Original Article

Hypoxia-induced miR-760-3p downregulation promotes cardiomyocyte apoptosis by regulating very-low-density lipoprotein (VLDL) receptor expression

Zheng Huang1,2, Guo-Qiang Zhong1, Rong-Hui Tu1, Cun-Ming Fang2, Xiao-Lin Ma2, Xue-Jun Hu2

1Department of Cardiology, The First Affiliated Hospital, Guangxi Medical University, Nanning 530021, Guangxi, China; 2Department of Cardiology, People’s Hospital of Xuancheng City, Xuancheng 242000, Anhui, China

Received November 19, 2018; Accepted January 7, 2019; Epub May 15, 2019; Published May 30, 2019

Abstract: Background: Previous studies have shown that downregulation of miR-760-3p or upregulation of very low-density lipoprotein receptor (VLDLR) expression are associated with hypoxia-induced cell apoptosis. The goal of this study was to explore the roles of miR-760-3p and VLDLR in hypoxia-induced cardiomyocyte injuries, examining their underlying relationship. Methods: Levels of miR-760-3p and VLDLR were detected using RT-PCR or Western blotting in H9C2 cells after exposure to hypoxia. miR-760-3p or miR-760-3p-inhibitors were transiently transfected into H9C2 cells to assess the effects of miR-760-3p on VLDLR expression and cardiomyocyte apoptosis during hypoxia. Dual-luciferase reporter assay was used to verify the relationship between miR-760-3p and VLDLR. Results: Present data shows that hypoxia reduced expression of miRNA-760-3p but induced VLDLR expression in H9C2 cells. Overexpression of VLDLR induced cardiomyocyte apoptosis by increasing caspase-3 activity and expression of pro-apoptotic gene Bax, as well as through inhibition of anti-apoptosis Bcl2 expression after cells were exposed to hypoxia. Molecularly, the dual-luciferase reporter assay showed that miR-760-3p was able to bind to the VLDLR 3’-untranslated region (3’-UTR) and inhibit levels of VLDLR mRNA and protein in H9C2 cells after exposure to hypoxia. In contrast, miR-760-3p inhibitors inversely affected gene expression and cardiomyocyte apoptosis. Conclusion: Hypoxia-induced downregulation of miR-760-3p contributes to apoptosis of hypoxic cardiomyocytes, whereas miR-760-3p overexpression protects cardiomyocytes from apoptosis by regulating VLDLR expression in vitro.

Keywords: miR-760-3p, hypoxia, very low-density lipoprotein, apoptosis, cardiomyocyte

Introduction

Cardiovascular disease, including ischemic heart disease, heart failure, and blood vessel disease, remains the leading cause of disability and death [1-3]. However, 90% of cardiovascular diseases are preventable or treatable [4, 5]. Risk factors for cardiovascular disease include aging, tobacco smoking, physical inactivity, excessive alcohol consumption, unhealthy diet, obesity, genetic predisposition, and family history [5]. Cardiovascular pathology is characterized by hypoxia, whereby oxygen supplies are reduced at the cellular, tissue, or whole body level [6]. Hypoxia transforms cell metabolism from aerobic oxidation of glucose and fatty acids to glycolysis, causing myocardial or cerebral ischemia. Severe hypoxia can induce cardiomyocyte and neuronal death. Hypoxia also changes expression of different hypoxia- and metabolism-related genes, such as hypoxia-inducible factor 1-alpha, vascular endothelial growth factor, very low-density lipoprotein receptor (VLDLR), and others [7-9]. VLDLR is a member of the low-density lipoprotein receptor family. It is the main receptor that regulates cell uptake of endogenous triglycerides [10, 11]. Thus, further examination of hypoxia-regulated cell changes and gene expression could identify novel strategies that prevent or treat hypoxia-induced organ damage in cardiovascular disease.

