Upregulation of miR-140-5p uncouples mitochondria by targeting Bcl-xL in vascular smooth muscle cells in angiotensin II-induced hypertension

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ABSTRACT

Angiotensin II–induced vascular smooth muscle cell (VSMC) remodeling and dysfunction is a major contributor to the development of hypertension. In spite of the low content of mitochondria and their low contribution to bioenergetics in VSMCs, recent studies have suggested that mitochondria play an important role in the regulation of VSMC function. However, the role of mitochondria in angiotensin II–induced VSMC dysfunction remains unknown. Here, we found that angiotensin II decreased the expression of Bcl-2-like protein 1 (Bcl-xL), a newly identified protein in inhibition of uncoupled proton flux in mitochondria through interaction with the β-subunit of ATP synthase, and uncoupled mitochondria in VSMCs both in vivo and in vitro. Overexpression of Bcl-xL restored the mitochondrial and VSMC function in response to angiotensin II treatment in vitro, suggesting that angiotensin II uncouples mitochondria through downregulation of Bcl-xL. Mechanistically, angiotensin II increased the expression of miR-140-5p, which targeted and downregulated Bcl-xL in VSMCs. Inhibition of miR-140-5p using antagomir-140-5p in vivo attenuated mitochondrial uncoupling and hypertension in angiotensin II–treated mice. These results suggested that upregulation of miR-140-5p uncouples mitochondria by targeting Bcl-xL in VSMCs in angiotensin II–induced hypertension, and miR-140-5p and Bcl-xL are potential targets for treatment of vascular dysfunction.

Introduction

It was estimated that more than 1 billion people all over the world were hypertensive [1]. Hypertension is one of the leading risk factors for cardiovascular disease and other diseases, and there are about 9.4 million deaths from hypertension-related diseases each year [2]. The mechanisms underlying hypertension are multifactorial and include the hyperactivity of the renin-angiotensin system [3]. Angiotensin II (Ang II) is the major effector peptide of the system, resulting in vascular smooth muscle cell (VSMC) remodeling and dysfunction including VSMC hypertrophy, proliferation, oxidation, altered vascular smooth muscle tone, and impaired VSMC responses to vasoactive signals [3–5]. Thus, Ang II receptor blockers are widely used in the treatment of hypertension.

VSMC contraction and relaxation are mainly regulated by mechanical stimuli and circulating vasoactive signals [6–8]. During hypertension, the VSMCs’ responses to vasoactive signals, especially vasodilators, are impaired, which contributes to the development of hypertension [4]. Recent studies have shown that changes in VSMC metabolism are involved in the induction of VSMC dysfunction [9–11]. Different from striated muscle cells including skeletal muscle cells and cardiomyocytes, VSMCs largely rely on glycolysis-derived ATP other than mitochondrial oxidation-derived ATP to maintain their biological function [12]. It is estimated that only 30% of the ATP is produced from mitochondrial oxidation in VSMCs [13]. In spite of the low content of mitochondria (<5% volume) and their low contribution to bioenergetics in VSMCs, studies have shown that mitochondria are extensively involved in regulation of VSMC function [14,15]. Mitochondria are reported to be involved in regulation of various processes in VSMCs, including proliferation, migration,
contraction, and secretion, and mitochondrial dysfunction plays a common causal role in induction of vascular pathologies such as hypertension [14,16–18]. For example, VSMC-specific deletion of family with sequence similarity 3 gene family member A, a mitochondrial protein that enhances ATP production, attenuated Ang II–induced hypertension and cardiovascular hypertrophy [5]. These advances suggest that mitochondria play an important role in regulation of VSMC function in both physiological and pathological conditions.

The study aimed to test the effects of Ang II on mitochondrial function in VSMCs and the underlying mechanisms. We hypothesized that Ang II uncouples mitochondria and miRNAs are involved in regulation of the process. We found that Ang II induced VSMC mitochondrial uncoupling. Mechanistically, upregulation of miR-140-5p contributes to mitochondrial uncoupling through targeting Bcl-2-like protein 1 (Bcl-xL), a newly identified protein in inhibition of uncoupled proton flux in mitochondria through interaction with the β-subunit of ATP synthase [19,20], in VSMCs. These findings suggested that miR-140-5p and Bcl-xL are potential targets for treatment of vascular dysfunction.

