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Functional role of NF-κB in expression of human endothelial nitric oxide synthase

Kyu-Sun Lee [a,1], Joohwan Kim [a,1], Su-Nam Kwak [a,1], Kwang-Soon Lee [a], Dong-Keon Lee [a], Kwon-Soo Ha [a], Moo-Ho Won [b], Dooil Jeoung [c], Hansoo Lee [d], Young-Guen Kwon [e], Young-Myeong Kim [a,*]

[a] Department of Molecular and Cellular Biochemistry, School of Medicine, Kangwon National University, Chuncheon, Gangwon-do 200-702, South Korea
[b] Department of Neurobiology, School of Medicine, Kangwon National University, Chuncheon, Gangwon-do 200-702, South Korea
[c] Department of Biochemistry, College of Natural Sciences, Kangwon National University, Chuncheon, Gangwon-do 200-702, South Korea
[d] Department Life Sciences, College of Natural Sciences, Kangwon National University, Chuncheon, Gangwon-do 200-702, South Korea
[e] Department of Biochemistry, College of Life Science and Biotechnology, Yonsei University, Seoul 120-752, South Korea

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ABSTRACT

The transcription factor NF-κB has an essential role in inflammation in endothelial cells. Endothelial nitric oxide synthase (eNOS)-derived nitric oxide (NO) prevents vascular inflammation. However, the molecular mechanism underlying NF-κB-mediated regulation of eNOS expression has not been clearly elucidated. We here found that NF-κB-activating stimuli, such as lipopolysaccharide, tumor necrosis factor-α (TNF-α), and interleukin-1β, suppressed eNOS mRNA and protein levels by decreasing mRNA stability, without affecting promoter activity. TNF-α-mediated suppression of eNOS expression, mRNA stability, and 3′-untranslated region (3′UTR) activity were inhibited by NF-κB inhibitors and Dicer knockdown, but not by p38 MAPK and MEK inhibitors, suggesting the involvement of NF-κB-responsive miRNAs in eNOS expression. Moreover, TNF-α increased MIR155HG expression and promoter activity as well as miR-155 biogenesis, and these increases were blocked by NF-κB inhibitors. Transfection with antagomiR-155 blocked TNF-α-mediated suppression of eNOS 3′UTR activity, eNOS mRNA and protein levels, and NO and cGMP production. These data provide evidence that NF-κB is a negative regulator of eNOS expression via upregulation of miR-155 under inflammatory conditions. These results suggest that NF-κB is a potential therapeutic target for preventing vascular inflammation and endothelial dysfunction induced by suppression of miR-155-mediated eNOS expression.

1. Introduction

Nitric oxide (NO) generated from L-arginine by three isoforms of nitric oxide synthase (NOS) plays an important role in both physiological and pathological conditions. A large amount of NO produced by inducible NOS (iNOS) is associated with immune reactions. However, small and physiological amounts of NO produced by constitutive Ca^{2+}-dependent NOS isoforms, endothelial NOS (eNOS) and neuronal NOS, act as a signal molecule for neurotransmission and vascular homeostasis, respectively [1]. Since eNOS-derived NO plays a key role in systemic blood pressure and vascular inflammation [1,2], decreased NO production in endothelial cells (ECs) leads to hypertension and atherosclerosis.

In contrast to the transcriptional induction of iNOS via activation of the transcription factor NF-κB by lipopolysaccharide (LPS) and tumor necrosis factor-α (TNF-α) [3,4], eNOS activity is regulated by multiple levels of post-translational mechanism to include: Ca^{2+}-dependent dimerization, protein–protein interaction, subcellular localization, and phosphorylation [5–8]. This data indicates that eNOS activity is tightly regulated in a biological system. Moreover, regulation of eNOS expression can occur at both the transcriptional and post-transcriptional levels in response to a wide variety of stimuli. Shear stress and estrogen increase the transcriptional activity of the eNOS promoter [9,10]. Conversely, inflammatory activators, including LPS and TNF-α, suppress eNOS expression by decreasing the half-life of the eNOS mRNA [11,12]. Thus, eNOS expression is regulated by modulating its promoter activity and mRNA stability. However, no study to date has convincingly established the possible involvement of NF-κB in the modulation of eNOS expression.

