means from three independent experiments, with standard deviations represented by vertical bars. * Mean value was significantly different from that of the control group (P < 0.05). † Mean value was significantly different from that of the glucosamine (GlcN)-treated group (P < 0.05). (A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn)
The degree of 7 are unabsorbed. Procyanidin polymers are believed to be fermented by intestinal microflora to produce more smaller molecular-weight phenolic acids and some other unknown metabolites of colonic origin \(^{(25)}\). In a study investigating the bioavailability of GSPB2 in plasma, rats were orally administered with procyanidin B2 \(^{(26)}\). The study found that procyanidin B2, epicatechin and 3'-O-methyl-epicatechin were present in the plasma and urine of rats, and procyanidin B2 reached a maximum plasma concentration at 30–60 min after its consumption. The peak concentration of procyanidin B2 in plasma was 0·5 mM after the administration of procyanidin B2 (50 mg/kg body weight). However, further studies are still needed to clarify the mechanisms of the absorption of GSPB2. Additionally, GSPB2 and its metabolites are specifically accumulated in different organs such as liver, adipose tissue and muscle.

A high concentration of glucose (30 mM) is used to induce the apoptosis of mesangial cells. It has been observed that

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Mannitol</th>
<th>GlcN</th>
<th>GSPB2 (µg/ml)</th>
<th>GlcN</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH-Px (units/mg protein)</td>
<td>11·95</td>
<td>0·32</td>
<td>11·77</td>
<td>0·43</td>
<td>9·22*</td>
</tr>
<tr>
<td>SOD (units/mg protein)</td>
<td>46·10</td>
<td>7·05</td>
<td>45·19</td>
<td>6·98</td>
<td>21·91**</td>
</tr>
<tr>
<td>MDA (nmol/mg protein)</td>
<td>0·73</td>
<td>0·06</td>
<td>0·74</td>
<td>0·09</td>
<td>1·17*</td>
</tr>
</tbody>
</table>

GlcN, glucosamine.

Mean value was significantly different from that of the control group: * \(P < 0·05\), ** \(P < 0·01\).

Mean value was significantly different from that of the GlcN-treated group: † \(P < 0·05\), †† \(P < 0·01\).

Fig. 3. Effects of grape seed procyanidin B2 (GSPB2) on the protein expression of poly(ADP-ribose) polymerase (PARP), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and receptor for advanced glycation end products (RAGE) in mesangial cells cultured under high-dose glucosamine. Values were normalised to those of \(\beta\)-actin as the control. Values are means (n 4), with standard deviations represented by vertical bars. * Mean value was significantly different from that of the control group \((P < 0·05)\). † Mean value was significantly different from that of the glucosamine (GlcN)-treated group \((P < 0·05)\).
Oxidative stress plays a pivotal role in the induction of mesangial cell apoptosis. SOD is an enzyme responsible for catalysing the conversion of superoxide anion radical into peroxides, which are then converted into water by GSH-Px, and MDA is the most abundant by-product of lipid peroxidation. It has been demonstrated that GSPB2 is a powerful antioxidant, and its antioxidative activity is much stronger than that of vitamins C and E. Consistently, the present results showed that the activities of GSH-Px and SOD were markedly increased after the oral administration of GSPB2. In addition, the production of ROS was increased under the condition of oxidative stress. High levels of ROS induce DNA strand breaks, thereby activating PARP. The enzyme PARP is involved in a number of cellular processes including DNA repair and programmed cell death. During apoptosis, PARP is rapidly cleaved, and thus the detection of cleaved PARP is a diagnostic test for apoptosis in cells. Subsequently, the activity of GAPDH that is inhibited by cleaved PARP results in the increased expression of RAGE, which ultimately leads to cellular damage. In the present study, we observed ROS-induced cellular damage in mesangial cells treated with high-dose GlcN. However, GSPB2 inhibited the cleavage of PARP, improved the activity of GAPDH and down-regulated the expression of RAGE, which provided more evidence on its protective effects on mesangial cells against cellular damage.

Mitochondrial dysfunction, involved in the pathogenesis of DN, is the terminal event in the process involving oxidative stress-induced programmed cell death in mesangial cells. Mitochondria are not only ‘the power plant’ of the cell, but also the major source of ROS generated as the by-products of oxygen metabolism. mtDNA is a primary target of attack by ROS because it is deficient in DNA repair capacity, lacks histone-like coverage, and is localised close to the electron transport chain. ROS may induce mtDNA and impair the electron transport chain, which leads to the production of high levels of ROS. GSPB2 has the capacity to scavenge free radical and reduce the production of ROS due to its powerful antioxidative activity, which provides the possibility for the improvement of mitochondrial dysfunction. To evaluate mitochondrial dysfunction, we selected the following indicators: NRF-1; TFAM; mtDNA copy number. NRF-1 is a positive regulator of TFAM, which plays a role in mitochondrial transcription and regulates the transcription and replication of mtDNA. The mtDNA copy number was identified as a surrogate marker of mitochondrial function.

Studies have found that PGC-1α, a major regulator of oxidative metabolism and mitochondrial biogenesis, could bind to and co-activate the transcriptional function of NRF-1 on the promoter for TFAM, which, in turn, promotes mtDNA transcription and replication. The activation of PGC-1α improves mitochondrial biogenesis following oxidant injury. Therefore, PGC-1α plays a key role in mitochondrial dysfunction and oxidative stress. Although there have been few studies describing the effect of GSPB2 on the expression of PGC-1α, some have showed that polyphenols (e.g. resveratrol) enhance the expression of PGC-1α, indicating that GSPB2, which is also one of the polyphenols, might have a...
role in promoting the expression of PGC-1α. Moreover, it has been found that GSPB2 activates AMPK and increases the protein levels of SIRT1, which are the regulators of PGC-1α activity and energy metabolism of mitochondria, in human umbilical vessel cells (45). It has been reported that the activity of the AMPK–SIRT1–PGC-1α axis is lowered in high glucose-induced mesangial cells, leading to cell damage, exacerbated oxidative stress as well as apoptotic cell death in diabetes (18).

In the present study, the inactivation of the AMPK–SIRT1–PGC-1α axis was found in mesangial cells treated with high-dose GlcN. Interestingly, our data showed that GSPB2 could restore the protein levels of phospho-AMPK, SIRT1 and PGC-1α, suggesting the activation of the AMPK–SIRT1–PGC-1α axis by GSPB2. Furthermore, the present data showed that the activation of the AMPK–SIRT1–PGC-1α axis increased the expression levels of the transcription factors NRF-1 and TFAM, and, subsequently, activated the transcription and replication of mtDNA. These protective effects of GSPB2 on mitochondrial function have also been reported in in vivo studies (41, 46).

In conclusion, the present study suggested that GSPB2 could inhibit high-dose GlcN-induced mesangial cell apoptosis in vitro, which may be due to the improvement of oxidative stress, mitochondrial dysfunction and the activity of the AMPK–SIRT1–PGC-1α axis. The AMPK–SIRT1–PGC-1α axis could be one of the molecular mechanisms involved in the renal protective effects of GSPB2. These results provide further evidence that GSPB2 may be a potential therapeutic agent for the treatment of DN.

Acknowledgements

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The authors’ contributions are as follows: Y. L. and Z. Z. formulated the research question and designed the study; L. B. and X. C. performed the analysis and analysed the data; L. B. wrote the paper; X. C. made some modifications in the manuscript. The authors have no conflicts of interest to disclose.
References


