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MicroRNA-200a improves diabetic endothelial dysfunction by targeting KEAP1/NRF2

Ziping Jiang¹, Junduo Wu², Fuzhe Ma³, Jun Jiang⁴, Linlin Xu⁵, Lei Du⁶, Wenlin Huang⁷, Zhaohui Wang⁸, Ye Jia⁹, Laijin Lu¹ and Hao Wu⁶

¹Department of Hand and Foot Surgery, The First Hospital of Jilin University, Changchun, Jilin, China

²Department of Cardiology, The Second Hospital of Jilin University, Changchun, Jilin, China

³Department of Nephrology, The First Hospital of Jilin University, Changchun, Jilin, China

⁴Department of Neurosurgery, The Second Hospital of Shandong University, Jinan, Shandong, China

⁵Department of Neurology, The Second Hospital of Shandong University, Jinan, Shandong, China

⁶Department of Nutrition and Food Hygiene, School of Public Health, Shandong University, Jinan, Shandong, China

⁷School of Science and Technology, Georgia Gwinnett College, Lawrenceville, Georgia, USA

⁸Department of Acupuncture and Tuina, Changchun University of Chinese Medicine, Changchun, Jilin, China

⁹Department of Diabetes Complications and Metabolism, Diabetes Metabolism Research Institute, Beckman Research Institute of City of Hope, Duarte, California, USA

Correspondence should be addressed to L Lu or H Wu: ljlu@jlu.edu.cn or hwu@sdu.edu.cn

Abstract

Over a half of the diabetic individuals develop macrovascular complications that cause high mortality. Oxidative stress (OS) promotes endothelial dysfunction (ED) which is a critical early step toward diabetic macrovascular complications. Nuclear factor erythroid 2-related factor 2 (NRF2) is a master regulator of cellular antioxidant defense system and combats diabetes-induced OS. Previously, we found that impaired NRF2 antioxidant signaling contributed to diabetes-induced endothelial OS and dysfunction in mice. The present study has investigated the effect of microRNA-200a (miR-200a) on NRF2 signaling and diabetic ED. In aortic endothelial cells (ECs) isolated from C57BL/6 wild-type (WT) mice, high glucose (HG) reduced miR-200a levels and increased the expression of kelch-like ECH-associated protein 1 (*Keap1*) – a target of miR-200a and a negative regulator of NRF2. This led to the inactivation of NRF2 signaling and exacerbation of OS and inflammation. miR-200a mimic (miR-200a-M) or inhibitor modulated KEAP1/NRF2 antioxidant signaling and manipulated OS and inflammation under HG conditions. These effects were completely abolished by knockdown of *Keap1*, indicating that *Keap1* mRNA is a major target of miR-200a. Moreover, the protective effect of miR-200a-M was completely abrogated in aortic ECs isolated from C57BL/6 *Nrf2* knockout (KO) mice, demonstrating that NRF2 is required for miR-200a's actions.

In vivo, miR-200a-M inhibited aortic *Keap1* expression, activated NRF2 signaling, and attenuated hyperglycemia-induced OS, inflammation and ED in the WT, but not *Nrf2* KO, mice. Therefore, the present study has uncovered miR-200a/KEAP1/NRF2 signaling that controls aortic endothelial antioxidant capacity, which protects against diabetic ED.

Key Words

- ▶ aorta
- ▶ diabetes
- ▶ endothelial dysfunction
- ▶ miR-200a
- ▶ NRF2

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Introduction

Macrovascular diseases develop in over a half of the diabetic individuals, posing a severe threat to public health (Forbes *et al.* 2004). Endothelial dysfunction (ED) is a critical early event in the pathogenesis of diabetic vascular complications (Sena *et al.* 2013, Sharma *et al.* 2017). Thus, it is essential to improve ED, the benefit from which may prevent or slow-down the progression of diabetic macrovascular complications.

Evidence has shown that hyperglycemia promotes oxidative stress (OS) (Giacco & Brownlee 2010) which contributes to ED (Ceriello *et al.* 2013). Under hyperglycemia, an aberrant production of reactive oxygen species (ROS) exceeds the scavenging capacity of the antioxidant defense system, enhancing OS (Betteridge 2000, Incalza *et al.* 2018). This has brought to light the activation of the antioxidant defense system as a viable strategy to improve diabetic ED (Laight *et al.* 2000).

Nuclear factor erythroid 2-related factor 2 (NRF2) is a master regulator of the cellular antioxidant defense system (Kaspar *et al.* 2009, Zheng *et al.* 2011). In the nucleus, NRF2 activates the transcription of a series of genes, such as NAD(P)H dehydrogenase quinone 1 (*Nqo1*) and heme oxygenase 1 (*Ho1*) (Ruiz *et al.* 2013), the protein products of which function as antioxidants that scavenge upon excessive diabetes mellitus (DM)-generated free radicals. In the cytoplasm, NRF2 is sequestered by kelch-like ECH-associated protein 1 (KEAP1) which restricts NRF2 from nuclear translocation and facilitates proteasomal degradation of NRF2 (Zhang & Hannink 2003). Thus, inhibition of KEAP1 may offer protection against diabetic ED by activation of the NRF2 antioxidant signaling.

