Delphinidin prevents high glucose-induced cell proliferation and collagen synthesis by inhibition of NOX-1 and mitochondrial superoxide in mesangial cells

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1. Introduction

Diabetic nephropathy (DN) is one of the most common complications of diabetes and a leading cause of end stage renal diseases. DN is characterized by an expansion of glomerular mesangium, which was caused by mesangial cell proliferation and excessive accumulation of extracellular matrix (ECM) (1,2). Although many clinical factors, such as hypertension and hyperlipidemia, affect the development of DN, hyperglycemia plays a major role in the pathological process of DN (2–4). One of the mechanisms whereby hyperglycemia induces DN is by increasing the generation of reactive oxygen species (ROS) (4) and clinical and animal studies have shown increases in the production of ROS and in the markers of oxidative stress in diabetic kidneys (5,6). Furthermore, the scavenging of ROS suppresses hyperglycemia-induced cell proliferation in mesangial cells (4).

Anthocyanins are abundant natural polyphenolics that contribute the intense colors of fruits and vegetables, including berries, red grapes, and red cabbage (7). Epidemiological studies have shown anthocyanins protect against the developments of cardiovascular diseases, cancer, and other chronic diseases, such as osteoporosis (8,9). Furthermore, it has been shown that anthocyanins exert beneficial effects on chronic diseases by reducing oxidative stress and inflammation (9). Delphinidin is a representative anthocyanidin (aglycon of anthocyanin), and was reported to exert the strongest anti-cancer effect among the common...
anthocyanidins (8,9). Delphinidin has also been shown to have antioxidant effects in the presence of oxidized low density lipoprotein (ox-LDL) (10), ultraviolet B (11), and carbon tetrachloride (12) in cells and mice. Moreover, among the common six anthocyanidins, delphinidin exerts the strongest antioxidant effects against 2,2-diphenyl-1-picrylhydrazyl (9). Nevertheless, the effect of delphinidin on high glucose-induced oxidative stress has not been previously investigated. Therefore, in the present study, we examined the effect of delphinidin on high glucose-induced cell proliferation and collagen synthesis in mesangial cells, and subsequently explored the mechanisms underlying the antioxidant effect of delphinidin.

2. Material and methods

2.1. Reagents

Delphinidin, cyanidin, malvidin, Mito TEMPO, L-NIL, allopurinol, N-acetyl-L-cysteine (NAC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), bromodeoxyuridine (BrdU), and 2',7'-dichlorodihydrofluorescein diacetate (H2DCFH-DA) were obtained from Sigma-Aldrich (St Louis, MO, USA). MitoSOX Red was obtained from Invitrogen (Carlsbad, CA, USA). Antibodies against phosphorylated ERK and total ERK were purchased from Cell Signaling Technology (Danvers, MA, USA), the TGF-β antibody was from Abcam (Cambridge, UK).

2.2. Cell culture

Mouse mesangial cells (CRL-1927, ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (GIBCO, Carlsbad, NY, USA) and 1% penicillin-streptomycin (GIBCO). High glucose treatment was performed by culturing cells in DMEM containing 25 mM (4500 mg/L) glucose for the indicated times and mannitol was used to enhance osmotic pressure.

2.3. Cell proliferation assay

Cell proliferation was determined using a MTT assay or by detecting BrdU incorporation using a commercial kit (Millipore, Billerica, MA, USA) according to manufacturer's instructions. Briefly, cells were seeded in the wells of a 24-well plate containing 250 μL of growth medium, and 25 μL MTT stock solution was then added to each well. After incubation for 4 h, dimethyl sulfoxide solution was added to lyse cells and dissolve the formazan crystals formed. After 5 min, 100 μL aliquots of the lysis solutions were transferred to the wells of a 96-well plate, and absorbance was read on a micro plate reader at 550 nm. For BrdU incorporation, cells were seeded in the wells of a 96-well plate and then treated with BrdU for 24 h. BrdU incorporation was detected using anti-BrdU antibody and a peroxidase substrate and absorbance was read at 450 nm.

