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Original Research

Inhibition of the oxidative stress-induced miR-125b protects glucose metabolic disorders of human retinal pigment epithelium (RPE) cells

Gang Liu, Chun-Di Zhang, Jing Wang, Wan-Cheng Jia*

Department of Ophthalmology, Shanghai Jiao Tong University Affiliated Sixth People's Hospital South Campus, Shanghai, 201400, China

Correspondence to: wchengjia109@163.com

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Abstract: The dysfunction of retinal pigment epithelium (RPE) with aging leads to age-related macular degeneration (AMD). Oxidative stress has been demonstrated as one of the causes of retinal pathological conditions. This study was conducted to investigate the mechanism of hydrogen peroxide (H_2O_2) induced human retinal pigment epithelial (RPE) cell dysfunction. We report miR-125b is induced by H_2O_2 treatments in RPE cells. In addition, we observed inhibited glucose metabolism under oxidative stress. Overexpression of miR-125b promotes the disorders of cellular glucose metabolism through direct targeting Hexokinase 2 (HK2). Restoration of HK2 in H_2O_2 treated RPE cells prevents the oxidative stress-suppressed glucose metabolism. Inhibition of the H_2O_2-induced miR-125b by inhibitor significantly prevented disorders of glucose metabolism. This study will contribute to the development of the miRNA based therapeutic approaches for against the oxidative stress-mediated human AMD.

Key words: Retinal pigment epithelium; miR-125b; Age-related macular degeneration; Oxidative stress.

Introduction

Age-related macular degeneration (AMD), a degenerative and progressive malignancy involving the dysfunctional retinal pigment epithelium (RPE), Bruch's membrane and the choriocapillaries is the leading cause of irreversible visual impairment in the elderly (1). Defects in RPE, photoreceptors, and the choriocapillaris are characteristic of AMD (2). "Dry AMD", the most common AMD phenotype, is characterized by an increase in the number and diameter of the extracellular sub-RPE deposits (3). Moreover, "dry AMD" accompanies with pigmentary irregularities and progressive atrophy of the RPE and retina (4). In contrast, "wet AMD" is associated with subretinal choroidal neovascularization (CNV) in the macular region, resulting in the rapid loss of visual acuity (5). The molecular events of AMD progression include altered cellular homeostasis, oxidative stress, necrosis, apoptosis of RPE cells, inflammation and DNA damage in the degeneration of photoreceptors (6). Currently, the pathogenesis is not clear and treatment options for AMD are limit. Therefore, understanding the molecular mechanisms of RPE dysfunction or death is essential for preventing AMD.

Hexokinases (HKs) are glycolysis enzymes which catalyze the first essential step in glucose metabolism through phosphorylation of glucose to glucose-6-phosphate (G-6-P) (7). There are four major isoforms (HK1, HK2, HK3, and HK4) characterized in mammalian tissue (7). Among them, HK2 is highly upregulated in human diseases such as cancers (8) and AMD (9). Recent studies revealed that increased anaerobic glycolysis levels was correlated with the pathogenesis of AMD (9, 10), suggesting inhibition of HK2 may be an effective approach against human AMD.

MicroRNAs (miRNAs) are a class of short, non-coding RNAs (~20-25 nt) that regulate gene expression at the posttranscriptional level through complementary binding to their target mRNA (11, 12). Recently, miRNA has been widely studied as an important regulator of biological pathological processes of AMD (13). It was reported that dysregulated miRNAs of RPE cells contribute to the development of the chronic atrophy, which can be observed in the early phases of AMD (14). Moreover, studies indicate that miRNAs have a crucial role in regulating vasculogenesis and angiogenesis in the choroid neovascularization of late AMD (15). It was reported that miR-125b could be induced by N-(4-hydroxyphenyl)-retinamide (4HPR), a retinoic acid derivative, which induces ROS generation in RPE cells (16), suggesting miR-125b might involve in regulating oxidative stress pathways and is important in maintaining RPE cell functions. In this study, we will study the roles of miR-125b in the oxidative stress-induced metabolic dysfunction RPE cells. The potential of inhibiting miR-125b as therapeutic approach against oxidative stress in RPE cells will be investigated.

