MicroRNA-218 promotes high glucose-induced apoptosis in podocytes by targeting heme oxygenase-1

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A B S T R A C T

Emerging evidence has demonstrated that microRNAs (miRNAs) play a mediatory role in the pathogenesis of diabetic nephropathy. In this study, we found that miR-218 was upregulated in high glucose (HG) treated podocytes, which are essential components of the glomerular filtration barrier and a major prognostic determinant in diabetic nephropathy. Additionally, up-regulation of miR-218 was accompanied by an increased rate of podocyte death and down-regulation in the level of nephrin, a key marker of podocytes. However, inhibition of miR-218 exerted the opposite effect. In addition, the dual-luciferase reporter assay showed that miR-218 directly targeted the 3'-untranslated region of heme oxygenase-1 (HO-1), and further study confirmed an increase of HO-1 in HG-treated podocytes transfected with anti-miR-218. Knockdown of HO-1 blocked the anti-apoptotic effect of anti-miR-218. Furthermore, inhibition of miR-218 was associated with decreased expression of the known pro-apoptotic molecule p38-MAPK activation. Following preconditioning with SB203580, an inhibitor of p38-MAPK, the stimulatory effect of HG on podocyte apoptosis was strikingly ameliorated. These findings suggested that miR-218 accelerated HG-induced podocyte apoptosis through directly down-regulating HO-1 and facilitating p38-MAPK activation.

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1. Introduction

Diabetic nephropathy (DN) is a major microvascular complication of diabetes and the leading cause of end-stage renal disease (ESRD) [1]. In the early stages of DN, the main clinical feature is development of persistent microalbuminuria, which is usually followed by a progressive decline in renal function [2]. Therefore, there is an imperative need to intervene in the early stages of DN. Recently, increasing evidence indicated that podocyte loss is a hallmark of early DN [3]. Podocytes constitute the glomerular filtration barrier together with endothelial cells and the glomerular basement membrane. Besides, podocytes are highly differentiated cells with poor proliferation ability, and the extent of their injury is a major prognostic determinant in DN as their loss is accompanied by increased proteinuria, progressively declining glomerular filtration rate (GFR) and glomerular dysfunction [3,4]. Therefore, there has been a great deal of interest in developing podocyte-based therapeutics for preventing DN.

MicroRNAs (miRNAs) are a class of approximately 22 nucleotide, small, noncoding RNAs that negatively regulate gene expression by binding to the 3'-UTR of their target messenger RNAs [5]. Emerging evidence demonstrated that a number of miRNAs have ectopic expression in the kidney and are involved in the progression of kidney disease. MicroRNA-29c was shown to be up-regulated in the kidney glomeruli of db/db diabetic mice and its knockdown remarkably reduced albuminuria and kidney mesangial matrix accumulation [6]. Another report showed that miR-216a and miR-217 were upregulated in TGF-β-treated mouse glomerular mesangial cells, and activated Akt kinase by downregulating phosphatase and tensin homologue (PTEN), an Ebox regulator [7]. In addition, miRNAs also play vital roles in podocyte homeostasis. Mice with podocyte-specific deletions of Dicer or Drosha, two critical RNAse III enzymes in the miRNA biogenesis pathway, sustained podocyte dysregulation, proteiunuria defects and collapsing glomerulopathy [8–10]. However, the roles of miRNAs in DN pathogenesis need to be further elucidated.

Heme oxygenase-1 (HMOX1 or HO-1) enzyme, a 32 kDa, rate-limiting enzyme in heme catabolism, is an antioxidant defense,
and key cytoprotective, enzyme [11]. The first human case of HO-1 deficiency revealed that in patients with severe growth retardation, HO-1 deficiency leads to oxidative stress and enhanced endothelial cell injury [12]. Interestingly, various reports indicate that HO-1 is upregulated in the kidney under negative conditions and exerts a renoprotective effect against renal injury [13,14]. In vivo and in vitro studies also demonstrated that in glomeruli of streptozotocin (STZ) treated rats and in mouse podocytes treated with high glucose, inhibition of HO-1 promotes podocyte apoptosis [15]. In addition, through two miRNA target prediction algorithms (miRanda and TargetScan), we found miR-218, a pro-apoptosis related molecule, could directly target HO-1, leading us to hypothesize that miR-218 may participate in the pathologic process of DN through regulating HO-1.

In this study, we show that miR-218 is upregulated in high glucose (HG)-induced podocytes, which accelerates cell apoptosis by suppressing its direct target gene, HO-1. We also found that HO-1 inhibited phosphorylation of p38-MAPK in HG-stimulated podocytes. Taken together, our study suggests that miR-218 may be a potential therapeutic target for DN treatment.