2.4. ROS generation

Intracellular or mitochondrial ROS levels were measured by flow cytometry. Mesangial cells were seeded in a 6-well plate at a density of 2 × 10^5 cells per well. After synchronization for 24 h, cells were pretreated with various agents for 1 h and then stimulated with 25 mM glucose for 24 h. For intracellular ROS, H2DCF-DA was added to each well at a final concentration of 20 μM and incubated for 20 min at 37 °C in the dark. For mitochondrial superoxide, 5 μM MitoSOX Red was added to each well. The plate was washed 3 times with PBS, and attached cells were trypsinized, re-suspended, and then immediately subjected to FACSCalibur flow cytometry (BD

![Fig. 1. Effects of glucose on cell proliferation and collagen synthesis in mouse mesangial cells.](image-url)
Bioscience, San Jose, CA, USA). ROS were detected using excitation and emission wavelengths of 514 nm and 529 nm, respectively for H2DCF-DA and 510 nm and 580 nm, respectively for MitoSOX Red. Cells treated with 1 mM hydrogen peroxide (H2O2) for 1 h were used as a positive control.

2.5. Real-time polymerase chain reaction (RT-PCR)

Total RNA from cells was isolated using Trizol reagent (SigmaAldrich), as directed by the manufacturer. To prepare cDNA, 1 μg of each total RNA sample was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was performed using Real-Time PCR 7500 System and Power SYBR Green PCR master mix (Applied Biosystems), according to the manufacturer’s instructions. RT-PCR was performed using the following conditions. Reactions were initiated at 95 °C for 10 min, and this was followed by 45 amplification cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 20 s, and hybridization-elongation at 72 °C for 35 s. Primers for β-actin, collagen-I and collagen-IV were based on NCBI’s Nucleotide database and designed using the Primer Express program (Applied Biosystems): β-actin (forward, 5'-TGG ACA GTG AGG CAA GGA TAG-3'; reverse, 5'-TAC TGC CCT GGC TCC TCC TAG CA-3'); collagen-I (forward, 5'-ACA TGC TCA GCT TTG TGG AT-3'; reverse, 5'-ATA TTG GCA TGT TCG TGT TAG GC-3'), collagen-IV (forward, 5'-GGG TCC TGT CGT GAA GAG TTT AGA-3'; reverse, 5'-GCC GTG GCA TCT GCA-3').

2.6. Western blot analysis

Mesangial cells were lysed and centrifuged at 700 g for 5 min. Protein concentrations in the supernatants were determined using
the Bradford assay (Sigma–Aldrich). Equal amounts of proteins were separated by 12% Sodium Dodecyl Sulfate (SDS)-polyacrylamide gel electrophoresis at 80 V, and then transferred to a 0.45 μm polyvinylidenedifluoride (Millipore). After destaining, membranes were blocked with 5% nonfat dry milk in TBST (10 mM Tris HCl, pH 7.4/150 mM NaCl/0.1% Tween 20) and incubated overnight at 4°C with primary antibodies (diluted 1:1000). Membranes were washed in TBST and incubated with a goat-anti-rabbit IgG secondary antibody (diluted 1:1000; Bio Rad, Hercules, CA, USA) for 1 h at room temperature. Blots were detected using a chemiluminescence reagent (Millipore).

2.7. Statistical analysis

Results are expressed as mean ± SEM. The differences among the groups were analyzed by one-way ANOVA followed by Tukey’s post hoc test. Statistical significance was accepted for p value < 0.05.

3. Results

3.1. Delphinidin prevented glucose-induced cell proliferation and collagen synthesis

Glucose treatment at 5.6, 10, 17, and 25 mM for 24 h increased cell numbers in a dose dependent manner, and cell numbers at 25 mM glucose were higher than at 17 mM (Fig. 1A and B). Furthermore, treatment with glucose for 24 h also dose dependently increased the mRNA levels of collagen I and IV and the mRNA levels at 25 mM glucose were significantly higher than those of osmotic control (Fig. 1C and D). These results suggest that glucose at 25 mM increases the cell proliferation and collagen synthesis in mesangial cells.

The pretreatment of cells with delphinidin suppressed high glucose-induced cell proliferation in a dose dependent manner, and 50 μM delphinidin completely normalized the cell proliferation (Fig. 2A). Delphinidin at a concentration of 50 μM was used in this experiment since it was the lowest dose to completely suppress high glucose-induced cell proliferation. Delphinidin alone had no effect on cell proliferation (Fig. 2B and C). The high glucose (25 mM glucose) also increased the mRNA levels of collagen I and IV, and this was suppressed by delphinidin (Fig. 2D and E). These results indicate that delphinidin inhibits cell proliferation and collagen synthesis induced by high glucose concentrations.

