Effect of endothelial microRNAs on blood pressure homeostasis

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Abstract Endothelial function is an important factor for maintenance of blood pressure (BP) homeostasis. Recently, microRNA (miRNA) has emerged as a potential regulator of endothelial function. However, the role of miRNAs in maintenance of BP homeostasis is unknown. In the present study, we investigated the potential role of endothelial miRNAs in BP regulation in vivo by using endothelial cell (EC)-specific Dicer knockout (KO) mice and measured BP before and after 8 weeks of high salt loading intervention. The EC-specific Dicer homozygous KO mice showed embryonic lethality. The EC-specific Dicer heterozygous KO mice (Het) showed a significant decrease in expression of Dicer mRNA in lung compared to wild-type (WT) mice. However, there were no differences between WT and Het mice in heart rates, systolic and diastolic blood pressures before and after 8 weeks of high salt loading. In this study, we demonstrated that the endothelial Dicer is essential for embryonic development. Additionally, we could not observe any phenotype in the hemodynamic parameters of EC-specific heterozygous Dicer KO mice.

Keywords: microRNA, Dicer, blood pressure, endothelium

Introduction

Elevated blood pressure (BP) is one of the causes of cardiovascular disease and leading to death. An increase in the prevalence of hypertension is a global issue and many therapeutic challenges for the treatment of hypertension have arisen worldwide1. Elucidation of the molecular mechanism of BP regulation might contribute to finding novel therapeutic targets for controlling BP. BP is tightly and coordinately controlled in many organs and systems. Among them, endothelial function such as endothelium dependent vaso-relaxation is important machinery for BP homeostasis2.

MicroRNAs (miRNAs) are a small non-coding RNA that represses gene expression by post-transcriptional gene regulation3,4. MicroRNA biogenesis and functionality are organized by multiple proteins including cytoplasmic ribonuclease III Dicer that is processed from ~70 nt hairpin structures (called pre-miRNA) to double-strand mature form. This mature miRNA is loaded onto Argonaute (AGO) proteins, and its AGO-miRNA complex binds to the complementary sequence of 3’ untranslated region (UTR) of target mRNA via seed region of miRNA (nucleotides 2-8)5. To date, miRNA mediated post-transcriptional gene regulation and its biology have developing an attractive field of research, and much attention has focused on the miRNA as a novel candidate of therapeutic targets.

Many lines of evidence suggest that miRNAs affect gene expression in endothelial cells (ECs) and implicate pathogenesis and the development of cardiovascular diseases6,7. It has been reported that the knockdown of Dicer increased endothelial nitric oxide synthase (eNOS) protein expression and affected cell proliferation and tube formation in human umbilical vein endothelial cells (HUVEC)8. Moreover, the EC-specific deletion of Dicer1 in atherosclerosis prone ApoE KO mice inhibited the progression of atherosclerosis9. These evidence suggests that the endothelial miRNAs are potential regulators of cardiovascular biology and disease. However, the role of endothelial miRNAs in the maintenance of BP is unclear. To investigate the role of miRNAs in BP homeostasis in vivo, we generated EC-specific Dicer KO mice and determined BP fluctuation before and after long-term high salt loading intervention.
Materials and Methods

Animal experiments. All mice were housed in temperature-controlled quarters (21°C) with a 12-h light/12-h dark cycle and provided with water and food ad libitum. Female mice were used for all experiments. In high salt loading experiments, mice were given salt water containing 2% NaCl or normal water. The animal protocols were approved by the Animal Care and Use Committee of the University of Tokyo (P14-047) and Waseda University (2017-A103a). EC-specific Dicer knockout mice were generated by cross breeding Tie2-Cre mice with Dicer1-floxed mice. The Dicer1-floxed mice were used after being backcrossed with B6 background mice at least 8 times. Genomic DNA samples from mouse tails were used for PCR-based genotyping with the following primers: Tie2-Cre-F (forward), 5’-CGCATAACCAGTGAAACAGCATTGC-3’; Tie2-Cre-R (reverse), 5’- CCCTGTGCTCAGACAGAAATGAGA-3’; Dicer flox Fp (forward), 5’- CCTGACAGTGACGGTCCAAAG-3’; Dicer flox Rp (reverse), 5’- CATGACTCTTCCAACACT-3’.