In recent years, NRF2 activation has been achieved by small molecule-induced structural inactivation of KEAP1 protein function. However, little attention has been paid to inhibition of *Keap1* gene expression, which may be an effective strategy to activate NRF2. Notably, inhibition of *Keap1* gene expression has not been previously studied for its effect on diabetic ED, encouraging the investigation of this effect in the present study.

MicroRNAs (miRNAs) are short non-coding RNAs that act as suppressors of protein expression by inducing translational arrest or degradation of mRNAs, affecting almost every key cellular function (Fabian *et al.* 2010). MicroRNA-200a (miR-200a) was primarily reported to target and degrade *Keap1* mRNA in breast cancer (Eades *et al.* 2011). We and others have found that miR-200a is upstream of KEAP1/NRF2 signaling in animal models of diabetic nephropathy (Wei *et al.* 2014, Wu *et al.* 2016).

In particular, specific inhibition of miR-200a increased renal *Keap1* mRNA and protein expression, which in turn blunted renal NRF2 antioxidant signaling and exacerbated diabetic nephropathy in mice (Wei *et al.* 2014, Wu *et al.* 2016). These findings urge the investigation of the expression and action of miR-200a/KEAP1/NRF2 signaling in the diabetic endothelium, as well as the impact of miR-200a supplementation on ED.

In the present study, we first explored the effect of high glucose (HG) on aortic endothelial cells (ECs), showing that HG altered miR-200a/KEAP1/NRF2 signaling, and enhanced OS and inflammation in a time-dependent manner. Moreover, the role of miR-200a in controlling the expression and function of the KEAP1/NRF2 signaling in ECs was confirmed by using specific mimic and inhibitor of miR-200a. As miR-200a may target multiple mRNAs, the role of KEAP1 in mediating miR-200a's actions in ECs was explored by using a siRNA (siRNA) for *Keap1* mRNA, in the presence of miR-200a mimic (miR-200a-M). Further, by using ECs isolated from C57BL/6 wild-type (WT) and *Nrf2* gene-knockout (KO) mice, NRF2 was investigated for its requirement for miR-200a's effects on HG-induced OS and inflammation. Finally, to assess the role of miR-200a/KEAP1/NRF2 signaling in diabetic ED, WT and *Nrf2* KO diabetic mice were treated with miR-200a-M.

Materials and methods

Cell culture and experiments

ECs were isolated from the aortas of 8-week-old C57BL/6 WT or *Nrf2* KO male mice and were cultured under normal glucose (NG, 1 g/L) conditions, as described in our previous studies (Wu *et al.* 2018a,b).

To observe the time-dependent impact of HG on the expression of miR-200a/KEAP1/NRF2 signaling, OS and inflammation, ECs isolated from the WT mice (WT ECs) were exposed to HG (4.5 g/L) or mannitol (3.64 g/L), for 1, 6, 12, 24 and 48 h. To study the role of miR-200a in controlling KEAP1/NRF2, OS and inflammation under HG conditions, WT ECs were challenged by HG with miR-200a-M (30 nM, Thermo Fisher Scientific) or a specific inhibitor of miR-200a (miR-200a-I, 30 nM, Thermo Fisher Scientific), for 48 h. To verify the role of KEAP1 in mediating miR-200a's action, WT ECs were co-treated with HG and miR-200a-M, with *Keap1* silenced using an siRNA (100 nM, GenePharma, Suzhou, China), for 48 h. The *Keap1*-siRNA was introduced into ECs using the transfection reagent

Lipofectamine® 2000 (Thermo Fisher Scientific). To investigate the requirement of NRF2 in miR-200a's action, ECs isolated from the *Nrf2* KO mice (KO ECs) were treated with HG and miR-200a-M (30 nM), for 48 h. The doses of miR-200a-M, miR-200a-I and *Keap1*-siRNA used were based on our preliminary work and were similar to those previously reported (Kim *et al.* 2012, Wang *et al.* 2014, Barwari *et al.* 2018).

Analysis of ROS and lipid peroxides

Cellular ROS and malondialdehyde (MDA) levels were measured using assay kits from Sigma-Aldrich, following the manufacturer's instructions.

Analysis of nitric oxide production

In order to evaluate endothelial function, the generation of NO was measured using 3-amino,4-aminomethyl-2',7'-difluorescein diacetate (DAF-FM DA, Sigma-Aldrich) as previously described (Yang *et al.* 2015).