2. Materials and methods

2.1. Podocyte culture

Conditionally immortalized mouse podocytes were obtained from the Cell Resource Center of Peking Union Medical College (Beijing, China). Podocytes were cultured as follows: firstly, podocytes were cultured in RPMI 1640 medium (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Sigma–Aldrich) and 100 U/ml of penicillin/streptomycin (Solarbio, Beijing, China) in the presence of 10 U/ml γ-interferon (γ-IFN) (Sangon Biotech, Shanghai, China) at 33 °C in a humidified atmosphere of 5% CO2 (growth permissive conditions). After culturing to a confluence of 80%–90%, the podocytes were then subcultured without γ-IFN at an atmosphere of 37 °C and 5% CO2 for 14 days to induce cell proliferation (growth restrictive conditions). After that, podocytes were serum-restricted for 24 h and then cultured in RPMI 1640 medium containing high glucose (HG, 25 mM D-glucose) or normal glucose (NG, 5 mM D-glucose) for 24, 48 or 72 h.

2.2. Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from cells using TRizol reagent according to the manufacturer’s instruction (Invitrogen, Carlsbad, CA, USA). Detection of miRNA and mRNA was performed by using one-step primescript miRNA cDNA synthesis kit (Takara, Dalian, China) and M-MLV reverse transcriptase (Promega, Madison, WI, USA), respectively. Gene expression was detected by RT-qPCR using SYBR Green qPCR Master Mix (Thermo Fisher, Shanghai, China) and incubated at 4 °C over-night. Subsequently, the membrane was washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:1000) (Santa Cruz Biotechnology) for 1 h at 37 °C. The protein bands were visualized and detected by an enhanced chemiluminescence detection system (Amersham, Little Chalfont, UK).

2.4. Cell transfection

Pre-miR-218, pre-miR-control, anti-miR-218 and anti-miR-control (GenePharma, Shanghai, China) were transfected into podocytes (1 × 105/well) at a final concentration of 30 nM using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. The same protocol was followed for the HO-1 siRNA and negative control siRNA (siNC) (Santa Cruz Biotechnology). After 48 h of transfection, cells were harvested and used for further analysis.

2.5. Apoptosis assay

After transfection with anti-miR-218 or anti-miR-control for 48 h, podocytes (1 × 105/well) were trypsinized and centrifuged at 1000 rpm for 5 min at room temperature. Then, 500 μl binding buffer were added to the precipitate followed by 10 μl Annexin V-fluorescein isothiocyanate (FITC) stock solution (BD Biosciences, San Jose, CA, USA), and the cells were incubated for 30 min at 4 °C. After that, 5 μl propidium iodide (PI) (BD Biosciences) was added and incubated at room temperature in the dark for another 10 min. Apoptotic podocytes were quantified by flow cytometry (BD Biosciences). For p38-MAPK inhibitor studies, podocytes were pre-incubated with the p38-MAPK inhibitor (SB203580, Invitrogen) at a final concentration of 5 μM in RPMI 1640 for 1 h and then incubated for an additional 48 h in the presence of HG medium.

2.6. Dual luciferase reporter assay

To validate whether miR-218 directly targets the HO-1 3’-untranslated region (3’-UTR), we performed a firefly luciferase reporter assay using pGL3 control vector (Promega). First, the wildtype 3’-UTR sequence of HO-1 containing putative miR-218 target sites or mutated 3’-UTR constructed by replacing some binding sites of miR-218 with other nucleotides were synthesized and sub-cloned into the XbaI and FseI sites of pGL3 control vector. For the reporter assay, 0.1 μg of pGL3 vectors containing wildtype or mutated 3’-UTR of HO-1 were co-transfected with pre-miR-218 or pre-miR-control into human embryonic kidney 293 (HEK293) cells using Lipofectamine 2000 (Invitrogen). After transfection for 48 h, cells were harvested and luciferase activity was analyzed with the Dual Luciferase Reporter Assay System (Promega).

2.7. Data analysis

All quantitative data were presented as means ± SD. Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, Shanghai, China) and the protein concentration was detected by BCA Protein Assay Kit (Beyotime Biotechnology). A total of 30 μg protein extracted from cells was separated by 12% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a PVDF membrane (Merck Millipore, Billerica, MA, USA). After incubating with blocking buffer (phosphate-buffered saline containing 3% non-fat milk and 0.1% Tween-20) for 1 h at 37 °C, the membrane was washed with PBST (phosphate buffer solution with Tween-20) and exposed to the primary antibodies, anti-HO-1 (1:1000), anti-nephrin (1:1500), anti-p38 (1:1000), anti-phospho-p38 (1:1000) and anti-β-actin (1:2000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and incubated at 4 °C overnight. Subsequently, the membrane was washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:1000) (Santa Cruz Biotechnology) for 1 h at 37 °C. The protein bands were visualized and detected by an enhanced chemiluminescence detection system (Amersham, Little Chalfont, UK).
USA) by one-way analysis of variance (ANOVA) or two-tailed Student's t-test. Statistical significance were set at \( p < 0.05 \).