Semi-quantitative RT-PCR. Total RNA was extracted from soleus and lung tissues using ISOGEN II (WAKO, Osaka, Japan) according to the manufacturer’s protocols. 1μg of RNA was reverse transcribed using ReverTra Ace (TOYOBO, Osaka, Japan) with oligo-dT. An aliquot of the RT reaction was used directly for PCR with Ex Taq HS (TaKaRa, Tokyo, Japan) and gene-specific primers. Primer sequences were as follows: Dicer, 5’-GGCTGCATCGGATAGTACACC-3’ and 5’- CACACGCCTCCTACCAAACAC-3’; eNOS, 5’- GACCCTCACCGCTACCATCAACAT-3’ and 5’- CTGGCCTTCTGCTCATTTTC-3’; GAPDH, 5’-GACCCCTTCATTGACCTCAAC-3’ and 5’-TAAGCAGTTGGTGTCAGGAA-3’. GAPDH was used as an internal control.

MicroRNA analysis. The TaqMan MicroRNA Reverse Transcription Kit and TaqMan MicroRNA assays (Applied Biosystems, Foster City, CA) were used according to the manufacturer’s protocols for real-time PCR quantification of mature miRNA expression. Each reverse RT reaction contained 10 ng of purified total RNA. The reaction mixtures were incubated for 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C. Real-time PCR reactions for each miRNA were performed in duplicate in a 10-μl reaction mixture that included 1μl of the RT product. Reactions were carried out on an Applied Biosystems StepOnePlus Real-Time PCR System in 96-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Small RNA, U6 was used as an internal control.

Blood pressure measurement. Heart rate, systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured by the tail-cuff method using BP-98A-L (Softmay, Tokyo, Japan). The mice were acclimated to the procedure from 1 week before measurements.

Statistics. Data are presented as mean ± standard error of the mean (SEM). Statistical significance (P < 0.05) was determined by a Student’s t-test for comparisons between two groups or a two-way ANOVA followed by Tukey-Kramer post-hoc test for multiple comparisons.

Results

Generation of endothelial cell-specific Dicer KO mice. To investigate the role of endothelial miRNAs in BP homeostasis in vivo, we inactivated Dicer, which is an essential enzyme for miRNA processing in ECs by cross-breeding Tie2-Cre mice and Dicerfl/fl mice. However, we could not obtain homozygous KO offspring. It has recently been reported that crossbreeding Tie2-Cre and Dicerfl/fl mice results in lethality due to developmental vascular dysfunction. We confirmed that the inactivation of Dicer in ECs results in embryonic lethality during the developmental stage. Because the EC-specific homozygous Dicer KO mice suffered early embryonic lethality, we then used the heterozygous Dicer KO mice (Het) for all experiments. To confirm the expression levels of Dicer mRNA and EC-enriched miRNA, these RNA expressions...
were quantified in lung, which contains abundant ECs. The Dicer mRNA expressions were significantly reduced in lung of Het mice compared to WT mice (Fig. 1A, B). On the other hand, EC-enriched miR-126 levels did not change significantly in Het mice (Fig. 1C).

**Blood pressure in endothelial cell-specific Dicer heterozygous KO mice.** To determine the effect of endothelial Dicer inactivation on BP homeostasis, we measured basal BP and BP changes in response to high salt water loading to induce hypertension. There were no differences in resting heart rates (HR), systolic blood pressure (SBP) and diastolic blood pressure (DBP) between heterozygous Dicer KO mice and WT mice. After 8 weeks of high salt water loading, HR, SBP and DBP did not increase in either WT or Het mice. Moreover, there were no differences in these hemodynamic parameters between genotypes (Fig. 2A-C).