**Methods**

**Animals**

Animal experiments were approved by Fourth Military Medical University Committee on Animal Care (No. 20,190,233). C57 mice (male, 8 weeks) were purchased from Animal Research Center of Fourth Military Medical University (China). Hypertension was induced by Ang II (T7040, Topscience) infusion (1 μg/kg/min, 4 weeks) with osmotic minipumps (Alzet model 2006, Direct Corp., Cupertino, CA, USA) as described previously [21]. Osmotic minipumps were implanted subcutaneously under 2% isoflurane anesthesia. Blood pressure was monitored using tail-cuff plethysmography (CODA, Kent Scientific) as previously described [21].

**Functional assessment of thoracic aorta**

Thoracic aorta function was assessed in vitro as described previously [21]. Briefly, mice were sacrificed and the thoracic aortas were excised and placed in ice-cold physiological saline solution (PSS). Thoracic aorta segments (1 mm) were mounted in a myograph system (DMT 610 M, Danish Myo Technology, Denmark), which was incubated in PSS continuously gassed with a mixture of 5% CO₂ and 95% O₂ at 37°C (pH 7.35–7.45). Aorta segments were precontracted with phenylephrine (PE, T17310, Topscience) (10 μM) and were evoked by cumulative sodium nitroprusside (SNP) (10⁻¹⁰ to 10⁻⁵ M) to induce vasodilation. Percentage of PE-induced contractile force was used to quantify vasodilation. Cumulative PE (10⁻⁹ to 10⁻⁵ M) was used to evoke vasoconstriction without PE pretreatment to assess vasoconstriction.

**VSMCs isolation and culture**

Primary VSMCs were isolated and cultured as described previously [22]. Briefly, the thoracic aortas were dissected, and the adhering periadventitial tissue and endothelium were removed. After incubation with Collagenase I solution (Sigma, 1 mg/ml) for 10 min, the aortas were minced into small pieces for second digestion with Collagenase I for 2 h. Isolated VSMCs were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin-glutamine. VSMC purity was determined by positive staining of smooth muscle-specific α-actin antibody (Santa). VSMCs between passages 2 (P2) to P5 were used for further experiments.

Polymerase chain reaction (PCR) Total RNA was extracted from either endothelium-denuded thoracic aortas or cultured VSMCs using RNA simple Total RNA Kit (TIANGEN Biotech; Beijing, China.) according to the manufacturer’s instruction. After quantification of total RNA, reverse transcription was performed using Prime Script RT Master Mix (cat. no. RR036A; Takara, Shiga, Japan). Quantitative real-time PCR was performed using Takara SYBR Premix Ex Taq II (cat. no. RR820A; Takara) according to the
manufacturer’s instruction. Data were normalized to the mRNA levels of actin or U6 and were analyzed by the $2^{-\Delta\Delta CT}$ method. The primers of mRNAs were synthesized by Ribobio Co. (China), and the sequences used are shown in Table 1.

Transfection

Transfection of miRNA mimics (50 nM), miRNA inhibitors (50 nM), and their negative controls (NC, 50 nM) were performed using Lipofectamine RNAiMAX (Invitrogen, USA) and Opti-MEM (Gibco, USA) according to the manufacturer’s instruction. After 6 h transfection in cells, the medium was changed to DMEM. The transfection efficiency was detected by qPCR at 60 h after transfection. All mimics and inhibitors were obtained from Guangzhou Ribobio Co., Ltd.

Adenovirus infection

Cultured VSMCs were infected with adenovirus carrying Bcl2l1 or green fluorescent protein (GFP) gene at a m.o.i. of 20 and experiments were performed after 60–72 hr in culture. GFP was used as NC. Adenovirus carrying Bcl2l1 gene was provided by Hanheng Biotechnology (Shanghai, China).