Animal housing and experiments

C57BL/6 WT (*Nrf2*^{+/+}) and *Nrf2* KO (*Nrf2*^{-/-}) mice were obtained through breeding of heterozygotes (*Nrf2*^{+/-}) (Zheng *et al.* 2011, Sun *et al.* 2017). The mice were housed in the Animal Center of Jilin University at 22°C, on a 12:12-h light-dark cycle, with free access to rodent feed and tap water. The Institutional Animal Care and Use Committee at Jilin University approved all the experimental procedures which meets the UK Animals (Scientific Procedures) Act, 1986 and associated guidelines.

Eight-week-old male WT and *Nrf2* KO mice were injected intraperitoneally with sodium citrate or streptozotocin (STZ, 50 mg/kg/day, dissolved in 0.1 M sodium citrate, pH 4.5; Sigma-Aldrich) once every day, for 5 consecutive days (Zheng *et al.* 2011, Kato *et al.* 2016, Wu *et al.* 2016). Fasting glucose levels (4-h fast) were determined 1 week after the last injection. Mice with fasting glucose levels above 13.89 mM were considered diabetic. Blood glucose levels were recorded 4, 8, 12, 16 and 20 weeks post DM onset.

The WT and *Nrf2* KO diabetic mice received s.c. injections of miR-200a-M or its negative control (both at 1 mg/kg, 3 times weekly; Thermo Fisher) immediately after DM was confirmed, for a total period of 20 weeks. At the end of the procedures, the mice were killed under anesthesia by intraperitoneal injection of chloral hydrate

(0.3 mg/kg) (Pan *et al.* 2017), with the aortas harvested for analysis.

Assessment of aortic ED

To assess aortic ED, contractility in response to phenylephrine (PE) and relaxation in response to acetylcholine (ACh) were determined using thoracic aortas, as previously described (Peng *et al.* 2015, Wu *et al.* 2018a). The doses of PE and ACh were 10⁻⁹, 10⁻⁸, 10⁻⁷, 10⁻⁶, 10⁻⁵ and 10⁻⁴ M.

Analysis of aortic morphology

The freshly harvested thoracic aortas were immediately fixed into 10% buffered formalin solution, embedded in paraffin, and sectioned into 5-μm-thick sections onto glass slides. Morphology of the aortas was evaluated using hematoxylin and eosin (H&E) staining. Selection of areas to photograph was done by people blind to the identity of the samples.

Immunohistochemical (IHC) staining

IHC staining was performed as previously described (Wu *et al.* 2015), using primary antibodies against KEAP1 (1:100; Santa Cruz Biotechnology), NRF2 (1:100; Santa Cruz Biotechnology) and 4-hydroxynonenal (4-HNE, 1:100; Alpha Diagnostic Int., San Antonio, TX, USA). Positive area of 4-HNE was quantified within the full-thickness of the artery wall. Selection of areas to photograph and scoring was done by people blind to the identity of the samples.

Quantitative real-time PCR (qPCR)

qPCR was performed using aortic tissue and lysate of ECs as previously described (Deshpande *et al.* 2013, Wu *et al.* 2015). For miR-200a, *U6* was used as an endogenous control, and *Gapdh* for the others. The primers for *Gapdh*, *Ho1*, inducible nitric oxide synthase (*iNos*), *Keap1*, *miR-200a*, monocyte chemoattractant protein 1 (*Mcp-1*), *Nqo1*, *U6* and *Vcam-1* were provided by Thermo Fisher Scientific.

Western blot

Western blot analysis was performed as previously described (Wu *et al.* 2015, 2018a, Yaribeygi *et al.* 2018),

using cell lysates and aortic tissue, with primary antibodies against GAPDH (1:3000; Santa Cruz Biotechnology), KEAP1 (1:1000; Santa Cruz Biotechnology), NRF2 (1:1000; Santa Cruz Biotechnology) and histone H3 (1:1000; Santa Cruz Biotechnology). Histone H3 was used as an endogenous control for nuclear NRF2 (n-NRF2), and GAPDH for the others.

Isolation of nuclei

In order to determine the level of n-NRF2, nuclei were isolated from ECs using a nuclear and cytoplasmic extraction kit from Thermo Fisher Scientific, following the manufacturer's instructions.

Statistical analysis

Cell experiments were performed in triplicate. Seven mice per group were studied. Western blot images were analyzed using Image Studio Lite (LI-COR Biosciences, Lincoln, NE, USA). IHC-positive area was quantified using Image Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA). One-way ANOVA was performed for the comparisons among the WT or KO groups. Two-way ANOVA was used for the comparisons among the WT

and KO groups. The measurements for each group were summarized as means $\pm$ s.d. A test is significant if $P < 0.05$.