3.2. Delphinidin suppressed high glucose-induced ROS generation

Treatment of cells with 25 mM glucose for 24 h increased ROS generation, and pretreatment with 50 μM delphinidin or 1 mM N-acetyl cysteine (NAC) suppressed the ROS generation (Fig. 3A). Pretreatment of apocynin (100 μM), NOX inhibitor, Fig. 3. Effect of delphinidin, cyanidin, and malvidin on high glucose-induced reactive oxygen species generation (ROS) in mouse mesangial cells. Cells were pretreated with 50 μM of delphinidin (Del) or 1 mM N-acetyl cysteine (NAC) for 1 h and then exposed to 25 mM glucose for 24 h (A). Cells were pretreated with 50 μM of Del, cyanidin (Cyan), or malvidin (Mal) for 1 h prior to 25 mM glucose exposure for 24 h (B and C). ROS levels were detected using a 2’,7’-dichlorofluorescein diacetate probe by flow cytometry and cell proliferation were measured using a MTT assay. Results are reported as the means ± SE of three to five separate experiments. Mannitol (glucose 5.6 mmol/L + mannitol 19.4 mmol/L) was used to enhance osmotic pressure. *p < 0.05 vs. Osmotic control (OC). #p < 0.05 vs. 25 mM glucose.
Fig. 4. Effect of delphinidin on high glucose-induced NADPH oxidase activation and mitochondrial superoxide generation in mouse mesangial cells. Cells were pretreated with 50 µM delphinidin (Del) for 1 h prior to 25 mM glucose exposure for 24 h. The mRNA level of NADPH oxidase 1 (NOX-1) was measured with real-time PCR (A). Cells were pretreated with 100 µM apocynin (APO) for 1 h and then incubated in the presence of 25 mM glucose for 24 h. H$_2$O$_2$ (1 mM) was treated for 1 h as positive control (B). Cells were pretreated with APO for 1 h in a dose dependent manner and then treated with 25 mM glucose for 24 h (C). Cells were pretreated with 50 µM Del for 1 h and then treated with 25 mM glucose for 24 h (D). Cells were pretreated with various doses (10–30 µM) of Mito TEMPO for 1 h prior to 25 mM glucose exposure for 24 h (E and F). Cells were pretreated with various doses of inducible nitric oxide synthase inhibitor L-NIL (0.1–1 mM; G) or xanthine oxidase inhibitor allopurinol (1–30 µM; H) for 1 h and then treated with 25 mM glucose for 24 h. Reactive oxygen species (ROS) levels were detected using 2',7'-dichlorofluorescin diacetate probe and mitochondrial (Mito) superoxide levels were detected using MitoSOX Red by flow cytometry. Cell proliferation was measured using a MTT assay. Results are reported as the means ± SE of three to five separate experiments. Mannitol (glucose 5.6 mmol/L + mannitol 19.4 mmol/L) was used to enhance osmotic pressure. *p < 0.05 vs. Osmotic control (OC). #p < 0.05 vs. 25 mM glucose.
Fig. 5. Effect of delphinidin on high glucose-induced ERK activation in mouse mesangial cells. Cells were treated with 25 mM glucose for 0, 5, 15, 30, and 60 min (A). Cells were pretreated with the 50 μM delphinidin (Del; B), 1 mM N-acetyl-L-cysteine (NAC; C), 100 μM apocynin (Apo; D), or 30 μM Mito TEMPO (Mito; E) for 1 h and then incubated in the presence of 25 mM glucose for 60 min. ERK phosphorylation was measured with western blotting (A–E). Cells were pretreated with ERK inhibitor PD98059 (10 μM) for 1 h and then exposed to 25 mM glucose for 24 h (F–H). Cell proliferation was measured using a MTT and the mRNA expression of collagen-I and collagen-IV was assessed by real-time PCR. Results are reported as the means ± SE of three to five separate experiments. Mannitol (glucose 5.6 mmol/L + mannitol 19.4 mmol/L) was used to enhance osmotic pressure. *p < 0.05 vs. Osmotic control (OC). #p < 0.05 vs. 25 mM glucose.
suppressed ROS generation (Fig. 4B), and does-dependently suppressed glucose-induced cell proliferation (Fig. 4C). Increases in mitochondrial superoxide levels by 25 mM glucose were also suppressed by delphinidin pretreatment (Fig. 4D). Pretreatment of cells with mitochondria-targeted antioxidant Mito TEMPO suppressed high glucose-induced superoxide generation in mitochondria and cell proliferation in a dose dependent manner (Fig. 4E and F). However, iNOS inhibitor L-NIL and xanthine oxidase inhibitor allopurinol had no effect on high glucose-induced ROS generation (Fig. 4G and H). These results suggest that delphinidin exhibits antioxidant effect through inhibition of NOX-1 activation and mitochondrial superoxide generation in high-glucose-treated cells.