AntagomiRNA injection

AntagomiR-140-5p (miR30000151-4-5, Ribobio, China) and antagomiR-NC (miR3N0000001-4-5, Ribobio, China) were obtained from Ribobio Co. (Ribobio, China). AntagomiR-NC or antagomiR-140-5p were injected (0.8 mg/kg in 0.2 ml saline for each mouse) into tail vein every 4 days during Ang II treatment.

Predicting the miRNAs which target Bcl-xL

The potential miRNAs in regulation of Bcl2l1 were predicted by miRDB (http://www.mirdb.org/), and TargetScan (http://www.targetscan.org/). The predicted miRNAs were identified by taking the intersection of these two databases. Then the miRNAs were verified by qPCR.

Confocal imaging

An inverted confocal microscope (Zeiss LSM 800, 40×, 1.3 NA oil-immersion objective) was used. Cells or blood vessels were incubated with Tyrode’s solution. For measurement of mitochondrial membrane potential, TMRM (20 nM) was loaded at 37°C for 10 min followed by 3 times washing with Tyrode’s solution. To obtain TMRM signals, images were captured by excitation at 573 nm and emission collection at 600–650 nm. All experiments were performed at room temperature (22–24°C).

Luciferase reporter assay

293 T cells (1 × 10⁶) were seeded into 6-well plates. Then, cells were transfected with psiCHECK-2-Bcl2l1 3’-UTR/muta and miR-140-5p mimic or mimic-NC following the manufacturer’s instructions (Invitrogen, USA). Luciferase assays were performed with the dual-luciferase reporter assay system (Madison, USA) according to the manufacturer’s instructions. Luminescent signals were quantified by a luminometer (Glomax, Promega, Madison, WI, USA) 48 h after transfection.

Measurement of VSMC respiration

VSMCs were cultured in XF24 cell-culture microplates (Seahorse Bioscience). The cells were incubated in Tyrode’s solution. Bioenergetics analyses were performed in an XF24 Extracellular Flux Analyzer (Seahorse Bioscience) with the injection of oligomycin (1 μM), FCCP (1 μM), and rotenone
(1 μM) and antimycin A (1 μM) sequentially. Basal respiration, respiration for ATP production and maximal respiration were calculated following the manufacturer’s instruction.

**Western blot**
Proteins were extracted from either blood vessels or VSMCs. The levels of protein expression or phosphorylation were measured using Western blot as described previously [5]. The immunoblots were probed with anti-p-myosin light chain (#3671, Cell Signaling Technology), anti-MLC (#3672, Cell Signaling Technology), anti-Bcl-xL (#2764, Cell Signaling Technology) and anti-β-actin antibodies (#4970, Cell Signaling Technology) overnight at 4°C followed by incubation with the corresponding secondary antibodies at room temperature for 1 h. The blots were visualized with ECL-plus reagent.

**Statistical analysis**
All values are presented as mean ± SEM. Data were compared with one-way ANOVA or two-way ANOVA, with all ANOVA tests followed by an unpaired t-test, as appropriate. Bonferroni’s correction for multiple comparisons was used. In all statistical comparisons, p value of less than 0.05 was considered to be statistically significant.

**Results**
Ang II–induced VSMC mitochondrial dysfunction is a major contributor to the development of hypertension. However, the underlying mechanisms are poorly understood. The study aimed to test the effects of Ang II on mitochondrial bioenergetics in VSMCs and the underlying mechanisms. We hypothesized that Ang II uncouples mitochondria and miRNAs are involved in regulation of the process. We found that Ang II induced VSMC mitochondrial uncoupling through upregulation of miR-140-5p, which targeted Bcl-xL in VSMCs.