Results

HG altered endothelial miR-200a/KEAP1/NRF2 signaling, enhancing OS and inflammation in a time-dependent manner

To investigate the expression of miR-200a, *Keap1* and NRF2 antioxidant signaling, and the levels of OS and inflammation in response to a time series of HG incubation, ECs were exposed to HG for 1 h, 6 h, 12 h, 24 h and 48 h. HG time-dependently decreased miR-200a levels (Fig. 1A), and enhanced *Keap1* mRNA (Fig. 1B) and protein (Fig. 1C) expression. This decreased whole cellular NRF2 (total NRF2, t-NRF2) at 24 h and 48 h (Fig. 1D). Different from the pattern of t-NRF2 expression, HG incubation increased n-NRF2 at 6 h and 12 h, but decreased n-NRF2 thereafter, ending with a drastic decrease in n-NRF2 level at 48 h (Fig. 1E). The mRNA levels of the NRF2 downstream antioxidant genes *Nqo1* and *Ho1* were elevated by HG at 12 h and 24 h and were downregulated by HG at 48 h. The levels of ROS and MDA, and the mRNA levels of

Figure 1

HG altered endothelial miR-200a/KEAP1/NRF2 signaling, enhancing OS and inflammation in a time-dependent manner. ECs isolated from C57BL/6 WT mice were cultured under NG conditions and were treated with mannitol or HG for 1 h, 6 h, 12 h, 24 h and 48 h. The levels of (A) miR-200a, *Keap1* (B) mRNA and (C) protein, (D) t-NRF2, (E) n-NRF2, and (F) the mRNA expression of *Nqo1* and *Ho1* were determined by qPCR or Western blot. (G) The mRNA levels of the pro-inflammatory genes *Vcam-1* and *Mcp-1* were measured by qPCR. (H) ROS and MDA levels were determined using commercial kits to assess OS. (H) The relative levels of these indices were summarized as a heatmap. The data was normalized to the NG group and presented as means $\pm$ s.d. ($n = 3$). * $P < 0.05$ vs NG. † $P < 0.05$ vs HG 24 h. H3, histone H3; HG, high glucose; *Ho1*, heme oxygenase 1; *Keap1*, kelch-like ECH-associated protein 1; M, mannitol; miR-200a, microRNA-200a; *Mcp-1*, monocyte chemoattractant protein 1; MDA, malondialdehyde; NG, normal glucose; NRF2, nuclear factor erythroid 2-related factor 2; n-NRF2, nuclear NRF2; *Nqo1*, NAD(P)H dehydrogenase quinone 1; OS, oxidative stress; qPCR, quantitative real-time PCR; ROS, reactive oxygen species; t-NRF2, total/whole cellular NRF2; *Vcam-1*, vascular cell adhesion molecule-1; WT, wild-type.

Vcam-1 and *Mcp-1* were increased by HG incubation for 24 and 48 h (Fig. 1G). Forty-eight-hour HG incubation dramatically enhanced OS and inflammation (Fig. 1G). This coincided with the HG-enhanced *Keap1* gene expression (Fig. 1B and C) and the HG-induced inhibition of miR-200a and NRF2 antioxidant system at 48 h (Fig. 1D, E and F), suggesting opposing effects of miR-200a and KEAP1 in the control of NRF2 antioxidant signaling, and in the modulation of OS and inflammation under HG conditions (Fig. 1H).

miR-200a controlled the KEAP1/NRF2 antioxidant system, combating HG-induced OS and inflammation

We aimed to verify whether miR-200a controls the endothelial KEAP1/NRF2 antioxidant system and whether miR-200a is capable of diminishing oxidative and inflammation in ECs under 48-h incubation with HG. miR-200a-M supplementation increased miR-200a levels (Fig. 2A) and blunted the HG-induced expression of *Keap1* (Fig. 2B and C). This elevated the levels of t-NRF2 (Fig. 2D), n-NRF2 (Fig. 2E), and the mRNA expression of *Nqo1* and *Ho1* (Fig. 2F), alleviating the HG-induced OS and inflammation (Fig. 2G). On the contrary, inhibition of miR-200a by miR-200a-I (Fig. 2A) further increased *Keap1* mRNA and protein levels compared with NC-I under HG

conditions (Fig. 2B and C), resulting in a further decrease in the levels of t-NRF2 (Fig. 2D), n-NRF2 (Fig. 2E), and mRNA levels of *Nqo1* and *Ho1* (Fig. 2F). Moreover, miR-200a-I exacerbated the HG-induced endothelial OS and inflammation (Fig. 2G). These results demonstrate that alteration of miR-200a levels remarkably changed the expression of KEAP1/NRF2 antioxidant system and the status of OS and inflammation under HG conditions, suggesting that miR-200a governs endothelial antioxidant activity that combats endothelial OS and inflammation.