**Gene expression of endothelial nitric oxide synthase in endothelial cell-specific Dicer heterozygous KO mice.** Endothelium-derived nitric oxide (NO), which is produced by endothelial nitric oxide synthase (eNOS), is a central regulator of endothelial function and blood pres-
sure homeostasis\textsuperscript{17,18}. The eNOS mRNA expression in soleus muscle of the endothelial cell-specific Dicer heterozygous KO mice was comparable to that of WT mice (Fig. 3A, B).

**Discussion**

Previous studies have demonstrated that endothelial miRNAs control endothelial functions and are implicated in the onset and development of cardiovascular disease\textsuperscript{9,19-22}. However, the role of endothelial miRNAs in BP homeostasis has been poorly understood. To investigate the role of endothelial miRNAs in BP homeostasis in the present study, we generated the endothelial cell-specific Dicer KO mice and measured the BP of these mice before and after 8 weeks of high salt loading intervention.

We generated the EC-specific Dicer KO mice using the Cre/loxP system because it has already been shown that the conventional KO of the Dicer gene leads to embryonic lethality\textsuperscript{23}. However, consistent with a recent report\textsuperscript{16}, the endothelial Dicer homozygous KO mice, generated by cross-breeding of Tie2-Cre mice and Dicer\textsuperscript{flon} mice, also showed embryonic lethality. Gauvrit et al. reported that the endothelial Dicer homozygous KO mice displayed impairment of blood and lymphatic vasculature separation, hemorrhage and edema from E12.5 to E14.5\textsuperscript{16}. These data highlighted that the Dicer-mediated miRNA processing in ECs is essential for normal embryonic development.

Here, we showed that the EC-specific heterozygous Dicer deletion did not alter BP in resting and 8 weeks of high salt-loaded conditions. On the other hand, a previous study demonstrated that the brown adipose tissue (BAT)-specific Dicer heterozygous KO mice exhibited reduced, levels of miR-193b and miR-378 were down-regulated approximately 50% in BAT. On the other hand, we showed that EC-enriched miR-126 was not decreased, whereas Dicer mRNA was significantly reduced in lung. It is possible that the remaining Dicer1 allele compensated for the heterozygous inactivation of Dicer-mediated miRNA processing. These results may suggest that there are tissue-specific differences in functional redundancy of Dicer-mediated miRNA biogenesis and functionality.

Previous studies demonstrated that a high salt diet (8% NaCl) did not affect blood pressure homeostasis in different strains of either male or female mice such as C57BL/6, 129 and NZW/LacJ\textsuperscript{23-27}. On the other hand, it has been shown that high salt containing water (2% NaCl) intake induced significant elevation of BP and HR two weeks after loading in C57BL/6 male mice\textsuperscript{20}. In the present study, when we adopted the high salt water loading (2% NaCl) intervention to our EC-specific heterozygous Dicer KO mice, we did not observe any changes in the hemodynamic parameters of WT C57BL/6 and EC-specific heterozygous Dicer KO female mice. These data may imply the existence of a gender difference in sensitivity to high salt loading-induced HR and BP elevation. Future study will focus on the mechanisms of such a difference.

In summary, we showed that the EC-specific Dicer homozygous KO mice were embryonically lethal and there was no obvious phenotype in BP homeostasis in the EC-specific Dicer heterozygous KO female mice. Our findings confirmed that an essential role of Dicer in normal embryonic development. However, the remaining Dicer1 allele might compensate for the phenotype of hemodynamics in EC-specific Dicer heterozygous KO mice. It has been reported that miRNAs are important regulators in cardiovascular diseases such as atherosclerosis and abdominal aortic aneurysms and potential therapeutic candidates regarding these diseases\textsuperscript{9,20}. Therefore, further studies are needed to investigate the role of endothelial miRNA-mediated post-transcriptional gene regulation in the maintenance of BP homeostasis.

**Conflict of Interests**

There is no conflict of interests regarding the publication of this article.

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