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules that are 17-22 nucleotides in length. They regulate expression of target genes by binding to the 3’-untranslated region (3’-UTR), which inhibits the translation of
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Figure 1. Hypoxia-induced injury to cardiomyocytes. H9c2 cells were grown in either normoxic or hypoxic conditions for 48 hours, then subjected to cell viability CCK-8 and flow cytometric apoptosis assays. A. The CCK-8 assay. Data are expressed as the mean ± standard error (n = 4). \( *P < 0.05 \) compared to normoxic cells. B. Flow cytometry. The graph shows quantified flow cytometry data.

Previous studies have demonstrated that hypoxia alters expression of various microRNAs that play a crucial role in cell metabolism, stress, and apoptosis, in response to hypoxia [14-17]. Zhang et al. revealed that hypoxia upregulated expression of several miRNAs, including miR-21-5p, miR-378-3p, miR-152-3p, and let-7i-5p, but downregulated expression of miR-760-3p in H9c2 cardiomyocyte cells [18]. Indeed, miR-760 miRNA has been reported to promote survival of different cancer cells. For example, overexpression of miR-760 miRNA promoted ovarian cancer cell proliferation [19] and also promoted breast cancer cell chemoresistance by upregulating epithelial-mesenchymal transition [20, 21]. Rat miR-760-3p and human miR-760 share the same sequence. In this regard, further examination of miR-760-3p and its target genes could provide new insight into hypoxia-induced cell damage, providing novel information for prevention of hypoxia-induced cardiomyocyte injuries. Therefore, the current study first established an in vitro model of cardiomyocyte injury by growing cardiomyocytes in hypoxic conditions. This study then performed bioinformatic analysis of miR-760-3p target genes using online tools (Target Scan and miRanda). Next, hypoxia-induced expression of these genes was examined, along with the underlying molecular events in vitro.

Results

Hypoxia induces cardiomyocyte injury

The current study first assessed hypoxia-induced cardiomyocyte injuries in vitro using cell viability CCK-8 and flow cytometric apoptosis assays. It was found that H9C2 cells growing in the hypoxic condition (1% O2) had significantly reduced viability, compared to cells growing in the normoxic condition (21% O2), indicating that hypoxia damaged cardiomyocytes.

Hypoxia downregulates miR-760-3p expression but upregulates VLDLR expression

To explore the underlying changes in gene expression, miR-760-3p and VLDLR mRNA and protein levels were measured using RT-PCR and Western blotting, respectively. Present da-
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Figure 2. Hypoxia downregulates miR-760-3p expression but upregulates VLDLR expression in H9c2 cells. H9c2 cells were grown in either normoxic or hypoxic conditions for 48 hours, then subjected to RT-PCR and Western blot analyses. A. RT-PCR analysis of miR-760-3p. B. RT-PCR analysis of VLDLR. C. Western blot analysis of VLDLR. The graph shows quantified Western blot data. Data are expressed as the mean ± standard error (n = 4). *P < 0.05 compared to normoxic cells.

VLDLR overexpression induces cardiomyocyte apoptosis in response to hypoxia

To explore the underlying molecular events contributing to hypoxia induction of cardiomyocyte apoptosis, this study overexpressed VLDLR in H9c2 cells using the adenovirus carrying VLDLR cDNA. VLDLR overexpression promoted cardiomyocyte apoptosis in the hypoxic condition (Figure 3) and altered expression of apoptosis-related proteins, such as Bcl2, Bax, and caspase 3, further indicating that VLDLR overexpression promotes cardiomyocyte apoptosis in response to hypoxia. However, the current study did not determine the effects of VLDLR overexpression in the normoxic condition.