KEAP1 predominantly mediated miR-200a's actions in ECs under HG conditions

Because miR-200a may target several mRNAs that might contribute to miR-200a's actions upon HG-induced endothelial injury, we investigated the role of KEAP1 in mediating miR-200a's actions in HG-treated ECs by using *Keap1*-siRNA, in the presence of miR-200a-M. The *Keap1*-siRNA effectively reduced *Keap1* mRNA and protein levels (Fig. 3A and B). miR-200a-M did not further decrease *Keap1* expression (Fig. 3A and B) despite its remarkable elevation of miR-200a (Fig. 3C). In the presence of the *Keap1*-siRNA, miR-200a-M did not further enhance the expression of NRF2 antioxidant signaling (Fig. 3D, E and F), nor did it further inhibit OS and inflammation (Fig. 3G).

Figure 2

miR-200a controlled the KEAP1/NRF2 antioxidant system, combating HG-induced OS and inflammation. ECs isolated from WT mice were treated with miR-200a-M, miR-200a-I and their negative controls respectively, in the presence of HG for 48 h. The expression of the miR-200a/KEAP1/NRF2 signaling, including (A) miR-200a, (B) *Keap1* mRNA and (C) protein, (D) t-NRF2, (E) n-NRF2, and (F) the mRNA levels of *Nqo1* and *Ho1* were determined. Additionally, (G) ROS and MDA levels, and mRNA levels of *Vcam-1* and *Mcp-1* were measured to assess OS and inflammation. The data were normalized to the NG group and presented as means $\pm$ s.d. ($n = 3$). * $P < 0.05$ vs NG. † $P < 0.05$ vs HG + NC-M, ‡ $P < 0.05$ vs. HG + NC-I. miR-200a-I, miR-200a inhibitor; miR-200a-M, miR-200a mimic; NC-I, negative control of miR-200a-I; NC-M, negative control of miR-200a-M. Other abbreviations are the same as Fig. 1.

Figure 3

KEAP1 predominantly mediated miR-200a's actions in ECs under HG conditions. To investigate the role of KEAP1 in mediating miR-200a's efficacies, HG-treated ECs that were isolated from WT mice were co-treated with *Keap1*-siRNA, in the presence of miR-200a-M or NC-M, for 48 h. The levels of *Keap1* (A) mRNA and (B) protein, (C) miR-200a, (D) t-NRF2, (E) n-NRF2 were measured. The (F) mRNA levels of *Nqo1* and *Ho1*, (G) levels of ROS and MDA, as well as mRNA levels of *Vcam-1* and *Mcp-1* were determined. The data were normalized to the NG group and presented as means $\pm$ s.d. ($n = 3$). * $P < 0.05$ vs NG. † $P < 0.05$ vs HG + NC-siRNA. NC-siRNA, negative control siRNA for *Keap1*; siRNA, small interfering RNA. Other abbreviations are the same as Figs 1 and 2.

These findings indicate that KEAP1 is a critical target of miR-200a and predominantly mediates miR-200a's effects on endothelial antioxidant activity, OS and inflammation under HG conditions.

NRF2 was required for miR-200a to attenuate HG-induced endothelial injury, and increase NO production

To address whether or not NRF2 plays a role in miR-200a's actions upon HG, ECs isolated from WT and *Nrf2* KO mice were subjected to HG for 48 h, in the presence of miR-200a-M. miR-200a-M increased miR-200a levels (Fig. 4A), decreased *Keap1* mRNA (Fig. 4B) and protein (Fig. 4C) levels in both the WT and *Nrf2* KO ECs. NRF2 protein was undetectable in the *Nrf2* KO ECs (Fig. 4D), confirming the deletion of the *Nrf2* gene in these mice. In the absence of NRF2, miR-200a-M failed to elevate *Nqo1* and *Ho1* mRNA levels (Fig. 4E) and was unable to restrain OS and inflammation (Fig. 4F), the effects of which were in contrast to those in the WT ECs. HG generated less NO in the *Nrf2* KO ECs compared with the WT ECs (Fig. 4G). Moreover, miR-200a supplementation was able to increase NO generation in the WT, but not *Nrf2* KO, ECs. These findings demonstrate that miR-200a's anti-oxidative and anti-inflammatory activities are dependent on NRF2,

and miR-200a drives endothelial NO production through activation of NRF2.

miR-200a supplementation manipulated the aortic KEAP1/NRF2 antioxidant system under hyperglycemic conditions