* Corresponding author. Fax: +82 33 244 3286.
E-mail address: ymkim@kangwon.ac.kr (Y.-M. Kim).
[1] These three authors contributed equally to this study.

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Diverse lines of evidence demonstrate that microRNA (miRNA) plays a key role in the negative regulation of post-transcriptional gene expression, mainly by promoting mRNA degradation or inhibiting mRNA translation via binding to the 3′-untranslated region (3′UTR) of its targeted mRNAs [14]. Recent studies showed that inflammatory cytokines increases the expression of some miRNAs, such as miR-146, miR-125b, and miR-155. These miRNAs suppress the post-transcriptional gene expression by specifically binding to the 3′UTRs of their target genes [15–17]. This evidence suggests that NF-κB-responsive miRNAs plays an important role in eNOS expression under inflammatory conditions.

We here found that NF-κB inhibitors and NF-κB p65 knockdown prevented TNF-α-induced decreases in eNOS expression and NO production in human ECs. Furthermore, these effects were accompanied by a decrease in miR-155 levels. These results indicate that NF-κB is critically involved in the negative regulation of eNOS expression at the post-transcriptional level via miR-155 expression.

2. Materials and methods

2.1. Materials

Cell culture media supplements and Lipofectamine RNAiMAX were purchased from Invitrogen Life Technologies (Carlsbad, CA), and 4-amino-5methylamino-2,7-difluorofluorescein (DAF-FM) diacetate was purchased from Molecular Probes (Eugene, OR). Antibodies against human eNOS, Dicer, and NF-κB p65 were purchased from BD science (San Jose, CA) and Cell Signaling Technology (Danvers, MA). miRNeasy Mini kit, miR-155 mimics, antagomiR-155, miScript SYBR Green PCR kit, and real-time PCR primers were purchased from QIAGEN (Hilden, Germany). Luciferase report assay kits were purchased from Promega (Madison, WI). Human recombinant TNF-α, interleukin-1β (IL-1β), and a cGMP assay kit were purchased from R&D system (Minneapolis, MN). All other reagents were purchased from Sigma–Aldrich (St. Louis, MO) unless indicated otherwise.

2.2. Human umbilical vein endothelial cells (HUVECs) isolation and cell culture

HUVECs were isolated from human umbilical cord veins by collagenase treatment, as previously described [18]. Cells used for all experiments were from passages 3 to 5. Cells were grown in M199 media supplemented with 20% fetal bovine serum, 100 U/ml penicillin, 100 ng/ml streptomycin, 3 ng/ml basic fibroblast growth factor, and 5 U/ml heparin at 37 °C in a humidified CO_2 incubator.

2.3. Transfection with miRNAs and siRNA

HUVECs were seeded into six-well plates coated with gelatin at a density of 2×10^5 cells/well and maintained for 1 day. After cells were cultured in serum-free media for 2 h, cells were transfected with antigomiR-155 (80 nM), miR-155 mimics (50 nM), negative control (80 nM) of antagomiR-101 and miR-101, Dicer siRNA (80 nM) or scrambled control (80 nM) in Opti-MEM reduced serum medium using Lipofectamine RNAiMAX according to the manufacturer's instructions. After 4 h incubation, fresh medium was provided and cells were further cultured for 48 h.

2.4. Reporter gene assay

HUVECs were transfected with 4 μg of pGL3-eNOS promoter-Luc construct (pGL3-Luc vector containing the 5′-flanking region of eNOS (−16,000 nt to +22 nt), pGL3-MIR155HG promoter-Luc constructs (pGL3-Luc vector containing 1 kb and 2 kb regions of MIR155HG promoter), psiCHECK-2-eNOS 3′UTR-WT and mutant reporter constructs (Supplementary Fig. 1) or each basic plasmid using Lipofectamine 2000. After 4 h incubation, fresh medium was provided and cells were cultured for 24 h, followed by incubation with TNF-α (10 ng/ml) for 24 h. Reporter gene activity was assayed by a luciferase assay system or a Dual-luciferase report assay kit.