VLDLR is a target gene of miR-760-3p

Present data demonstrates that hypoxia induces H9c2 cell apoptosis and decreases miR-760-3p expression. However, VLDLR overexpression rescued the effects of hypoxia on apoptosis and miR-760-3p. It was, therefore, speculated that miR-760 might silence VLDLR expression in hypoxic H9c2 cells. Bioinformatic analysis of miR-760-3p targeting genes was performed using an online tool, TargetScan. Analysis showed that VLDLR, indeed, is a predicted target gene of miR-760-3p (Figure 4A). Dual-luciferase reporter assay was then conducted to verify this result. Transfection with miR-760-3p mimics suppressed the luciferase activity of the WT-3’-UTR reporter gene in HEK293T cells (Figure 4B). However, the luciferase activity of the MUT-3’-UTR reported gene did not significantly change in HEK293T cells (Figure 4B). Western blot data further supports that transfection of a miR-760-3p mimic can...
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inhibit VLDLR protein expression in HEK293T cells (Figure 4C).

miR-760 reduces cardiomyocyte apoptosis by targeting VLDLR expression

To further confirm the effects of miR-760 on cardiomyocyte apoptosis and VLDLR expression in response to hypoxia, this study transiently transfected miR-760-3p mimics or an miR-760-3p-inhibitors into H9c2 cells. miR-760-3p overexpression significantly suppressed VLDLR expression in hypoxic cardiomyocytes. Apoptosis-related proteins, such as caspase-3 and the pro-apoptotic protein Bax, were significantly reduced. Anti-apoptotic protein Bcl-2 was increased (Figure 5). Apoptosis of hypoxic cardiomyocytes was significantly reduced (Figure 5). In contrast, transfection of miR-760-3p inhibitors increased expression of VLDLR and apoptosis-related proteins and increased the rate of cell apoptosis (Figure 5).

Discussion

Cardiovascular disease significantly contributes to death, worldwide, and affects patient quality of life [5]. Cardiovascular disease has been associated with an increase in cardiomyocyte hypoxia, which promotes cell apoptosis [6]. The current study first determined hypoxia-induced cardiomyocyte injuries in vitro, then explored the underlying molecular mechanisms. It was found that hypoxia induced cardiomyocyte apoptosis and downregulated miRNA-760-3p expression, but upregulated VLDLR expression. Moreover, VLDLR overexpression induced cardiomyocyte apoptosis and increased caspase-3 activity and Bax expression, but inhibited Bcl2 expression in response to hypoxia. Moreover, miR-760-3p was able to bind to the VLDLR 3'-UTR and decrease VLDLR mRNA and protein levels in H9c2 cells following hypoxia. However, miR-760-3p inhibitors rescued the effects of hypoxia on cardiomyocyte apoptosis and gene expression. In vitro data from the current study demonstrates that hypoxia-induced downregulation of miR-760-3p induces apoptosis of hypoxic cardiomyocytes, whereas miR-760-3p overexpression protects cardiomyocytes from apoptosis by regulating VLDLR expression. Future studies should investigate
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Figure 4. Prediction and verification of VLDLR as a miR-760-3p-targeting gene. A. Prediction of the miR-760 binding site to VLDLR 3’-UTR. B. Luciferase reporter assay. HEK293T cells were grown and transiently transfected with the wild type or mutated VLDLR 3’-UTR, together with miR-760-3p mimics and inhibitors. Total cellular protein was lysed and subjected to luciferase reporter assay. C. Western blot: HEK293T cells were grown and transiently transfected with miR-760-3p mimics and inhibitors for 48 hours, then subjected to Western blot analysis to measure VLDLR expression. The graph shows quantitative data of the Western blot. *P < 0.05 compared to the negative control.

how VLDLR regulates cardiomyocyte apoptosis and whether targeting VLDLR expression could be a novel strategy to protect cardiomyocytes against hypoxia.

Pathological hypoxia is the main cause of myocardial ischemia-induced myocardial cell injury and death. Chronic hypoxia can trigger an adaptive response to hypoxia, such as increased production of red blood cells or induced tissue hyperplasia. In contrast, acute hypoxia, like myocardial infarction and strokes, induces cell damage or apoptosis [6, 22]. In the acute situation, it is crucial to effectively protect cardiomyocytes or neurons from injury or death. Hypoxia induces changes in cell metabolism and gene expression [22-26]. Previous studies have provided evidence that hypoxia-induced alteration of miRNA expression is an important endogenous protection mechanism against hypoxia-induced cell injury [24, 25]. Expression of miR-499, a cardiac specific miRNA, inhibited cardiomyocyte apoptosis induced by myocardial infarction and inhibited expression of dynamin-related protein-1 (Drp1) in rats [26], while miR-210 induced tissue angiogenesis and inhibited apoptosis to improve cardiac function in a murine model of myocardial infarction [27]. The current study found that hypoxic cardiomyocytes expressed a reduced level of miR-760-3p, which further confirmed a previous study [18]. Furthermore, the current study revealed that downregulated expression of miR-760-3p suppressed VLDLR expression and promoted cardiomyocyte apoptosis following hypoxia. Since the current data is novel and has not been previously reported, further studies are necessary to understand the underlying molecular mechanisms by which VLDLR regulates cell apoptosis and survival.