3. Results

3.1. miR-218 is upregulated in HG-treated podocytes

To investigate the potential role of miR-218 in podocytes, we analyzed the expression of miR-218 at different time points (0 h, 24 h, 48 h and 72 h) following stimulation with HG (25 mM) by RT-qPCR. The results demonstrated that miR-218 levels were up-regulated following HG treatment in podocytes in a time-dependent manner (Fig. 1A), suggesting that miR-218 might be involved in the response to hyperglycemic conditions.

3.2. Down-regulation of miR-218 suppresses apoptosis of podocytes

Because DN is characterized by increased apoptosis in podocytes [3,16], and miR-218 has been reported as a pro-apoptosis factor in many cancer cells [17,18], we further investigated the functional role of miR-218 in podocyte apoptosis. We first analyzed the effect of miR-218 on the expression of nephrin, one of the key markers of podocytes in the slit diaphragm [19] by RT-qPCR and Western blot. The results indicated that compared with NG-treated podocytes, HG-treated podocytes exerted a marked decrease in the mRNA (21%) and protein (32%) levels of nephrin, whereas anti-miR-218 treatment significantly reversed this effect (Fig. 1B–D). Subsequently, flow cytometry was performed to examine the effect of miR-218 down-regulation on podocyte cell apoptosis. As shown in Fig. 1E and F, compared with NG-treated podocytes, HG-treated podocytes exhibited a significant increase in apoptotic cells. However, the percentage of apoptotic cells induced by HG decreased when the podocytes were transfected with anti-miR-218. These results indicated that miR-218 functions as a negative regulator of HG-induced cell apoptosis in podocytes.

3.3. HO-1 is a target of miR-218

To explore the mechanisms by which miR-218 promotes HG-induced podocyte apoptosis, we used two miRNA target prediction algorithms (miRanda and TargetScan) to identify the potential target(s) of miR-218 under hyperglycemic conditions. Interestingly, some of the targets have already been reported as the targets for miR-218 in other cell lines [20]. In addition to these targets, we found that HO-1 is another putative target of miR-218, which has not been previously experimentally validated but was known to be involved in the kidney pathogenesis process [21,22]. In addition, we found a highly conserved binding site for miR-218 in the 3'-UTR region of HO-1 in several species, including has, mmu, rno and cfa (Fig. 2A). To verify whether miR-218 targets the HO-1 3’-UTR, HO-1 3’-UTR (wild type and mutated type with miR-218 binding sites) were cloned into pGL3/luc reporter vectors and a luciferase reporter assay was performed. The results showed that transient co-transfection of pre-miR-218 with luciferase expression plasmids in HEK293 cells significantly inhibited HO-1 3’-UTR luciferase activity relative to pre-miR-control (pre-miR-Ctrl), while HO-1 3’-UTR containing mutations at miR-218 binding sites lost this response (Fig. 2B). These results confirmed that HO-1 3’-UTR is a direct target of miR-218.
of miR-218.

To further examine the effect of miR-218 on HO-1, we transfected podocytes with anti-miR-218 and treated them with HG (25 mM) for 48 h. Then, RT-qPCR and Western blot analysis were performed to assess HO-1 mRNA and protein levels. The results showed that HG treatment significantly decreased HO-1 expression in podocytes, both at the mRNA and protein levels (Fig. 2C and D). However, the effect of HG on HO-1 expression was reversed when podocytes were transfected with anti-miR-218 (Fig. 2C and D). Taken together, these results suggested that miR-218 mediates HG-induced HO-1 expression, and that HO-1 is the direct target of miR-218.

3.4. Knockdown of HO-1 blocks the anti-apoptotic effect of anti-miR-218 in podocytes

To investigate whether miR-218 functions through HO-1, we further examined the function of HO-1 siRNA on miR-218-mediated podocyte apoptosis. To this end, both anti-miR-218 and HO-1 siRNA harboring no specific binding sites of miR-218 were co-transfected into HG-induced podocytes. The results showed that the anti-apoptotic effect of anti-miR-218 on HG-induced podocytes was largely reversed by HO-1 siRNA (Fig. 2C and D), same as the nephrin level (Fig. 3C and D). Our results suggested that miR-218 functionally promotes podocyte apoptosis via a HO-1-dependent pathway.

