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MicroRNA1 modulates oxLDL-induced hyperlipidemia by down-regulating MLCK and ERK/p38 MAPK pathway

Hua-Qing Zhu [a,b,*], Feng Wang [a], Liu-Yi Dong [c], Qing Zhou [a,b], Yuan Wang [a,b,*]

[a] Laboratory of Molecular Biology and Department of Biochemistry, Anhui Medical University, Hefei, Anhui, People's Republic of China
[b] Key Laboratory of Gene Research of Anhui Province, Hefei, Anhui, People's Republic of China
[c] Department of Pharmacology, Anhui Medical University, Hefei, Anhui, People's Republic of China

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ABSTRACT

Aims: This study was aimed to determine whether microRNA1 (miR1) plays a role in the activation of myosin light chain kinase (MLCK) mediated by oxLDL in human umbilical vein endothelial cells (HUVECs).
Main methods: HUVECs were treated with oxLDL along with a control miR or miR1 mimic. MiR1 expression was assayed by miRNA plate assay kit and mirVana™ miRNA isolation kit. The MLCK protein, transcript, and kinase activity were measured by Western blot, real-time-polymerase chain reaction and γ-^{32}P-ATP phosphate incorporation, respectively. In addition, phosphorylation of MLC, ERK and p38 was analyzed by Western blot.
Key findings: The results showed that upon treatment with oxLDL, miR1 expression was decreased, whereas MLCK expression was increased, in a time- and dose-dependent manner. Consistent with this, miR1 mimic prevented MLCK expression and activation and attenuated the phosphorylation of MLC and ERK/p38 in oxLDL-treated HUVECs. Furthermore, we showed that miR1 was able to bind a site located at the 3′un-translational region of MLCK mRNA and inhibited its expression.
Significance: Taken together, this study demonstrated that the effect of miR1 on hyperlipidemia is mediated through down-regulation of MLCK and the ERK/p38 MAPK pathway.

Introduction

Oxidized low-density lipoprotein (oxLDL) is a critical factor in the initiation and progression of hyperlipidemia and atherosclerosis (AS) and contributes to endothelial dysfunction and plaque destabilization through multiple mechanisms (Landmesser and Harrison, 2001). Human studies have confirmed that oxLDL and oxidized lipid byproducts are present within atherosclerotic plaques (Lee et al., 2001; Navab et al., 2012). Multiple investigations have established an essential role of the vascular endothelium in the regulation of the diffusion integrity of the intravascular space (Luissint et al., 2012). A major function of the endothelial cell is to serve as a barrier to fluid and solute flux across the blood vessel inner wall (Hirase and Node, 2012).

Myosin light chain kinase (MLCK) has been shown to play an important role in regulating the contractile state and barrier function of endothelium (Sun et al., 2011). MLCK is essential for endothelial cell contraction (Isotani et al., 2004). In endothelial cells, phosphorylation of myosin light chain (MLC) by activated MLCK plays a critical role in the development of AS (Kramerov et al., 2012; Tinsley et al., 2000).

MicroRNAs (miRs) are a class of small non-coding RNAs (20–24 nucleotides), which primarily bind to the 3′ untranslated region of a target mRNA and negatively regulate gene expression at posttranscriptional level (Tan et al., 2012). MiRs are involved in a wide range of pathophysiological cellular processes including development, differentiation, growth, metabolism, and tumor formation (Bushati and Cohen, 2007; Chang, 2007; Duan et al., 2012). Aberrant expression of miRs has been linked to a number of cardiovascular pathological conditions, including AS. Therefore, miRs have been suggested to be novel therapeutic targets for cardiovascular diseases (Weber et al., 2010; Nazari-Jahantigh et al., 2012; Qin and Zhang, 2011). Indeed, studies have demonstrated that miR1 is associated with cardiac hypertrophy and heart failure (Elia et al., 2009; Hua et al., 2012). However, it remains unclear whether miR1 plays a role in hyperlipidemia. The mitogen-activated protein kinase (MAPK) signaling pathway plays a significant role in a wide range of diseases associated with miRs. For example, the miR21 expression pattern is found to be correlated with ERK/MAPK activity (Mei et al., 2013). In addition, miR155 is found to be involved in the MAPK pathway by targeting MAPK kinase kinase 10 (Zhu et al., 2012). Finally,

* Corresponding authors at: Laboratory of Molecular Biology and Department of Biochemistry, Anhui Medical University, Hefei 230032, Anhui Province, People's Republic of China. Tel.: +86 551 5161140.
E-mail addresses: aydzhq@126.com (H.-Q. Zhu), wangyuan@ahmu.edu.cn (Y. Wang).

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MAPK/ERK/miR31/LATS2 may represent a novel signaling pathway in VSMC growth (Liu et al., 2011).

Our previous study showed that miR1 prevents high-fat diet-induced endothelial permeability in apoE knock-out mice (Wang et al., 2013), However, it is not clear whether miR1 plays a role in the MLCK activation and expression mediated by oxLDL and even less is known about the signaling pathway. Therefore, the present study was to investigate the role of miR1 in MLCK expression and activity induced by ox-LDL in HUVECs and to assess the function of the ERK/P38 MAPK signaling pathway in hyperlipidemia.

Materials and methods

Cell culture

Primary HUVECs were purchased from Cloneticse, Biowhitttaker, East Rutherford, NJ. HUVECs at passage 3–4 were pre-incubated with various reagents in EGMe supplemented with 10% FBS. HUVECs were cultured for 24 h, oxLDL was incubated and miR1 mimic or a scrambled oligonucleotide was co-transfected by using Attractene Transfection Reagent (Qiagen). Each experiment was repeated at least three times with different batches of primary HUVECs.