2.5. Measurements of NO and cGMP

Intracellular NO levels were measured *in situ* by using DAF-FM diacetate (Molecular probe) according to the manufacturer's instructions. HUVECs were transfected with miR-155 mimics and antigomiR-155 for 24 h and treated with 10 ng/ml of TNF-α for 24 h. Cells were incubated with 5 μM (final concentration) DAF-FM diacetate for 30 min in a CO_2 incubator. Intracellular NO levels were determined using a confocal laser microscope, as previously described [18]. cGMP was determined using a cGMP assay kit (R&D systems).

2.6. Polymerase chain reaction (PCR) analysis

miRNAs were isolated from HUVECs treated with TNF-α for 12 h using a miRNeasy mini kit. cDNAs for determining miRNAs were obtained from 1 μg of total RNAs using a miScript II RT kit. Quantitative real-time PCR (qRT-PCR) was performed with miScript SYBR Green PCR Kit according to the manufacturer's instructions. The levels of miR-155 were analyzed by miScript Primer Assay with a target miR-155-specific primer and universal primer. The relative levels of miRNA-155 were normalized to the housekeeping gene SNORD-95. In addition, thee NOS mRNA levels were determined by using iTaqTM SYBR Green Supermix with ROX (BioRad, Hercules, CA) with ABI PRISM 7000 Sequence Detection System (Applied Biosystem) using target-specific primers. The fold-changes for miR-101 and target gene mRNAs were calculated using $2^{-\Delta\Delta Ct}$ method as previously described [19]. In addition, MIR155HG mRNA levels were determined by RT-PCR analysis. The primers used in this study were listed in the Supplementary Table 1.

2.7. Western blot analysis

Cells were treated with TNF-α for 24 h and suspended in RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS). Cell suspensions were incubated on ice for 30 min for complete cell lysis. Next, cell debris was removed by centrifugation at 12,000*g* for 15 min. Cell lysates (20 μg) were separated by SDS–PAGE and target protein levels were determined by Western blot analysis, as previously described [18].

2.8. Chromatin immunoprecipitation (ChIP)

HUVECs were treated with TNF-α for 12 h. DNA/protein cross-linking was obtained by incubating cells for 20 min at 37 °C in 1% formaldehyde. After sonication, chromatin was immunoprecipitated overnight with 10 μl of anti-NF-κB antibody. Targeted promoter sequences of MIR155HG were identified by PCR using primer pairs spanning MIR155HG-specific promoter regions containing the NF-κB binding sequence (Supplementary Table 1). The products (about 150–180 bp) were identified on a 2% agarose gel.

2.9. Statistical analysis

Quantitative data are expressed as mean ± standard deviation (SD) of at least three separate experiments. Statistical significance was determined using either one-way ANOVA or the unpaired Student's *t* test, depending on the number of experimental groups analyzed. Significance was established at a p value <0.05.

3. Results

3.1. NF-κB inhibitor reverses TNF-α-mediated suppression of eNOS expression

As shown in a previous study [11,12], treatment of HUVECs with NF-κB-activating inflammatory stimuli, such as LPS, TNF-α, and IL-1β, effectively suppressed eNOS mRNA and protein levels (Fig. 1A and B), suggesting that NF-κB negatively regulates eNOS expression. We next examined the effects of NF-κB inhibitor on eNOS expression. TNF-α-mediated suppression of eNOS mRNA and protein levels were effectively reversed by the NF-κB inhibitors Withaferin A and Bay11-7082. However, these levels were not reversed by the p38 MAPK inhibitor SB203580, the JNK inhibitor SP600125, and the MEK inhibitor PD98059 (Fig. 1C and D). Moreover, eNOS promoter activity was not affected in HUVECs by treatment with TNF-α alone or in combination with Withaferin A and Bay11-7082 (Fig. 1E). Interestingly, TNF-α treatment resulted in a significant decrease in the half-life of eNOS mRNA, from 24.6 to 7.8 h, compared with control, and this decrease was blocked by co-treatment with Bay11-7082 (Fig. 1F). These results suggest that NF-κB plays an important role in negatively regulating eNOS mRNA stability.