Materials and Methods

Cell culture and oxidative stress treatment

ARPE-19 cells were obtained from the American Type Culture Collection (ATCC) and grown in DMEM/F12 containing 10% heat-inactivated fetal bovine serum (FBS) and 2.5 mM glutamine. Cells were incubated in a 37°C incubator with 5% CO_2. ARPE-19 cells were treated with H_2O_2 for 24 h at the indicated concentrations of each experiment.

MiRNAs precursor, inhibitor and plasmid DNA transfection

The miR-125b precursor, inhibitor and control miRNAs were synthesized by GenePharma, Co, Ltd. (Shanghai, China) and transfected with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) in accordance with manufacturer's instruction. The control miRNAs, miR-125b precursor or inhibitor was transfected at 100 nM for 48 hours. Cells were transfected with plasmids encoding Hexokinase 2 at 2 μg for 48 hours. The empty plasmid, pcDNA3.1, was used for mock transfection.

Luciferase assay

A total of 2 x 10^5 ARPE-19 cells were seeded in 12-well dishes for overnight before transfection. pGL3 vectors containing wild type 3'UTR or mutant 3'UTR of Hexokinase 2 and indicated amount of miR-125b precursor or control miRNAs were co-transfected using Lipofectamine 3000. Luciferase activities of cellular extracts were measured by a Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA) 24 hours post transfection. Efficiency of transfection was normalized using Renilla luciferase activity. All the experiments were repeated three times.

qRT-PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen) according to the manufacturer's protocol. RNA was reverse transcripted with the PrimerScript RT-PCR kit (TaKaRa Biotechnology, Dalian, China) for cDNA synthesis. To evaluate expressions of miRNAs, TaqMan MiRNA assay kits from ABI (Foster City, CA, USA) was performed to detect miR-125b, miR-143, miR-26, miR-21, miR-200c, miR-100, miR-17, miR-28, miR-185 and internal control RNA (snoRNAU6). The qPCR primers were miR-125b: sense, 5'-GCUCCCUGAGACCCUAAC-3'; antisense, 5'-CAGTGCAGGGTCCGAGGT-3'; miR-143: sense, 5'-TGAGGTGCAGTGCTGCATC-3'; antisense, 5'-GCTACAGTGCTTCATCTCAGACTC-3'; miR-26: sense, 5'-ATGGCTTCAAGTAATCC-AGGA-3'; antisense, 5' -GTGCAGGGTCCGAGGT-3'; miR-21: sense, 5'-GTCGTATCCAGTGCAGG-3'; antisense: 5'-CGAGGTATTCGCACTGGATACGACTCAACA-3'; miR-200c: sense, 5'-TAATACTGCCGGGTAATGATGG-3'; antisense, 5'-TCGTATCCAGTGCAGGGTC-3'; miR-17: sense, 5'-CAGTAAAGGTAAGGAGAGCTCAATCTG-3'; antisense, 5'-CATACAACCACTAAGCTAAAGAATAATCTGA-3'; miR-28: sense, 5'-CAAGGAGCTCACAGTC-3'; antisense, 5'TATTGAGTTACCCTCCAGGAGCTCACAAT-3';

miR-185: sense, 5'-CAATGGAGAGAAAGGCAGTTCC-3': antisense, 5'-AATCCATGAGAGATCCCTACCG-3'; U6: sense, 5'-CTCGCTTCGGCAGCACATATACT-3', antisense, 5'-ACGCTTCACGAATTTGCGTGTC-3'. Quantitative RT-PCR was conducted using a standard SYBR Green PCR kit (Promega, Madison, WI, USA) protocol with a LightCycler 480 real-time instrument (Roche). The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. All experiments were repeated three times.