**Ang II decreased Bcl-xL expression and induced mitochondrial uncoupling in VSMCs in vivo**
Ang II infusion for 4 weeks showed no significant effects on body weight, but increased systolic blood pressure in mice (Figure 1a-b). To test the function of vascular smooth muscle, vascular relaxation, and constriction in response to SNP and PE were detected in isolated thoracic aortas ex vivo. Vascular relaxation in response to SNP was impaired, while vascular constriction in response to PE showed no significant differences in isolated thoracic aortas from Ang II-treated mice (Figure 1 C-D). Next, the VSMCs were isolated from both Vehicle- and Ang II-treated mice to test for changes in mitochondrial bioenergetics. Cellular ATP showed no differences between VSMCs isolated from Ang II-treated mice (Figure 1e). Ang II treatment increased the basal respiration and proton leak in VSMCs isolated from Ang II-treated mice (figure 1f-g). Meanwhile, Ang II treatment increased the mitochondrial membrane potential in VSMCs in isolated thoracic aortas (Figure 1h). These results suggested that Ang II uncoupled mitochondrial respiration in VSMCs. Expressions of genes involved in mitochondrial uncoupling were detected, including Slc25a4, Slc25a5, Bcl2l1, Ucp1, Ucp2 and Ucp3. Among these genes, Bcl2l1 (encoding Bcl-xL) was downregulated in isolated VSMCs from Ang II-treated mice (Figure 1i). In addition, the protein content of Bcl-xL was also decreased in in VSMCs isolated from Ang II-treated mice, suggesting that Bcl-xL may contribute to mitochondrial uncoupling in VSMCs.

**Ang II uncoupled mitochondria through downregulating Bcl-xL in VSMCs**
To test whether Ang II decreases Bcl-xL directly in VSMCs, isolated VSMCs were treated with Ang II in vitro. Ang II decreased Bcl-xL expression in a dose-dependent manner in VSMCs (Figure 2a). Bcl-xL was overexpressed using adenovirus in VSMCs to test whether Bcl-xL downregulation contributes to Ang II–
induced VSMC dysfunction (Figure 2b). Bcl-xL overexpression restored Bcl-xL content and mitochondrial membrane potential in VSMCs treated with Ang II (Figure 2b-c). Bcl-xL overexpression abolished the effects of Ang II on upregulation of mitochondrial respiration and proton leak in isolated VSMCs (Figure 2d-e). In addition, Bcl-xL overexpression restored the VSMC function as evidenced by the levels of MLC phosphorylation in response to SNP in Ang II-treated VSMCs (Figure 2f). These results suggested that Bcl-xL downregulation contributes to Ang II–induced mitochondrial uncoupling and dysfunction.

**Ang II increased miR-140-5p expression in VSMCs**

Recent studies have suggested that miRNAs, small (19–24 nucleotides), endogenous, non-coding RNA, play an important role in regulation of gene expression at the transcriptional or post-transcriptional level through binding to the complementary 3′-untranslated regions (3′-UTR) of their target mRNAs [23,24]. Next, we tested whether miRNA is involved in regulation of Bcl-xL expression in VSMCs. The potential miRNAs in regulation of Bcl-xL expression were predicted by bioinformatic analysis. A total of 29 and 57 miRNAs were...
predicted from the TargetScan and miRDB databases, respectively. Six miRNAs were shared with these two databases. Among these miRNAs, three miRNAs were significantly increased in isolated VSMCs from Ang II-treated mice (Figure 3a). Among these three upregulated miRNAs, only miR-140-5p mimic decreased Bcl2l1 transcription, suggesting that miR-140-5p is a potential regulator of Bcl2l1 transcription (Figure 3b). In fact, miR-140-5p inhibitor increased Bcl2l1 transcription and Bcl-xL expression, while miR-140-5p mimic decreased Bcl-xL expression in VSMCs (Figure 3c-d). The binding sites for miR-140-5p in the 3’-UTR of Bcl-xL were further confirmed by luciferase reporter assay (Figure 3e). In addition, we detected the level of pre-miR-140-5p in isolated VSMCs, and found that pre-miR-140-5p was increased in isolated VSMCs from Ang II-treated mice (Figure 3f). The circulating level of miR-140-5p in Ang II-treated mice showed no significance difference (Figure 3g), suggesting that miR-140-5p was upregulated locally in VSMCs. These results suggested that miR-140-5p may contribute to
Ang II decreased Bcl-xL through upregulation of miR-140-5p