3.2. Knockdown of Dicer increases TNF-α-mediated eNOS mRNA instability

Recent studies have conclusively demonstrated that Dicer is a key cytosolic endonuclease of miRNA maturation from its precursors and that miRNAs are involved in the negative regulation of mRNA stability of their target genes [14]. To confirm the functional involvement of miRNAs in the negative regulation of eNOS expression, we examined whether Dicer knockdown would recover TNF-α-mediated post-transcriptional suppression of eNOS expression. Transfection with Dicer siRNA reversed the suppressive effects of TNF-α on eNOS mRNA and protein levels in HUVECs (Fig. 2A). In addition, Dicer knockdown significantly improved the decreased half-life of eNOS mRNA in HUVECs treated with TNF-α (Fig. 2B). Since miRNA binds to complementary sequences within the 3′UTRs of its target genes and destabilizes their mRNAs, we next examined whether Dicer knockdown would regulate the function of eNOS 3′UTR. TNF-α inhibited the activity of reporter gene bearing the 3′UTR of eNOS mRNA, and this inhibition was reversed by Dicer knockdown and Bay11-7082, but not by PD98059 (Fig. 2C). These data suggest that TNF-α responsive miRNAs are involved in eNOS expression via destabilization of eNOS mRNA.

3.3. NF-κB-dependent induction of miR-155 suppresses eNOS expression

Among the miRNAs induced in human ECs by TNF-α [17], miR-155 was found to target the 3′UTR of human eNOS by using TargetScan (data not shown). We reconfirmed miRNA-155 induction in HUVECs treated with TNF-α by qRT-PCR. TNF-α increased miR-155 expression in a time-dependent manner (Supplementary Fig. 2). Moreover, TNF-α-induced increases in miR-155 were significantly suppressed by Withaferin A, Bay11-7082, and NF-κB p65 knockdown (Fig. 3A). Treatment with TNF-α decreased eNOS 3′UTR-based reporter activity, but not mutant eNOS 3′UTR reporter activity, and this effect was abolished by Bay11-7082, NF-κB p65 knockdown, and antagomiR-155 (Fig. 3B). The reverse effect of Bay11-7082 was abolished by transfection with miR-155 mimics. Furthermore, TNF-α-mediated decreases in eNOS mRNA and protein levels were blocked by Bay11-7082, NF-κB p65 knockdown,