Western blot

After transfection with miRNAs or treatments with H_2O_2, ARPE-19 cells were harvested. Total protein was extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) with 1× protease inhibitor cocktail (Roche, Nutley, USA). Protein concentration was determined by Bradford assay. Equal amount of proteins was resolved on 10% sodium dodecyl sulfate–polyacrylamide gels (SDS-PAGE) and electrophoretically transferred to a polyvinylidene fluoride membrane. Membranes were blocked with 5% non-fat milk followed by incubation with primary rabbit monoclonal antibodies specific for Hexokinase 2 (#2867, Cell Signaling, Danvers, MA, USA), LDHA (#3582, Cell Signaling, Danvers, MA, USA) and α-Tubulin (#2148, Cell Signaling, Danvers, MA, USA). The blots were then incubated with goat anti-rabbit secondary antibody (Cell Signaling, Danvers, MA, USA) and visualized using enhanced chemiluminescence. Experiments were repeated three times.

Glucose metabolism assays

The glucose uptake assay was performed using the Glucose Uptake Assay Kit (#ab136955, Abcam, Cambridge, United Kingdom) according to the manufacturer's instructions. The lactate product assay was performed using the L-Lactate Assay Kit (Colorimetric) (#ab65331, Abcam, Cambridge, United Kingdom) according to the manufacturer's instructions. Extracellular acidification rate was detected using the Seahorse XFp Analyzer (Agilent, Santa Clara, CA, USA) according to the manufacturer's instructions. Equal number of cells from each treatment were analyzed. Results were normalized by protein concentrations and repeated three times.

Detection of ARPE-19 cell death

The viability of ARPE-19 cells from each treatment was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells were plated in 96-well plates at 1.5-2 x 10^5 cells/well for overnight. The cells were treated with indicated concentrations of H_2O_2 for 24 hours, culture medium was removed after treatment and 100 μl MTT solution was added for incubation at 37°C for one hour in a tissue culture incubator. The MTT solution was then removed and 100 μl dimethyl sulfoxide (DMSO) was added for one hour at room temperature. The cell viability was measured by a 96-well plate reader with a filter setting at 570 nm (reference filter setting was 630 nm). Experiments were repeated three times.

Statistical analysis

Data were presented as the means ± SD from three separate experiments. The differences between groups were analyzed using Student's t-test by Graphpad Prism 5.0 software. $P < 0.05$ was considered statistical significance.

Results

Oxidative stress induces miR-125b expressions in ARPE-19 cells

Previous study demonstrated that oxidative stress induces both primary human RPE cultures and ARPE-19 cells death (17). To investigate the roles of miRNAs

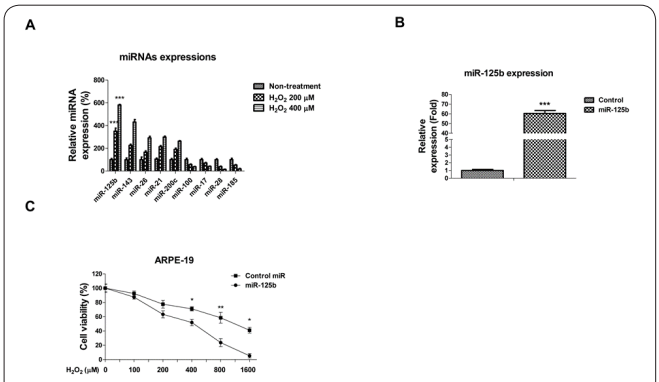

Figure 1. MiR-125b involves in the oxidative stress-induced RPE cell death. (A) Expressions of miRNAs by H_2O_2 treatments at 0, 200 or 400 μM. (B) ARPE-19 cells were transfected with control miRNAs or miR-125b precursor for 48 hours, the expression of miR-125b was detected by qRT-PCR. (C) ARPE-19 cells were transfected with control miRNAs or miR-125b for 48 hours, followed by the treatments with H_2O_2 at the indicated concentrations for 24 hours. Cell viability was measured by MTT assays. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

during the oxidative stress induced RPE cell death, we first examined the effects of oxidative stress on miRNAs expressions in human RPE cells. ARPE-19 cells were treated with H_2O_2, an oxidative stress inducer at 200 and 400 μM for 24 hours. Among the ten microRNAs in this experiment, we found miR-125b, miR-143, miR-26, miR-21 and miRNA-200c were significantly upregulated (Fig. 1A). In addition, miR-100, miR-17, miR-28 and miR-185 were found downregulated by H_2O_2 treatments (Fig. 1A). To examine the effects of miR-125b on the oxidative stress induced cell death, we measured the cell viability of RPE cells with or without exogenous overexpression of miR-125b. As we expected, overexpression of miR-125b (Fig. 1B) significantly enhanced the cytotoxicity of H_2O_2 on RPE cells (Fig. 1C). Taken together, these data suggest miR-125b plays important roles in RPE cells during the exposure of oxidative stress.