Reagents and antibodies

Methionine-free medium (RPMI 1640) was purchased from Invitrogen (Carlsbad, CA). Anti-MLCK and pMLC monoclonal antibody (mAb) were purchased from Sigma-Aldrich Corporation (St. Louis, MO). γ-^{32}P-ATP was from Yahui Biomedical Engineering Co. (Beijing, China). Calmodulin and myosin regulatory light chain were the gifts from Dr. Zhi at University of Texas Southwestern Medical Center, USA. OxLDLs were prepared by copper: LDLs were dialyzed with 0.01 mol/L phosphate-buffered saline (pH 7.4). Copper-oxidized LDLs were prepared under sterile conditions by incubating 500 μg/mL of LDL with 2.5 μmol/L $CuCl_2$ for 48 h at 37 °C. At the end of the incubation period, oxLDLs were extensively dialyzed at 4 °C against PBS (pH 7.4). OxLDLs were characterized by measuring thiobarbituric acid reactive substances and lipoperoxides. All other chemicals used were of the purest commercially available grade.

Modulation of miR1

The miR1 mimic synthesized from Qiagen was used to overexpress miR1 expression. A scrambled oligonucleotide (GenePharm Co. Ltd.) was used as a control. Transfection was performed by using TransMessenger transfection reagent (Qiagen) according to the manufacturer's instructions as described previously (Shen et al., 2009).

MiR1 expression assay

Total RNA was extracted from HUVECs using TRIzol reagent (Invitrogen). MiR1 expression was determined using the miRNA plate assay kit (Signosis, Inc.) and mirVana™ miRNA isolation kit according to the manufacturer's instructions. U6 was used as an internal control.

Cell proliferation assay

MTT assay was used to measure cell proliferation. In short, HUVECs were seeded at a density of 5000 cells/cm^2 in 96-well plates. The cells were incubated for 24 h in oxLDL. At the end of treatment, MTT at 0.25 mg/mL was added to the plates, and incubation continued for another 4 h at 37 °C. The supernatant was then carefully removed, and 150 μL of DMSO was added to dissolve the formazan crystals. The absorbance of the solubilized product at 490 nm (A490) was measured with an ELISA reader (Thermo Scientific Varioskan Flash, USA). All determinations were confirmed in at least three identical experiments.

MLCK mRNA assay

Total RNA was extracted from HUVECs in different groups using the TRIzol reagent (Invitrogen) following the manufacturer's instructions. Real-time reverse transcription-PCR of MLCK and β-actin mRNA was performed. The primers for MLCK andβ-actin were described previously (Zhu et al., 2008).

Western blot analysis

The protein levels of MLCK, pMLC, ERK, pERK, p38, pp38 andβ-actin were determined by Western blot analysis using respective specific antibodies (Zhu et al., 2011a, 2011b).

MLCK activity assay

MLCK activity was measured by rates of γ-^{32}P-ATP phosphate incorporation into MLC as described previously (Zhu et al., 2011a, 2011b).

Plasmids

The luciferase vector (wt-Luc-MLCK), which contains MLCK-miR1 response elements in the 3′UTR of MLCK, was purchased from Addgene Inc. The vector (mu-Luc-MLCK) with a mutation in the MLCK-miR1 response elements was generated by using site-directed gene mutagenesis. The reporter vector consisting of a luciferase gene followed by the miR1 binding consensus sequence was purchased from Signosis, Inc. (Sunnyvale, CA, USA).

Luciferase assays

HUVECs were cultured for 24 h. Two hundred nanograms of plasmid DNA (wt-Luc-MLCK or mu-Luc-MLCK) along with a miR1 mimic or a scrambled oligonucleotide was co-transfected by using Attractene Transfection Reagent (Qiagen) according to the manufacturer's instructions. The pRL-CMV vector expressing Renilla luciferase was used as an internal control. Luciferase assays were performed by using the dual luciferase reporter assay system (Promega) 24 h after transfection.

Statistical analysis

The data are expressed as the means ± SD. A comparison among each group was performed by one-way analysis of variance followed by the Newman–Keuls test to evaluate the statistical significance between two groups. P value of <0.05 was considered as significant.

Results

MiR1 and MLCK expression in oxLDL-treated HUVECs

To examine the role of oxLDL in endothelial function, we examined the effect of oxLDL on miR1 expression using miRNA plate assay kit. As shown in Fig. 1, oxLDL markedly inhibited miR1 expression upon treatment with 10 μg/mL oxLDL. The inhibition was enhanced in a dose and time-dependent manner (Fig. 1A, B). Next, as it is known that MLCK is important in regulating the contractile state of the endothelium, thus, we evaluated the MLCK expression in oxLDL-treated HUVECs and found that oxLDL stimulated MLCK expression in a dose and time-dependent manner (Fig. 1C, D). OxLDL is shown to enhance MLCK expression in smooth muscle cell (Augé et al., 1996). In agreement with this, we found that the levels of MLCK protein were increased in oxLDL-treated HUVECs, concomitantly with a decreased expression of miR1. HUVEC proliferation was also in a dose and time-dependent manner (Fig. 1E, F). This result suggests that miR1 may down-regulate MLCK protein expression. Since significant effects of oxLDL (150 μg/mL)