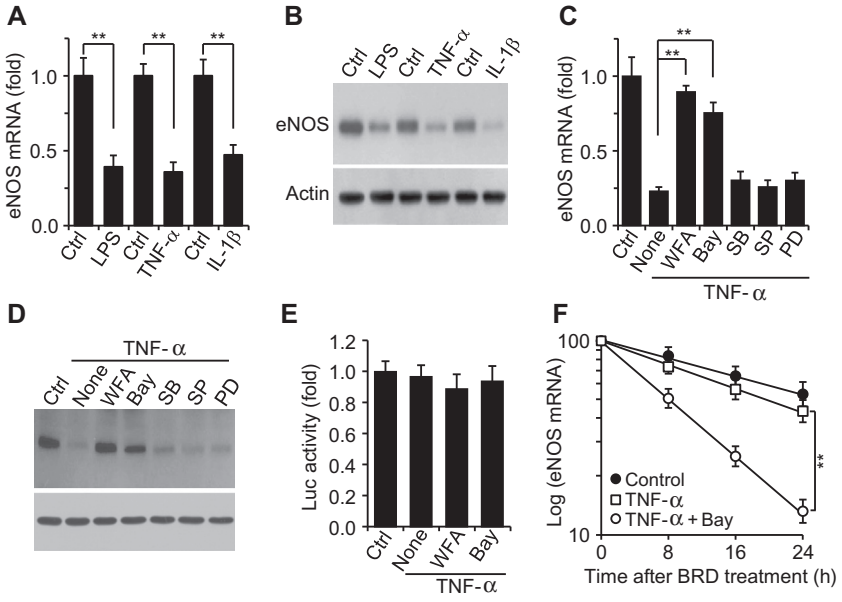

Fig. 1. NF-κB inhibitor reverses inflammatory stimulant-induced suppression of eNOS expression. HUVECs were stimulated with LPS (1 μg/ml), TNF-α (10 ng/ml), and IL-1β (10 ng/ml) in the presence or absence of Withaferin A (WFA, 1 μM), BAY11-7082 (Bay, 5 μM), SB203580 (SB, 10 μM), SP600125 (SP, 10 μM), and PD98059 (PD, 10 μM). (A and C) eNOS mRNA levels were determined by qRT-PCR. (B and D) eNOS protein was determined by Western blotting. (E) Cells transfected with pGL3-eNOS promoter construct were treated with TNF-α in the presence or absence of Withaferin A and BAY11-7082. Luciferase activity was determined. (F) Cells were stimulated with TNF-α in the presence or absence of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB, 20 μg/ml) for the indicated time periods. eNOS mRNA levels were determined by qRT-PCR. Data shown in graphs are the mean ± SD (n = 3). $^{**}p < 0.01$.

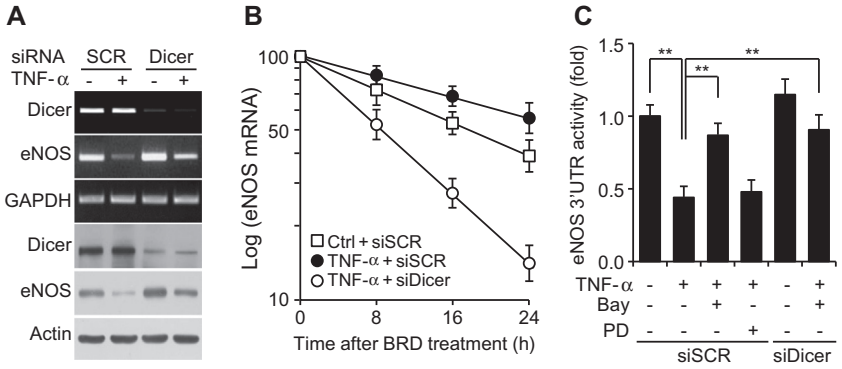

Fig. 2. Dicer knockdown reverses inflammatory stimulant-induced suppression of eNOS expression. (A and B) HUVECs were transfected with Dicer or scrambled siRNA and treated with TNF-α in the presence or absence of BRD. (A) mRNA and protein levels of Dicer and eNOS were determined by qRT-PCR and Western blotting. (B) eNOS mRNA levels were determined by qRT-PCR. (C) Cells were transfected with psiCHECK-2-eNOS 3′UTR-reporter construct and Dicer siRNA, and scrambled siRNA, followed by stimulation with TNF-α in the presence or absence of BAY11-7082 and PD98059 for 24 h. Luciferase activity was determined using a Dual-luciferase report assay kit. Data shown in graphs are the mean ± SD ($n = 3$). $^{**}p < 0.01$.