Glucose metabolism of RPE cells is suppressed by Oxidative stress

Recent studies reported the dysregulated glycolytic pathway is highly correlated with the progression of AMD (18), intriguing us to investigate the effects of oxidative stress on the glucose metabolism of RPE cells. ARPE-19 cells were treated with 200 and 400 μM H_2O_2 for 24 hours, followed by the measurements of glycolysis. We found the glucose uptake, lactate product and extracellular acidification rate (ECAR) were decreased by H_2O_2 treatments in RPE cells (Fig. 2A-2C). Moreover, the expressions of glycolysis enzymes, Hexokinase 2 and lactate dehydrogenase-A (LDHA) were downregulated under oxidative stress (Fig. 2D). These results demonstrate the glucose metabolism is suppressed by oxidative stress, suggesting restoration of the dysregulated glucose metabolism might be a new approach for the anti-oxidant agent development in AMD.

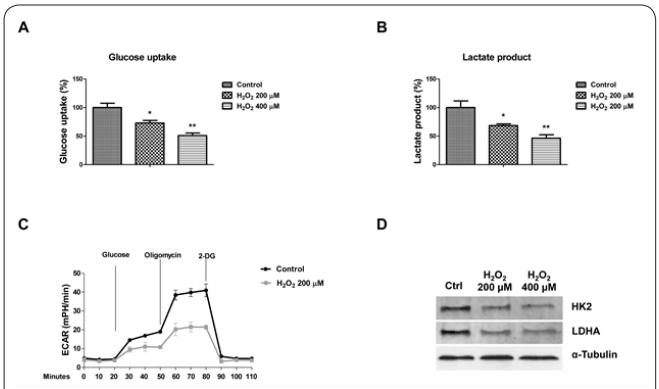

Figure 2. H_2O_2 suppresses glucose metabolism. ARPE-19 cells were treated with H_2O_2 for 24 hours, then the (A) glucose uptake, (B) lactate product, (C) Extracellular acidification rate and (D) expressions of glucose metabolism enzymes were measured. * $P < 0.05$; ** $P < 0.01$.

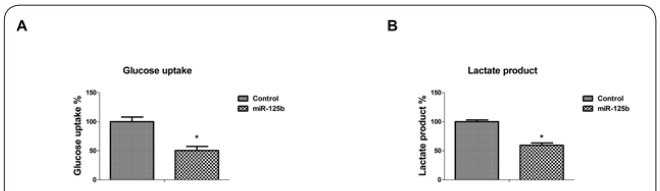

Figure 3. Overexpression of miR-125b inhibits glucose metabolism. ARPE-19 cells were transfected with control miRNAs or miR-125b precursor for 48 hours, the (A) glucose uptake and (B) lactate product were measured. (C) ARPE-19 cells were transfected with control inhibitor or miR-125b inhibitor for 48 hours, the glucose uptake and (D) lactate product were measured. * $P < 0.05$.

