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Original Contribution

7-Ketocholesterol inhibits isocitrate dehydrogenase 2 expression and impairs endothelial function via microRNA-144

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ABSTRACT

Oxysterol is associated with the induction of endothelial oxidative stress and impaired endothelial function. Mitochondria play a central role in oxidative energy metabolism and the maintenance of proper redox status. The purpose of this study was to determine the effects and mechanisms of 7-ketocholesterol (7-KC) on isocitrate dehydrogenase 2 (IDH2) and its impact on endothelial function in both human aortic endothelial cells (HAECs) and C57BL/6J mice. HAECs treated with 7-KC showed significant reductions of IDH2 mRNA and protein levels and enzyme activity, leading to decreased NADPH concentration and an increased ratio of reduced-to-oxidized glutathione in the mitochondria. 7-KC induced the expression of a specific microRNA, miR-144, which in turn targets and downregulates IDH2. *In silico* analysis predicted that miR-144 could bind to the 3′-untranslated region of IDH2 mRNA. Overexpression of miR-144 decreased the expression of IDH2 and the levels of NADPH. A complementary finding is that a miR-144 inhibitor increased the mRNA and protein expression levels of IDH2. Furthermore, miR-144 level was elevated in HAECs in response to 7-KC. Anti-Ago1/2 immunoprecipitation coupled with a real-time polymerase chain reaction assay revealed that 7-KC increased the functional targeting of miR-144/IDH2 mRNA in HAECs. Infusion of 7-KC *in vivo* decreased vascular IDH2 expression and impaired vascular reactivity via miR-144. 7-KC controls miR-144 expression, which in turn decreases IDH2 expression and attenuates NO bioavailability to impair endothelial homeostasis. The newly identified 7-KC–miR-144–IDH2 pathway may contribute to atherosclerosis progression and provides new insight into 7-KC function and microRNA biology in cardiovascular disease.

Introduction

The oxidative hypothesis for atherosclerosis has been critical in the development of our current understanding of the molecular mechanisms of this disease [1]. Oxidative changes in low-density lipoprotein (LDL) induced by various mechanisms are widely regarded as a critical trigger in inducing endothelial damage and plaque development [2,3]. Several lipid-derived bioactive molecules are thus generated in LDL, including oxysterols [4–7]. Among the oxysterols that have been identified, those oxidized at the C7-position, such as 7-ketocholesterol (7-KC), are detected at high levels in human atherosclerotic plaques and in the plasma of patients with a high cardiovascular risk, and they are abundant in oxidized LDL [6–8]. 7-KC is a highly toxic oxysterol found in abundance in atherosclerotic plaques and is believed to play a critical role in atherosclerosis. The major percentage of oxysterol is derived from dietary cholesterol-rich food sources, such as egg and egg-derived products, and meat products, which usually could be found in a typical western-type diet [9,10].

Isocitrate dehydrogenase 2 (IDH2) is a critical component of the mitochondrial antioxidant pathway through its ability to generate NADPH from the oxidative decarboxylation of isocitrate to α-ketoglutarate [11,12]. NADPH is necessary for regenerating reduced glutathione (GSH), the major antioxidant responsible for preventing reactive oxygen species (ROS) damage [13]. Thus, IDH2

Abbreviations: Ago, Argonaute; ECs, endothelial cells; eNOS, endothelial nitric oxide synthase; GSH, reduced glutathione; GSSG, glutathione disulfide; HAECs, human aortic coronary artery endothelial cells; IDH, isocitrate dehydrogenase; 7-KC, 7-ketocholesterol; LDL, low-density lipoprotein; miRNA, microRNAs; MOI, multiplicity of infection; NADP, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; ROS, reactive oxygen species; RISC, RNA-induced silencing complex; 3′-UTR, 3′-untranslated region

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activation plays a critical role in enhancing the mitochondrial glutathione antioxidant defense system and protects cells against oxidative damage [13,14]. To date, no studies to our knowledge have examined the effects of oxysterol on IDH2 and the consequences for endothelial function.

MicroRNAs (miRNAs) are noncoding small RNAs that regulate gene expression at the posttranscriptional level [15,16]. The mature miRNAs are small, noncoding molecules (20–25 nucleotides) that incorporate into the RNA-induced silencing complex (RISC) and specifically guide it to bind to target mRNA sequences, usually in the 3′-untranslated region (UTR), leading to suppressed protein translation or enhanced mRNA degradation [17]. RNA sequencing experiments showed that more than 50 microRNAs are highly expressed in cultured endothelial cells (ECs) [18,19].

Accumulating studies indicate an integrated miRNA network in the molecular mechanisms that control blood vessel development, cellular homeostasis, vascular inflammation, angiogenesis, and metabolism [20–23]. Several miRNAs have been identified to regulate redox status. In particular, miR-200 family members play a crucial role in oxidative stress-dependent endothelial dysfunction and in cardiovascular complications of diabetes and obesity. In addition, different miRNAs, such as miR-210, play a key role in mitochondrial metabolism, therefore modulating ROS production and sensitivity [24]. Thus, miRNAs that are modulated by ROS or involved in ROS production have been implicated in vascular diseases in which redox imbalance has a pathogenetic role.