Indeed, previous studies have demonstrated that hypoxia-induced gene alterations play a vital role in regulating hypoxia-induced cell injury [28-32]. VLDLR is abundantly expressed in adipose, heart, and skeletal muscle [33]. Previous studies have also indicated that VLDLR expression was significantly upregulated in various cells during hypoxia, promoting cell apoptosis [34-37]. The current study also
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Figure 5. miR-760 reduction of cardiomyocyte apoptosis by inhibiting VLDLR expression. H9C2 cells were grown and transiently transfected with either the miR-760-3p or miR-760-3p-inhibitors for 48 hours in a hypoxic condition. (A) RT-PCR. Transfected H9C2 cells were subjected to qRT-PCR analysis to measure miR-760-3p and VLDLR mRNA levels. (B) Western blot. Transfected H9C2 cells were subjected to Western blot analysis of caspase-3, Bax, Bcl-2, and VLDLR proteins. (C) Quantitative data of (B). (D) Flow cytometric apoptosis assay. Transfected H9C2 cells were subjected to flow cytometry. (E) Quantified data of (D). Data are expressed as the mean ± standard error (n = 4). *P < 0.05 vs. the control or mimics-NC group and *P < 0.05 vs. the control or inhibitors-NC group.
showed that VLDLR overexpression enhanced cardiomyocyte apoptosis, consistent with previous studies [10, 27-29]. Furthermore, another study demonstrated that knockdown of VLDLR improved survival and decreased cardiac infarction size in mice after myocardial infarction [38]. The current study revealed that VLDLR was a direct target gene of miR-760-3p, validated with the luciferase reporter assay and Western blot analysis. In addition, it was found that miR-760-3p overexpression effectively protects hypoxic cardiomyocytes from apoptosis.

In conclusion, the current study demonstrates that hypoxia-induced downregulation of miR-760-3p contributes to cardiomyocyte apoptosis, while overexpression of miR-760-3p attenuates hypoxia-induced cardiomyocyte apoptosis by decreasing VLDLR expression. However, the current study is just proof-of-principle. Future studies are necessary to determine whether targeting miR-760-3p-VLDLR could be a novel strategy for prevention of hypoxia-induced injury to cardiomyocytes, bettering understand how VLDLR overexpression induces cardiomyocyte apoptosis.

Materials and methods

Cell line, culture, and hypoxic model to induce cell injury

The rat cardiac muscle-derived H9c2 cell line was obtained from Cell Bank, Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (all from Gibco, Gaithersburg, Maryland, USA) in a humidified incubator with 5% CO₂ at 37°C. To establish the hypoxia cell injury model, this study cultured H9c2 cells in serum-free DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (all from Gibco, Gaithersburg, Maryland, USA) in a humidified incubator with 5% CO₂ at 37°C. To establish the hypoxia cell injury model, this study cultured H9c2 cells in serum-free DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (all from Gibco, Gaithersburg, Maryland, USA) in a humidified incubator with 5% CO₂ at 37°C.
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CAGGACCAGATGTGAT-3'; VLDLR, 5'-TCAACGAAT-GCTTGGTCAATAAC-3' and 5'-ATCTCAGACTTGGCTTTTGGCCTACT-3'. U6 mRNA was used as a control for miR-760 and β-actin was used as a control for VLDLR. Relative levels of miR-760-3p and VLDLR mRNA were calculated using the method of \(2^{-\Delta\Delta Ct}\).