MiR-125b inhibits glucose metabolism through directly targets Hexokinase 2

We next investigated whether the oxidative stress-induced miR-125b expression correlates with the suppressed glucose metabolism. As we expected, overexpression of miR-125b significantly suppressed glucose uptake and lactate product in RPE cells (Fig. 3A-3B). In addition, inhibition of miR-125b significantly promoted glucose uptake and lactate product in RPE cells (Fig. 3C-3D), suggesting the oxidative stress-induced miR-125b might directly target glucose metabolism. As an effort to find the potential targets of miR-125b involving in the glycolysis pathway, we performed a bioinformatics research through online miRNA target prediction database Targetscan.org. Among the potential targets of miR-125b, we found Hexokinase 2 contains the putative miR-125b binding site on the 3'UTR region (Fig. 4A). To assess whether miR-125b could target HK2 in RPE cells, we transfected miR-125b precursor or control miRNAs into RPE cells. As we expected, miR-125b inhibited the HK2 protein expression (Fig. 4B upper). In addition, our results showed inhibition of miR-125b significantly upregulated HK2 protein expression (Fig. 4B lower). To identify whether HK2 is a direct target of miR-125b in RPE cells, a dual-luciferase assay was performed to test whether miR-125b binds directly to the 3'-UTR of HK2. Co-transfection of miR-125b with expressing vector containing the wild-type HK2 3'-UTR significantly inhibited luciferase activity but not the mutant HK2 3'-UTR (Fig. 4C). These results demonstrate that miR-125b suppressed the expression of HK2 by directly targeting the HK2 3'-UTR.

Oxidative stress suppresses glucose metabolism through inhibition of HK2

We next studied whether restoration of HK2 in the

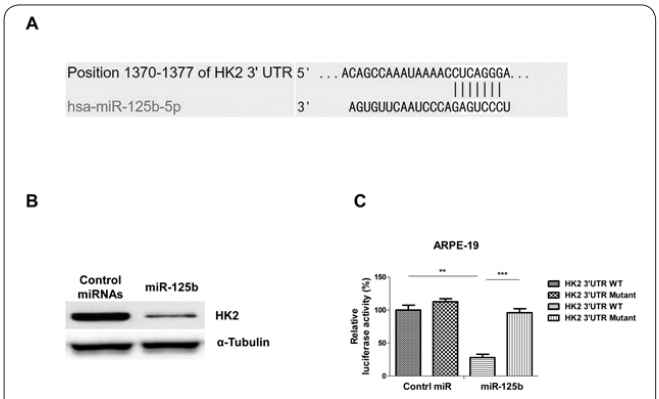

Figure 4. Hexokinase 2 is a direct target of miR-125b. (A) Target prediction from Targetscan.org. (B) Cell with overexpression of miR-125b showed downregulation of HK2 protein expression (upper). RPE cells with miR-125b inhibition showed upregulation of HK2 protein expressions (lower). α-Tubulin was an internal control. (C) Luciferase assay showed the inhibited activity of luciferase reporter with wild type 3'UTR of HK2 by mir-125b. * $P < 0.05$; ** $P < 0.01$.

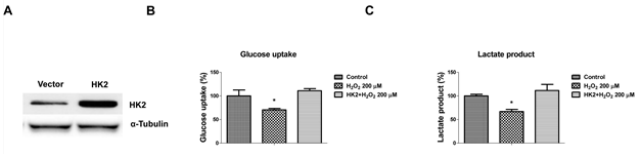

Figure 5. Restoration of HK2 prevents the oxidative stress mediated glucose metabolic disorders. (A) ARPE-19 cells were transfected with control vector or HK2 for 48 hours, cells were analyzed by Western blot. α-Tubulin was an internal control. (B) Cells without or with overexpression of HK2 were treated with H_2O_2 at 0 or 200 μM for 24 hours. Glucose uptake and (C) lactate product were measured. * $P < 0.05$.

H_2O_2 treated RPE cells could rescue the suppression of glucose metabolism. HK2 overexpression vector or control empty vector was transiently transfected into RPE cells (Fig. 5A). Moreover, with the overexpression of HK2, H_2O_2 treatments did not significantly suppress glucose uptake and lactate product (Fig. 5B-5C), suggesting the oxidative stress suppressed glucose metabolism was through the miR-125b-mudulated HK2 expression.

Inhibition of the oxidative stress-induced miR-125b attenuates glucose metabolic disorders

The above results demonstrated the molecular mechanisms for the oxidative stress-suppressed glucose metabolism through upregulation of miR-125b. To explore whether the miR-125b-mediated glucose metabolism under oxidative stress has biological significance, we transfected RPE cells with miR-125b inhibitor (Fig. 6A). We observed transfection of miR-125b inhibitor prevented the H_2O_2-induced miR-125b upregulation (Fig. 6B). Importantly, the glucose uptake and lactate production were significantly rescued by miR-125b inhibition under oxidative stress (Fig. 6C-6D), suggesting targeting miR-125b might be a new approach for maintenance of the cellular glucose metabolism under oxidative stress.