In the current study, we present evidence that 7-KC posttranscriptionally regulates IDH2 expression via miR-144 and thus promotes oxidative damage in vascular ECs. miR-144 overexpression inhibits IDH2 expression in vascular ECs, thereby attenuating NO availability. Most importantly, *in vivo* delivery of miR-144 to mice represses IDH2 expression in the aorta, reducing endothelium-dependent vasorelaxation. Conversely, silencing miR-144 in mice prevents the inhibition of IDH2 activity by 7-KC. These data reveal how an inducible miRNA comprises a negative feedback loop to ensure the tight regulation of endothelial function.

Materials and methods

Materials

7-Ketocholesterol was purchased from Sigma Chemical (St. Louis, MO). The eNOS inhibitor N^G-nitro-L-argine methyl ester (L-NAME) was obtained from Alexis (Farmingdale, NY).

Cell culture

Human aortic endothelial cells (HAECs) were purchased from Cell Applications Inc. (San Diego, CA) and cultured in M199 medium supplemented with FBS (20% vol/vol), penicillin (100 U/ml), streptomycin (100 μg/ml), heparin (90 μg/ml), and endothelial cell growth supplement (20 μg/ml). The cells were grown at 37 °C in humidified 5% CO_2 and used for experiments between passages 3 and 5 [25]. Then, cells were subjected to 7-ketocholesterol (Sigma-Aldrich) treatment.

Bovine aortic endothelial cells (BAECs, JCRB0155) were obtained from Health Science Research Resources Bank (HSRRB, Japan Health Sciences Foundation). The cells were cultured in Dulbecco's modified Eagle media (DMEM) with 10% fetal calf serum (FCS, Gibco).

Animal studies

Male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were used for this study. The animals were fed standard chow, maintained in a 22 °C room with a 12-h light/dark cycle, and received drinking water ad libitum. All procedures were reviewed and approved by the institutional Animal Care and Use Committee of Sun Yat-Sen University. Eight-week-old male mice were randomly divided into 3 groups: vehicle-treated (control group, $n = 8$), 7-KC-treated groups ($n = 8$), and 7-KC plus anti-miR-144 groups ($n = 8$). 7-KC was administered by Alzet minipump via tail vein for 5 consecutive days at a dose of 50 μg/kg bw/day), and control mice received 0.9% physiological saline injection. In selected studies, 7-KC-treated mice ($n = 8$) were also injected with lentiviral constructs expressing anti-miR-144. Lentiviruses encoding miR-144 precursor (miR-144), anti-sense miR-144 (anti-miR-144), and scrambled control miRNA (Ctrl miRNA) were obtained from System Biosciences Inc. (SBI, Mountain View, CA). The mirVana miRNA inhibitors and mimics (Life Technologies) were complexed with Invivofectamine 2.0 reagent (Invitrogen) to form the nanoparticles suitable for *in vivo* applications. miRNA oligonucleotides (3 mg/ml in water, 750 μl) were mixed with manufacturer's complexation buffer (750 μl), and then Invivofectamine 2.0 reagent was added (1500 μl). After 30 min incubation at 50 °C, dialysis was performed in PBS to remove excessive salts and solvents. Each mouse was injected with 4×10^7 infectious units in 150 μl PBS via the retro-orbital vein, resulting in a miRNA dose approximately ≈ 20 nM per mice). The mice were euthanized with inhaled isoflurane. At euthanasia, tissues were harvested and immediately stored at −80 °C.

miRNA and mRNA real-time polymerase chain reaction

For IDH2 mRNA analysis, total RNA from cells and aorta was isolated using TRIzol (Invitrogen). First-strand cDNA was synthesized with the Omniscript RT kit (Qiagen), used for quantitative real-time polymerase chain reaction (qRT-PCR). qRT-PCR was performed on ABI Prism 7900-HT Sequence Detection System, and the C_t values for each mRNA were normalized to NADPH.

For miRNA analysis in mice, cDNA was synthesized with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and used for qRT-PCR. For miRNA analysis in HAECs (First Choice Human Total RNA Survey Panel, Applied Biosystems), cDNA was synthesized with the RT2 First Strand Synthesis Kit (SABiosciences). For miR-144 analysis, proprietary primers from Applied Biosystems were used. Data were analyzed using the $\Delta\Delta C_t$ method, with normalization to U6, and are presented in arbitrary units. Primers used for mRNA quantification were designed using Primer Express 3.0 (Applied Biosystems). Primers specific for miR-144, miR-183, and U6 and primers used to measure RNA in human samples were obtained from SABiosciences. Primers specific for mouse miR-144 and U6 were obtained from Applied Biosystems [26,27].

Mitochondrial isolation

HAECs were washed two times with the isolation buffer containing 225 mM mannitol, 75 mM sucrose, 20 mM MOPS, 1 mM EGTA, 0.1% BSA (pH value was adjusted to 7.2 with Tris). The cells were harvested and centrifuged at 937 *g* for 5 min. The pelleted cells were incubated for 5–10 min in a hypotonic 100 mOsm medium containing 100 mM sucrose, 10 mM MOPS, 1 mM EGTA, 0.1% BSA (pH 7.2), and disrupted by 25–30 sharp strokes of the tight pestle in a Dounce homogenizer. The medium's tonicity was adjusted to 300 mOsm with 1.25 M sucrose, and the volume tripled with the isolation buffer. The disrupted cells were centrifuged for 5 min at 1075 *g*. The supernatant was spun at 9300 *g* for 10 min. The supernatant was removed and used for the cytoplasmic fraction assay to assess the purity of our mitochondrial preparation. The remaining sediment was resuspended in isolation medium and centrifuged at 1075 *g* for 5 min. The final