**Western blotting**

Total cellular protein was lysed on ice using the radioimmunoprecipitation assay buffer (RIPA; Beyotime, Shanghai, China) supplemented with 1% phenylmethanesulfonyl fluoride (Solarbio, Beijing, China). Protein concentrations were assayed using the bicinchoninic acid (BCA) protein assay kit (Beyotime), according to manufacturer instructions. Equal amounts of these protein samples were then separated in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and transferred onto polyvinylidene fluoride membranes (PVDF; Millipore, Bedford, MA, USA). For Western blotting analysis, the membranes were incubated in 5% skim milk at room temperature for 1 hour, then incubated with primary antibodies against VLDLR (Abcam, Cambridge, UK), Bcl-2 (Abcam), Bax (Abcam), caspase-3 (Abcam), and β-actin (Abcam), at a dilution of 1:100 and at 4°C overnight. The next day, the membranes were briefly washed with Tris-based saline-Tween 20 (TBS-T) three times. They were then incubated with the infrared dye-conjugated secondary antibody at room temperature for 1 hour. The membranes were finally scanned for positive protein bands using the Odyssey Infrared Imaging System (Licor, Lincoln, NE, USA) and relative levels of protein expression were quantified using Image J software (National Institute of Heath, Bethesda, MD, USA).

**Cell transfection**

An adenoviral vector carrying VLDLR cDNA or control cDNA was constructed using the AdEasy system. The adenovirus was produced in and collected from HEK293-T cells, according to a previous study [23]. To overexpress VLDLR in H9C2 cells, cells were grown and infected with the adenovirus to reach an infection efficiency up to 80%. miR-760 mimics and inhibitors were purchased from Ribobio Co. (Guangzhou, China) and transfected into H9C2 or HEK 293T cells using Lipofectamine 2000 (Invitrogen), according to manufacturer instructions. Present experiments were divided into five groups: H9C2 cells negative control, miR-760 mimics negative control (mimics-NC), miR-760-3p-mimics, miR-760 inhibitors negative control (inhibitors-NC), and miR-760-3p inhibitors. Transfected cells were then subjected to different assays.

**Luciferase reporter assay**

To determine target genes of miR-760-3p, this study first cloned the 3'-UTR DNA sequence of VLDLR (WT-VLDLR) covering the predicted miR-760-3p binding site or mutated the miR-760-3p binding site (MUT-VLDLR) into psiCHECK-2 vector (Ribobio). After DNA sequencing confirmation, these vectors were transiently transfected into HEK293T cells that were seeded into 24-well plates and cultured overnight. The next day, cells were co-transfected with either the miR-760-3p mimics or miR-760-3p inhibitors plus the luciferase reporter vector comprising wild-type or mutant VLDLR 3'-UTR using Lipofectamine 2000 (Invitrogen) for 24 hours. Total cellular protein was lysed using RIPA (Solarbio) and relative luciferase activity was determined using the Dual-Luciferase Reporter Analysis System (Promega, Madison, WI, USA), according to manufacturer protocol. Firefly luciferase activity was normalized to the Renilla luciferase activity.

**Acknowledgements**

This study was supported in part by grants from the National Natural Science Foundation of China (#81560068) and Natural Science Foundation of Guangxi Province (#2016GXNSFAA139198).

**Disclosure of conflict of interest**

None.

**Address correspondence to:** Dr. Guo-Qiang Zhong, Department of Cardiology, The First Affiliated Hospital, Guangxi Medical University, 22 Shuangyong Road, Nanning 530021, Guangxi, China. Tel: +86771-5356618; Fax: +86771-5350031; E-mail: guoq_zhong@163.com; Dr. Cun-Ming Fang, Department of Cardiology, People’s Hospital of Xuancheng City, 15 North Huancheng Road, Xuancheng 242000, Anhui, China. Tel: +86563-3033505; Fax: +86563-3033505; E-mail: sshfang@126.com
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