Next, we tested whether miR-140-5p contributes to the downregulation of Bcl-xL in response to Ang II in VSMCs. As shown in Figure 4a, miR-140-5p mimic increased mitochondrial membrane potential in VSMCs. Inhibition of miR-140-5p using miR-140-5p inhibitor showed no significant effects on mitochondrial membrane potential in untreated VSMCs, but abolished the effects of Ang II on upregulation of mitochondrial membrane potential and downregulation of Bcl-xL expression in Ang II-treated VSMCs (Figure 4b-c). In addition, miR-140-5p inhibitor restored the VSMC function as evidenced by MLC phosphorylation in response to SNP in isolated VSMCs treated with Ang II (Figure 4d). These results suggested that Ang II decreases Bcl-xL expression through upregulation of miR-140-5p.

Inhibition of miR-140-5p improved VSMC function in vivo

Next, we tested whether inhibition of miR-140-5p improves vascular function in Ang II-treated mice in vivo. miR-140-5p was downregulated
using antagonmir-140-5p in vivo (Figure 5a). Both Ang II and antagonmiR-140-5p treatment showed no significant effects on body weight of the mice (Figure 5b). AntagomiR-140-5p treatment decreased the blood pressure in Ang II-treated mice (Figure 5c). In addition, it improved the vascular smooth muscle function as evidenced by the improved relaxation in response to SNP, while their response to PE showed no significant changes (Figure 5d-e). In addition, antagonmiR-140-5p treatment blocked the effects of Ang II on upregulation of mitochondrial membrane potential and downregulation of Bcl-xL in VSMCs from Ang II-treated mice (figure 5f-g). These results reinforced the notion that Ang II uncouples mitochondria in VSMCs through miR-140-5p-mediated Bcl-xL downregulation.

**Discussion**

In spite of the low content of mitochondria and their low contribution to bioenergetics in VSMCs, more and more evidence suggests that mitochondria play an important role in regulation of vascular smooth muscle function. Here, mitochondria uncoupling was observed in VSMCs from Ang II-treated mice, which contributes to the impaired response of VSMCs to vasodilators. Mechanistically, upregulation of miR-140-5p contributes to mitochondrial uncoupling through targeting Bcl-xL. These findings suggested that miR-140-5p and Bcl-xL are potential targets for treatment of hypertension.

Mitochondria are reported to be involved in regulation of VSMC proliferation, migration, contraction, and secretion, and mitochondrial dysfunction contributes to vascular pathologies such as hypertension [14,16–18]. Declining
mitochondrial function may lead to oxidative stress, impaired ATP production and dysregulated signaling [25,26]. As mitochondria use oxidative phosphorylation in oxygen replete conditions for energy generation, we measured the oxygen respiration of VSMCs as a marker of mitochondrial function in our study. Ang II treatment induced mitochondrial dysfunction as manifested by mitochondrial hyperpolarization and uncoupling. Ang II increased mitochondrial basal respiration and proton leak, leading to impaired efficient of mitochondrial ATP production. Restoration of mitochondrial coupling improved mitochondrial function in VSMCs. These results reinforced the notion that although mitochondria’s contribution to bioenergetics is low, they play an important role in regulation of VSMC function. Our work implies that novel therapeutics aimed at improving vascular mitochondrial bioenergetics in VSMCs may improve vascular function in hypertension.

Mitochondrial uncoupling is mediated by a series of proteins, including adenine nucleotide translocator, uncoupling proteins (UCPs) and Bcl-