Discussion

Oxidative stress has been demonstrated as one of the causes of retinal pathological conditions (19). The retina is in a favorable environment for the generation of reactive oxygen species since it is exposed to both sunlight and high levels of oxygen. Studies showed that H_2O_2 could induce oxidative stress, resulting cytotoxic effects on RPE cells (20). Our results illustrated mechanisms for the H_2O_2 induced RPE cell dysfunction through the increased miRNAs expressions, suggesting inhibition of the H_2O_2-induced miRNAs might be an effect way for anti-oxidant treatments.

Previous studies revealed miRNAs played essential functions in the processes of AMD (14), intriguing us to explore the roles of miR-125b in the oxidative stress-induced RPE cells dysfunction. In this study, we observed miR-125b expressions were induced by H_2O_2 treatments. Meanwhile, H_2O_2 treatments suppressed glucose metabolism, suggesting a H_2O_2-miR-125b-glucose metabolism axis for the RPE cells dysfunction. The detailed mechanisms for the H_2O_2 induced miR-125b are under studying.

It has been reported that oxidative stress block glycolysis pathway in human lymphoma cells (18). However, the H_2O_2-modulated glycolysis of human RPE cells have not been described. Glycolysis is a critical pathway not only for the generation of adenosine triphosphate (ATP), but also other important metabolic intermediates for important cellular processes such as proliferation and migration (21). Glycolysis pathway produces pyruvate and lactate from glucose (21). It has been described recently the important influence of the glycolytic pathway on the progression of AMD (9, 10). Physiologically, lactate produced by glycolysis is transferred from retinal glial (Müller) cells to the photoreceptors (22). Moreover, human Müller cells mainly obtain ATP mainly from glycolysis even with enough oxygen (22), suggesting that dysregulated glycolysis may lead to a reduction in energy metabolism of retinal cells, leading to AMD. Our *in vitro* results demonstrated miR-125b suppressed glycolysis of RPE cells through direct targeting glycolysis key enzyme, Hexokinase 2, indicating targeting abnormally upregulated miR-125b

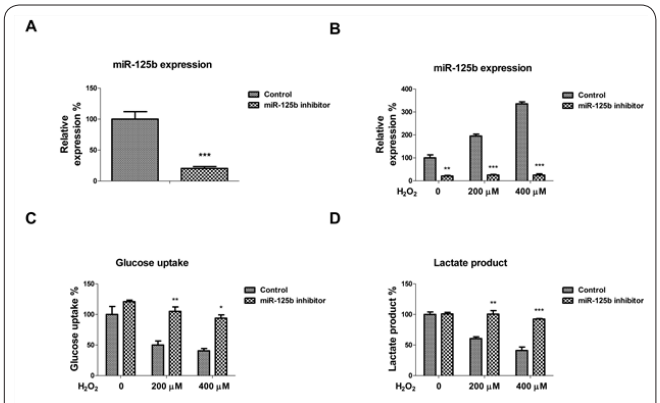

Figure 6. Inhibition of the oxidative stress induced miR-125b attenuates glucose metabolic disorders. (A) ARPE-19 cells were transfected with miR-125b inhibitor or control miRNAs for 48 hours, expressions of miR-125b were detected by qRT-PCR. ARPE-19 cells were transfected with miR-125b inhibitor or control miRNAs for 48 hours, cells were treated with H_2O_2 at 0, 200 or 400 μM for 24 hours, then (B) expressions of miR-125b, (C) glucose uptake and (D) lactate product were measured. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

might be an approach for maintenance of cellular metabolism. In summary, our results showed H_2O_2 induced miR-125b expression in human RPE cells. In addition, we observed inhibited glucose metabolism under oxidative stress. Hexokinase 2 is a direct target of miR-125b and inhibition of the H_2O_2-induced miR-125b by inhibitor significantly prevented disorders of glucose metabolism. This study will contribute to the development of the miRNA-based therapeutic approaches for against the oxidative stress-mediated human AMD.

Conflicts of Interests

The authors had no Conflicts of Interests.

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