To further explore the effect of miR-200a supplementation on the aortic KEAP1/NRF2 antioxidant system under hyperglycemic conditions in the presence or absence of NRF2, STZ-induced WT and *Nrf2* KO diabetic mice were treated with miR-200a-M for 20 weeks. The diabetic mice developed higher blood glucose levels compared with the non-diabetic control mice (Fig. 5A). miR-200a-M and its negative control did not have a significant impact on blood glucose levels (Fig. 5A). H&E staining showed a mild derangement of smooth muscle cells and thickening of tunica media in both the WT and *Nrf2* KO diabetic mice (Fig. 5B). Hyperglycemia resulted in reduction of miR-200a and elevation of *Keap1* mRNA levels in the aortas of the WT and *Nrf2* KO mice, the effects of which were reversed by miR-200a supplementation (Fig. 5C and D). Evident KEAP1 protein expression was observed in the aortas of the WT and *Nrf2* KO diabetic mice, as shown by IHC staining (Fig. 5E) and Western blot (Fig. 5F). miR-200a-M decreased aortic KEAP1 protein in both WT

Figure 4

NRF2 was required for miR-200a's capabilities to attenuate HG-induced endothelial injury and increase NO production. ECs isolated from either C57BL/6 WT or *Nrf2* KO mice were challenged with HG, in the presence of miR-200a-M or NC-M, for 48 h. The expression of (A) miR-200a, (B) *Keap1* mRNA, (C) KEAP1 protein, (D) t-NRF2, (E) *Nqo1* mRNA and *Ho1* mRNA were determined. OS and inflammation were assessed by measuring the levels of (F) ROS and MDA, as well as the mRNA levels of *Vcam-1* and *Mcp-1*. (G) NO production was measured to assess endothelial function. The data were normalized to the WT HG group and presented as means $\pm$ s.d. ($n = 3$). * $P < 0.05$ vs WT HG + NC-M. † $P < 0.05$ vs KO HG + NC-M. KO, knockout; NO, nitric oxide. Other abbreviations are the same as Figs 1 and 2.

and *Nrf2* KO diabetic mice (Fig. 5E and F). Moreover, miR-200a elevated NRF2 protein levels (Fig. 5G and H), and increased *Nqo1* and *Ho1* mRNA levels (Fig. 5I and J) in the aortas of the WT diabetic mice, the effects of which were completely abrogated in the *Nrf2* KO diabetic mice (Fig. 5G, H, I and J).

miR-200a-M improved hyperglycemia-induced aortic OS, inflammation and ED through activation of NRF2

We investigated the effect of miR-200a-M on hyperglycemia-induced aortic OS, inflammation and ED. In WT diabetic mice, miR-200a-M reduced aortic *iNos* mRNA levels (Fig. 6A), positive stain of 4-HNE (Fig. 6B), and mRNA levels of *Vcam-1* and *Mcp-1* (Fig. 6C and D). Additionally, miR-200a-M alleviated the hyperglycemia-provoked aortic contraction in response to PE at doses of 10^{-7} , 10^{-6} , 10^{-5} and 10^{-4} M (Fig. 6E, left panel) and improved the hyperglycemia-impaired aortic relaxation in response to ACh at 10^{-7} , 10^{-6} , and 10^{-4} M in the WT mice (Fig. 6F, left panel). These effects of miR-200a-M were completely abolished in the *Nrf2* KO diabetic mice (Fig. 6A, B, C and D; right panels of E, F). These results demonstrate that miR-200a protects against hyperglycemia-induced aortic OS, inflammation and ED via activation of NRF2.

Discussion

As ED contributes to the pathogenesis of diabetic macrovascular complications, the present study has researched the role of miR-200a/KEAP1/NRF2 in the regulation of diabetic ED. The present study has found that (1) aortic endothelial miR-200a expression was suppressed under HG/hyperglycemic conditions, leading to overexpression of KEAP1 and inactivation of NRF2 antioxidant signaling; (2) inhibition and miR-200a supplementation modulated the expression and function of KEAP/NRF2 under HG conditions, and altered HG-induced endothelial OS and inflammation, suggesting that miR-200a is a key upstream regulator of KEAP1/NRF2 signaling that combats HG-induced endothelial injury; (3) by silencing *Keap1* mRNA and using *Nrf2* KO mice, KEAP1 and NRF2 were shown to predominantly mediate miR-200a's actions; (4) *in vivo* delivery of miR-200a attenuated hyperglycemia-induced aortic OS, inflammation and ED through activation of NRF2.

The most innovative finding of the present study is the identification of miR-200a in controlling hyperglycemia-induced ED. Mechanistically, this effect was through miR-200a-induced manipulation of the KEAP1/NRF2 antioxidant signaling, since miR-200a's actions were

almost completely abolished when *Keap1* mRNA was knocked down or when the *Nrf2* gene was deleted (Figs 3, 4, 5 and 6). These findings demonstrate that the miR-200a/KEAP1/NRF2 pathway controls endothelial antioxidant activity under HG/hyperglycemic conditions and regulates endothelial function, providing miR-200a and *Keap1* mRNA as novel targets for the intervention of diabetic macrovascular diseases. Previously, we and others have reported that renal miR-200a was decreased under diabetic conditions, resulting in increased *Keap1* expression and blunted NRF2 antioxidant signaling. Despite these prior work on diabetic nephropathy, the role of miR-200a/KEAP1/NRF2 pathway in diabetes-induced endothelial dysfunction is unknown. Because endothelial dysfunction is a critical pathophysiological event prior to diabetic macrovascular complications, in the present study, we have characterized an important role of this pathway during diabetes-induced endothelial dysfunction. In support of our findings, a recent study showed that inhibition of histone deacetylase 3 prevented blood-brain barrier permeability in a mouse