3.5. MiR-218 regulates p38-MAPK signaling

Previous reports demonstrated that down-regulation of HO-1 in type I and type II diabetic rats resulted in an increase in the phosphorylation of p38-mitogen-activated protein kinase (p38-MAPK) [23]. We hypothesized that miR-218 might affect podocyte apoptosis via the p38-MAPK phosphorylation. To verify this hypothesis, we detected protein levels of phosphorylated p38-MAPK (p-p38) at 24 h after anti-miR-218 transfection. Western blot assay indicated that there was no effect on the expression of p38-MAPK. However, the phosphorylation of p38-MAPK, which was triggered by HG stimulation, was significantly inhibited by knockdown of miR-218 (Fig. 4A-B). Further investigation indicated that blockade of p38-MAPK phosphorylation via p38-MAPK inhibitor SB203580 significantly inhibited HG-induced podocyte apoptosis (Fig. 4C–D). These results indicated that miR-218 promoted the podocyte apoptosis via facilitating p38-MAPK phosphorylation.

4. Discussion

In this study, we determined that miR-218 expression increased in cultured podocytes exposed to HG. Our results also suggested that through directly targeting HO-1, miR-218 promoted p38-MAPK activity, thus facilitating podocyte apoptosis. We concluded that miR-218 may act as a potential therapeutic target for DN treatment. Previous studies showed that miR-218 plays important roles in cancers. In patients with medulloblastoma, miR-218 was down-regulated and acted as a tumor suppressor in medulloblastoma cases [24]. In oral squamous cell carcinoma, miR-218 was silenced by DNA hypermethylation, which targeted the rapamycin-insensitive component of mTOR (Rictor) and inhibits Akt phosphorylation [25]. Additionally, increasing evidence identified
miRNAs as essential regulators and important therapeutic targets in DN [7–10]. A recent study showed that miR-218 regulates TGF-β signaling [26], which was increased in various renal cells in diabetes and mediates the extracellular matrix deposition and is also involved in DN [27,28]. Our experiment identified an up-regulation of miR-218 expression in HG-treated podocytes. Our results are consistent with recent research which demonstrated that miR-218 was significantly increased in renal cells exposed to hyperglycemic conditions [29]. Indeed, we identified the role of miR-218 as a positive regulator of podocyte apoptosis. Podocytes play an essential role in DN and their loss is irreversible [30]. Stimulation of podocytes with HG resulted in an increased expression of nephrin, a biomarker of podocytes, and an increased podocyte death rate, while inhibition of miR-218 reversed this increase.

Another important finding of this study was the identification of HO-1 as a novel target of miR-218 in hyperglycemic conditions. Previous studies investigating miRNAs and HO-1 have mainly concentrated on the transcriptional repressor Bach1 [21,22], a member of the bZIP transcription factor family that represses HO-1 gene transcription through binding to Maf-recognition elements [31]. Interestingly, using two miRNA target prediction algorithms (miRanda and TargetScan), we found a highly conserved binding site for miR-218 in the 3′-UTR region of HO-1 in several species, making HO-1 of great interest. Our studies verified this prediction that miR-218 directly targets the 3′-UTR of HO-1 and represses HO-1 expression in HG-induced podocytes. Importantly, an inverse correlation between miR-218 and HO-1 expression was detected in podocytes. With the observation that CDK6, Rictor and cathepsin B are direct targets of miR-218 [20], it is becoming increasingly clear that miR-218 exerts its effects through modulating multiple targets and could therefore be a therapeutic target in DN.

Our study also revealed the role of miR-218 as a regulator of the p38-MAPK phosphorylation. The effect of miR-218 on p38-MAPK protein activation is of significant importance because previous studies have indicated that the activation of p38-MAPK was associated with cell apoptosis [32]. In addition, it was reported that phosphorylated p38-MAPK was increased in DN patients and mice, and pharmacologic inhibition of p38-MAPK normalized the proliferation rate of fibroblasts from patients with DN [33,34]. Our data suggested that the level of phosphorylated p38-MAPK was downstream of the HG-induced miR-218/HO-1 pathway because anti-miR-218 was able to decrease p38-MAPK activity. HO-1 has previously been identified as a negative regulator of p38-MAPK [35], our findings suggested for the first time that HO-1 inhibited p38-MAPK activation under hyperglycemic conditions, which had potential in the management of DN.

In summary, we propose that miR-218 is a critical regulator in HG-induced podocytes apoptosis. Our findings suggest that up-regulation of miR-218 promotes the progression of DN through inhibiting HO-1. These findings shed new light onto the regulation
of miR-218 in DN and provide a potential therapeutic target for DN treatment. Further studies will identify the role of miR-218 in db/db mice or STZ-induced mice in vivo and evaluate methods of providing cytoprotection to podocytes in DN.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2016.02.028.

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