3.3. Delphinidin suppressed high glucose-induced ERK1/2 activation and TGF-β expression

Treatment of mesangial cells with 25 mM glucose time-dependently increased ERK1/2 phosphorylation, and significantly increased ERK1/2 phosphorylation after 60 min of treatment versus the osmotic control (Fig. 5A). Increased ERK phosphorylation by 25 mM glucose at 60 min was suppressed by delphinidin (50 μM) pretreatment. Delphinidin alone had no effect on ERK phosphorylation (Fig. 5B). Furthermore, the increased ERK phosphorylation induced by 25 mM glucose was inhibited by 1 mM NAC pretreatment or 100 μM apocynin (Fig. 5C and D). Mito TEMPO at 30 μM also suppressed high glucose-induced ERK activation (Fig. 5E). In addition, increased cell numbers by 25 mM glucose was suppressed by pretreatment of ERK inhibitor PD98059 (10 μM). Increased mRNA levels of collagen I and IV by 25 mM glucose was also suppressed by PD98059 (Fig. 5F and H).

Treatment of cells with 25 mM glucose for 24 h increased the protein levels of TGF-β at 24 h and this was suppressed by pretreatment of 50 μM delphinidin, although delphinidin alone had no effect on TGF-β protein levels (Fig. 6A and B). Increased TGF-β protein levels induced by high glucose were inhibited by PD98059 (Fig. 6C). These results suggest that high glucose activates ERK1/2 and TGF-β, and thus, induces cell proliferation and collagen synthesis, and these adverse effects of glucose are suppressed by delphinidin.

4. Discussion

The present study demonstrates delphinidin prevents high glucose-induced cell proliferation and collagen synthesis in mesangial cells, and these anti-proliferative and anti-fibrotic effects of delphinidin are mediated by suppressing NOX-1 activation, mitochondrial superoxide generation, ERK1/2, and TGF-β. Thus,
this study elucidates the anti-oxidant effects of delphinidin in the presence of high glucose in mesangial cells and the mechanism responsible.

Hyperglycemia increases oxidative stress in peripheral tissues, and oxidative stress is closely associated with hyperglycemia-induced fibrosis in human and animal studies (13,14). High glucose also induces cell proliferation and collagen synthesis in mesangial cells in vitro, and increased ROS generation is responsible for the high glucose-induced effects (15). In the present study, high glucose was found to increase cell proliferation and collagen synthesis, and these effects were suppressed by the antioxidant NAC, which suggests ROS is an important mediator of glucose-induced mesangial cell activation. Like NAC, delphinidin also inhibited high glucose-induced ROS generation, and suppressed collagen synthesis and cell proliferation, which suggests that delphinidin counteracts the oxidant effect of high glucose. The anti-oxidant effect of delphinidin was more potent than two common anthocyanidins, cyanidin and malvidin, in this study and these results suggest delphinidin counteracts the oxidant effect of high glucose.

ROS generation in cells occurs with mitochondria, peroxisomes, plasma membrane NADPH oxidase (NOX), and other cellular elements (18). Among the sources of ROS, high glucose-induced enhanced ROS generation is primarily mediated by mitochondria and NOX (19). Mitochondrial ROS generation is enhanced in the kidney of diabetic subjects (20) and mitochondrial targeted anti-oxidant treatment prevents kidney damage in type 1 diabetic mice model (21). NOX has also been reported as important source of ROS generation in the podocytes (22) and mesangial cells (23). Moreover, NOX is a main contributor of increased ROS generation in the glomeruli of diabetic rats (24). The present study demonstrates that delphinidin suppressed increases in NOX-1 mRNA levels and mitochondrial ROS generation induced by high glucose. These results suggest that delphinidin counteracts the oxidant effect of glucose by suppressing NOX-1, which is consistent with the findings of previous studies that delphinidin suppresses NOX1 activation by ultraviolet B and ox-LDL (25). Furthermore, although cyanidin is known to inhibit glutamate-induced neuronal cell death through suppression of mitochondrial ROS (26), this study is firstly showing that delphinidin also suppresses mitochondrial ROS generation by high glucose.