model of type 2 DM, at least in part attributed to miR-200a/KEAP1/NRF2 pathway (Zhao *et al.* 2019). However, different from Zhao's study, our work has demonstrated a central role of the miR-200a/KEAP1/NRF2 pathway in control of diabetes-induced aortic endothelial dysfunction, shedding light upon the intervention of diabetes-induced macrovascular diseases, such as atherosclerosis. Given the protective effects of miR-200a on multiple organs/tissues under diabetes, such as the aorta, kidney and brain, miR-200a supplementation might produce benefits in the prevention of several complications of diabetes. This should urge further study of this pathway in diabetes and its various complications.

NRF2 plays a crucial role in protection against diabetic ED. Previously, we found that *Nrf2* gene deletion exacerbated DM-induced aortic endothelial OS, inflammation and dysfunction, demonstrating the requirement of NRF2 in self-protection against DM-induced endothelial injury (Wu *et al.* 2018b). In our previous (Wu *et al.* 2018b) and present work (Figs 1D, E, F and 5G, H, I, J), impaired NRF2 antioxidant signaling was

Figure 6

miR-200a-M improved hyperglycemia-induced aortic OS, inflammation and ED through activation of NRF2. To assess OS in the aortas of the WT and *Nrf2* KO mice, (A) *iNos* mRNA levels were measured by qPCR, and (B) 4-HNE were detected by IHC staining, with positively stained area quantified. To evaluate aortic inflammation, (C) *Vcam-1* and (D) *Mcp-1* mRNA levels were determined by qPCR. To analyze ED, vascular (E) contraction in response to PE, and (F) relaxation in response to ACh were determined using aortic rings from the WT and *Nrf2* KO mice. For (A-D), the data were normalized to WT Ctrl. All the data were presented as means $\pm$ s.d. ($n = 7$). * $P < 0.05$ vs WT Ctrl. † $P < 0.05$ vs KO Ctrl. ‡ $P < 0.05$ vs WT DM + NC-M. 4-HNE, 4-hydroxynonenal; ACh, acetylcholine; ED, endothelial dysfunction; iNOS, inducible nitric oxide synthase; PE, phenylephrine. Other abbreviations are the same as Figs 1, 2, 4 and 5.

found under the conditions of hyperglycemia/HG, and contributed to endothelial injuries, such as ED, oxidative damage and inflammation. These findings urge more potent and alternative approaches to enhance the NRF2 antioxidant capacity in protection against diabetic ED.

In recent years, attempts have been made to activate NRF2 in the endothelium. Baicalin, a functional component in *Scutellaria baicalensis* root, ameliorated HG-induced endothelial OS and inflammation through AKT/GSK-3 β /Fyn-mediated NRF2 activation (Chen *et al.* 2018). Resveratrol activated NRF2, attenuated HG-induced OS and inflammation in coronary arterial endothelial cells, and improved ED in high-fat diet-fed mice (Ungvari *et al.* 2010). Recently, we reported that sodium butyrate – a fatty acid derivative found in human diet and

also an inhibitor of histone deacetylase – improved diabetic ED through P300-mediated transcriptional activation of the *Nrf2* gene (Wu *et al.* 2018b). As KEAP1 is a key negative regulator of NRF2, in a previous report, activation of NRF2 in the diabetic endothelium was achieved by dh404 – a small molecule inhibitor of KEAP1 (Sharma *et al.* 2017). Decreasing *Keap1* mRNA levels or hampering the translation process of the KEAP1 protein (as opposed to the structural inactivation of KEAP1) may provide an alternative and viable approach for activating NRF2. Our findings support this notion, showing that miR-200a activated NRF2 through degradation of *Keap1* mRNA (Figs 2B, C, D, E, F, 3A, B, C, D, E, F and 5C, D, E, F, G, H, I, J).

In the present study, miR-200a levels were decreased under HG in a time-dependent manner (Fig. 1A).

In accordance with this observation, a remarkably lower miR-200a level was found in the aortas of the diabetic mice compared with the non-diabetic mice (Fig. 5C). To date, it is still unclear how HG or hyperglycemia reduces miR-200a levels in ECs or the endothelium. Aldose reductase was previously reported to be increased in the diabetic kidney, and accounts for the decrease in miR-200a level, providing a clue for the regulation of miR-200a in the endothelium under hyperglycemic conditions (Wei *et al.* 2014). This speculation needs to be investigated in future work.