Figure 5. Inhibition of miR-140-5p improved VSMC function in vivo. A. miR-140-5p was downregulated using antagomir-140-5p in vivo. B. Both angiotensin II (Ang II) and antagomir-140-5p treatment showed no significant effects on body weight of the mice. C. Antagomir-140-5p treatment decreased the blood pressure in Ang II-treated mice. D. Phenylephrine (PE)-induced vascular contraction in isolated thoracic aortas from antagomir-140-5p- and Ang II-treated mice. E. Sodium nitroprusside (SNP)-induced vascular relaxation in isolated thoracic aortas from antagomir-140-5p- and Ang II-treated mice. F. Antagomir-140-5p treatment blocked the effects of Ang II on upregulation of mitochondrial membrane potential in vascular smooth muscle cells (VSMCs) from Ang II-treated mice. G. Antagomir-140-5p treatment blocked the effects of Ang II on downregulation of Bcl-2-like protein 1 (Bcl-xL) expression in VSMCs from Ang II-treated mice. n = 6. **, P < 0.01.
xL. Among these proteins, UCPs have been well studied [27]. It has been shown that overexpression of UCP2 protected VSMCs against hypertension and other cardiovascular diseases through upregulation of mitochondrial uncoupling [28,29]. Mitochondrial uncoupling plays an important role in regulation of cell function, which decreases mitochondrial ROS generation and metabolic stress [30]. Here, we found that Ang II treatment increased mitochondrial membrane potential and mitochondrial uncoupling in VSMCs, which was associated with the downregulation of Bcl-xL. Bcl-xL, a member of the Bcl-2 family of proteins, is a newly identified protein in inhibition of uncoupled proton flux in mitochondria through interaction with the β-subunit of ATP synthase [19,20]. The mitochondrial F1F0 ATP synthase is one of the most abundant proteins of the mitochondrial inner membrane, which catalyzes the final step of oxidative phosphorylation to synthesize ATP from ADP and Pi. ATP synthase uses the electrochemical gradient of protons across the mitochondrial inner membrane to synthesize ATP. Bcl-xL is bound directly to the ATP synthase β subunit, where it increases ATP synthase activity rate and reduces inner mitochondrial membrane leak, enhancing the coupling and efficiency of oxidative phosphorylation [19,20,31]. Overexpression of Bcl-xL abolished the effects of Ang II on induction of mitochondrial uncoupling and cell dysfunction in VSMCs. These results suggested that Bcl-xL deficiency contributes to VSMC dysfunction in response to Ang II.

Recent studies have shown that miRNAs play an important role in many biological processes including development, cell proliferation and differentiation, apoptosis, and immune regulation by negatively regulating the expression of target genes in cells [32]. In addition, miRNAs are also involved in the occurrence and development of various cardiovascular diseases, including hypertension, coronary heart disease, myocardial infarction, and heart failure, and can therefore be used as potential therapeutic strategies [33–35]. Thus, we tested whether miRNAs were involved in regulation of Bcl-xL expression in the study. miR-140-5p was identified as an upstream regulator of Bcl-xL in VSMCs in response to Ang II treatment using bioinformatic analysis combined with other evidence. Ang II increased the expression of miR-140-5p, and inhibition of miR-140-5p abolished the effects of Ang II on downregulation of Bcl-xL expression and mitochondrial uncoupling. In addition, inhibition of miR-140-5p attenuated the hypertension and vascular smooth muscle dysfunction in Ang II-treated mice in vivo, suggesting that miR-140-5p is a potential target for treatment of vascular dysfunction. miR-140-5p was reported to be involved in regulation of cartilage matrix remodeling [36], cancer metastasis [37–39], odontoblastic differentiation [40] and others. The regulation of Bcl-xL expression by miR-140-5p has also been reported previously [41]. Our study extended these findings that miR-140-5p is also involved in regulation of mitochondrial uncoupling.

**Conclusion**

We found that upregulation of miRNA-140-5p uncouples mitochondria by targeting Bcl-xL in VSMC from Ang II-treated mice. Inhibition of miRNA-140-5p improves mitochondrial bioenergetics in VSMCs and attenuates hypertension in Ang II-treated mice. These findings suggested that mitochondrial bioenergetics plays an important role in regulation of VSMC function, and miR-140-5p and Bcl-xL are potential targets in treatment of VSMC dysfunction.

**Highlights**

- Angiotensin II uncouples mitochondria through decreasing Bcl-xL in VSMCs
- Overexpression of Bcl-xL restores VSMC function in response to angiotensin II
- Angiotensin II downregulates Bcl-xL through increasing miR-140-5p
- miR-140-5p and Bcl-xL are potential targets for treatment of vascular dysfunction

**Disclosure statement**

No potential conflict of interest was reported by the author(s).
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**Ethics approval**

Animal experiments were approved by Fourth Military Medical University Committee on Animal Care.

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