Although the antioxidant effect of anthocyanin has received considerable attention, it has not been examined extensively in the context of high glucose. A chokeberry anthocyanin fraction suppresses high glucose-induced oxidative stress in pancreatic beta-cells (27), and cyanidin-3-glucoside, a common anthocyanin, protects hepatocytes from high glucose-induced apoptosis and ROS generation (28,29). The present study is the first to report that delphinidin also suppresses high glucose-induced ROS generation in mesangial cells. This result is consistent with previously reported findings that delphinidin exhibits antioxidant effects against physical and chemical factors. In particular, delphinidin exhibits antioxidant effect in human immortalized HaCaT keratinocytes and primary human umbilical vein endothelial cells and in animal tissues, such as, liver and skin in mice, exposed to ultraviolet B, ox-LDL, and carbon tetrachloride (10–12).

ERK1/2 is activated by oxidative stress in a variety of cell types, including mesangial cells (30,31). Although ERK1/2 activation plays a protective role in many cases against oxidative stress-induced cell damage, ERK signaling pathways also mediate oxidative stress-induced adverse responses, such as, collagen synthesis and extracellular matrix accumulation (32,33). High glucose also activates ERK1/2, which has been associated with increased fibronectin and collagen IV levels in primary cultured rat mesangial cells (34). Furthermore, it has been shown that attenuation of oxidative stress suppresses high glucose-induced ERK1/2 activation, and thereby reduces cell proliferation and fibrosis (35). In the present study, high-glucose-induced ERK1/2 activation was inhibited by the antioxidants, such as, NAC, apocynin, Mito TEMPO, and delphinidin, and these results suggest ERK1/2 was stimulated by ROS. In addition, inhibition of high glucose-induced cell proliferation and collagen synthesis by PFD98059 indicates that ERK1/2 mediates high glucose-induced mesangial expansion. Moreover, inhibition of ERK1/2 by apocynin or Mito TEMPO suggests that ROS stimulates ERK1/2 phosphorylation. NOX- or mitochondrial ROS-induced ERK1/2 activation has been reported previously (36,37).

TGF-β constitutes an important signaling pathway for extracellular matrix accumulation and fibrosis (38). Diabetic rats and cells treated with high glucose levels demonstrate high levels of collagen synthesis and TGF-β activation, and inhibition of TGF-β attenuates the extracellular matrix accumulation (34). Consistent with this previous study, we also observed that increased TGF-β levels in high glucose-treated cells and it appears that TGF-β is a mediator of glucose-induced collagen synthesis. This notion is supported by our findings that delphinidin suppressed increase of TGF-β levels as well as collagen synthesis in high glucose-treated
mesangial cells. Furthermore, it seems that ERK1/2 activation is responsible for the increase in TGF-β protein levels observed in the present study, because pretreatment with PD98059 prevented glucose-induced TGF-β increases. Consistent with our data, PD98059 suppresses increased TGF-β mRNA levels and promoter activity induced by high glucose in mesangial cells (39).

Based on our findings and previously reported data, we postulate high glucose enhances the release of ROS from activated NOX-1 and mitochondria, which activates ERK1/2 and TGF-β, and thereby, mesangial cell proliferation and extracellular matrix accumulation. Delphinidin blocks the glucose-induced NOX-1 activation and mitochondrial superoxide generation, and thereby collagen synthesis and matrix accumulation (Fig. 7). Delphinidin may be a potential therapeutic treatment for diabetic nephropathy.

Conflict of interest

None declared

Acknowledgment

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(10) Sørensen S, Reddy KM, Johnson EC, Anderberg RJ, Johnson EK, Clifton GD, et al. Novel findings and previously reported data, we postulate high glucose enhances the release of ROS from activated NOX-1 and mitochondria, which activates ERK1/2 and TGF-β, and thereby, mesangial cell proliferation and extracellular matrix accumulation. Delphinidin blocks the glucose-induced NOX-1 activation and mitochondrial superoxide generation, and thereby collagen synthesis and matrix accumulation (Fig. 7). Delphinidin may be a potential therapeutic treatment for diabetic nephropathy.