Specific antagomirs are chemically engineered oligonucleotides that prevent other molecules from binding to a desired miRNA. In this regard, an antagomir is supposed to form a dimer with the target miRNA, blocking the target miRNA's ability to bind its target mRNA. However, antagomirs often led to decrease in the levels of target miRNAs, as shown in the present study (Fig. 2A) and many others (Putta *et al.* 2012, Wei *et al.* 2014, Wu *et al.* 2016). This might be caused by binding-induced instability of the target miRNAs, the effect of which should contribute to the inhibition of the target miRNA's efficacies.

A notable difference in the expression pattern was found between endothelial t-NRF2 and n-NRF2 under the time-course incubation with HG (Fig. 1D and E). Particularly, HG time-dependently decreased t-NRF2 levels, whereas HG increased n-NRF2 firstly and lowered n-NRF2 thereafter (Fig. 1D and E). This effect could be explained by the expression of KEAP1 protein under HG. KEAP1 is known to induce degradation of NRF2 via proteasome. As KEAP1 protein accumulated under HG (Fig. 1C), t-NRF2 level probably declined through degradation (Fig. 1D). n-NRF2 - a portion of t-NRF2 - translocated into the nuclei in response to HG for 6 and 12 hours (Fig. 1E), serving as a protective compensatory response to the stimulus HG. However, when KEAP1 was dramatically increased under HG for 24 and 48 hours (Fig. 1C), it significantly restricted NRF2 from nuclear translocation, resulting in less n-NRF2 (Fig. 1E). As a consequence of the decrease in n-NRF2 levels at 48 h, the mRNA levels of *Nqo1* and *Ho1* were drastically decreased (Fig. 1F). This had resulted in a significant elevation of OS and inflammation at 48 h (Fig. 1G), as a decompensatory stage during a longer period of stimulation with HG. In accordance with this decompensatory effect *in vitro*, our *in vivo* study has found that aortic NRF2 antioxidant signaling was blunted under long-term hyperglycemic conditions for 20 weeks (Fig. 5G, H, I and J). The remaining level of NRF2 antioxidant signaling under hyperglycemic conditions

(Fig. 5G, H, I and J), although basal, still offered protection, since hyperglycemia resulted in more severe aortic OS and inflammation in the *Nrf2* KO mice compared with the WT mice (Fig. 6A, B, C and D).

In previous studies, DM-induced OS and inflammation were evident in both the intima and adventitia (Tan *et al.* 2014, Wu *et al.* 2018a,b, 2019), where in the present study 4-HNE was found to be predominantly expressed (Fig. 6B). The enhanced OS and inflammation in the adventitia might be caused by adventitial ECs generated from neovascularization (O'Brien *et al.* 1993), and adventitial fibroblasts that is known to produce substantial amounts of ROS in response to vascular injury (Csanyi *et al.* 2009). It would be interesting to study the role of the adventitia in diabetic macrovascular diseases in future studies.

NRF2 has proven to be a viable drug target as evidenced by the use of dimethyl fumarate (also known as BG-12) – an inhibitor of KEAP1 – for the treatment of multiple sclerosis (Gold *et al.* 2012). More recently, sulforaphane – another inhibitor of KEAP1 – was shown in a pilot clinical trial to prevent carcinogen-induced oral cancer by activating NRF2 antioxidant signaling (Bauman *et al.* 2016). To date, activation of NRF2 has not been investigated in clinical trials for diabetic ED. The present study may provide an experimental basis for future clinical trials for diabetic ED. Notably, different from structural inhibition of the KEAP1 protein, targeting *Keap1* mRNA and its upstream miR-200a has shown novel strategies toward the activation of NRF2 in the endothelium.

Taken together, the present study has found that miR-200a improved diabetic ED via modulating KEAP1/NRF2 antioxidant signaling, demonstrating a critical role of NRF2 in protection against diabetic ED, and providing miR-200a and *Keap1* mRNA as potential targets in future management of diabetic macrovascular complications.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Hao Wu conceived and designed the project. Ziping Jiang, Junduo Wu, Fuzhe Ma, Jun Jiang, Linlin Xu, Lei Du, Wenlin Huang, Zhaohui Wang, Ye Jia, Laijin Lu and Hao Wu researched/interpreted the data. Hao Wu, Ziping Jiang and Laijin Lu wrote the manuscript. Hao Wu, Laijin Lu, Ziping Jiang, Junduo Wu, Fuzhe Ma, Jun Jiang, Linlin Xu, Lei Du, Wenlin Huang, Zhaohui Wang and Ye Jia discussed, reviewed and revised the manuscript. Hao Wu, Ziping Jiang, Jun Jiang, Linlin Xu, Lei Du, Zhaohui Wang and Laijin Lu provided funding. All the authors approve the version to be published.

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