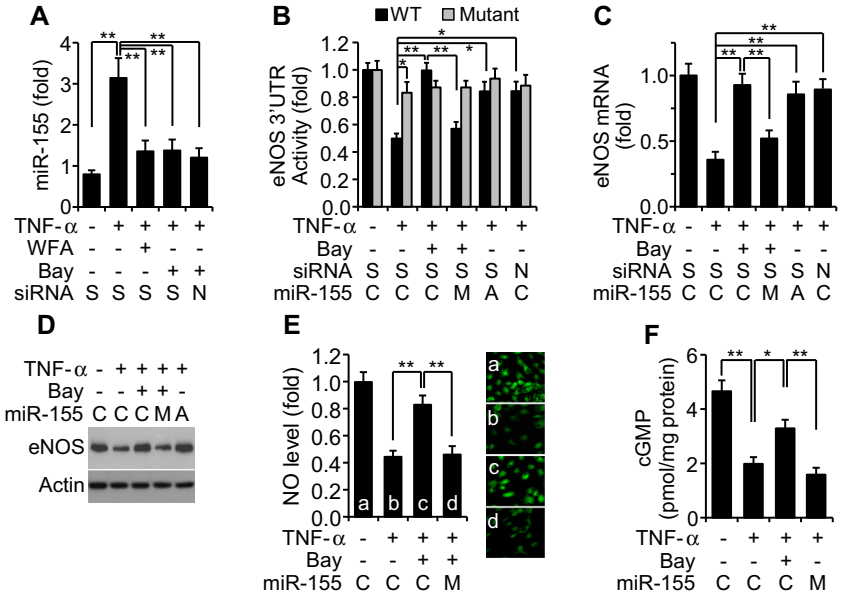

Fig. 3. NF-κB inhibition suppresses TNF-α-induced expression of miR-155, which targets the 3′UTR of eNOS. (A) HUVECs were transfected with NF-κB p65 (N) or scrambled siRNA (S) and treated with TNF-α in the presence or absence of Withaferin A and BAY11-7082 for 12 h. The levels of miR-155 were determined by qRT-PCR. (B–F) Cells were transfected with miR-155mimics (M), antagomiR-155 (A), negative control (C) for miR-155 and antagomiR-155, NF-κB p65 siRNA (N), scrambled siRNA (S), or psiCHECK-2-eNOS 3′UTR-reporter constructs (wild type and mutant), followed by stimulation with TNF-α alone or in combination with BAY11-7082. (B) Luciferase activity was determined using a Dual-luciferase report assay kit. (C and D) eNOS mRNA and protein levels were determined by qRT-PCR and Western blotting. (E and F) Intracellular NO and cGMP levels were determined by confocal microscopy and ELISA, respectively. Data shown in graphs are the mean ± SD ($n = 3$). $^{*}p < 0.05$ and $^{**}p < 0.01$.

and antagomiR-155. The protective effects of Bay11-7082 on eNOS expression were also significantly attenuated by transfection with miR-101 mimics (Fig. 3C and D). As expected, the protective effects of Bay11-7082 on TNF-α-mediated inhibition of NO production and cGMP synthesis were also blocked by miR-155 mimics (Fig. 3E and F). These results suggest that NF-κB-responsive miR-155 contributes to TNF-α-mediated suppression of eNOS expression and inhibition of the NO/cGMP pathway.

3.4. NF-κB inhibition blocks TNF-α-mediated transcriptional expression of MIR155HG that codes miR-155

miR-155 is encoded by the miR-155 host gene (MIR155HG, also known as *BIC*, B-cell integration cluster), and its biogenesis is regulated at both the transcriptional level and Dicer-mediated maturation process [20].We first examined whether a NF-κB inhibitor regulates TNF-α-induced expression of MIR155HG. TNF-α increased MIR155HG mRNA levels, which were inhibited by Bay11-7082 and NF-κB p65 knockdown (Fig. 4A). Treatment with TNF-α increased both mature and precursor miRNA-155 in HUVECs, and these increases were simultaneously inhibited by Bay11-7089 and NF-κB p65 knockdown (Fig. 4B). This suggests that the NF-κB inhibitor is not involved in the maturation of miR-155. Since MIR155HG is upregulated in macrophages and B-cells by NF-κB activation [20,26], we examined the effect of a NF-κB inhibitor on TNF-α-stimulated transcriptional activity of long and short MIR155HG promoter construct, containing four and two NF-κB binding sites, respectively (Fig. 4C). TNF-α significantly increased the transcriptional activity of the long MIR155HG promoter construct, but only minor effects, but statistically significant, were observed with the short MIR155HG promoter construct. Both promoter activities were significantly inhibited by Bay11-7089 and NF-κB p65 knockdown, but the long promoter activity was more effectively inhibited (Fig. 4D). Similarly, a ChIP

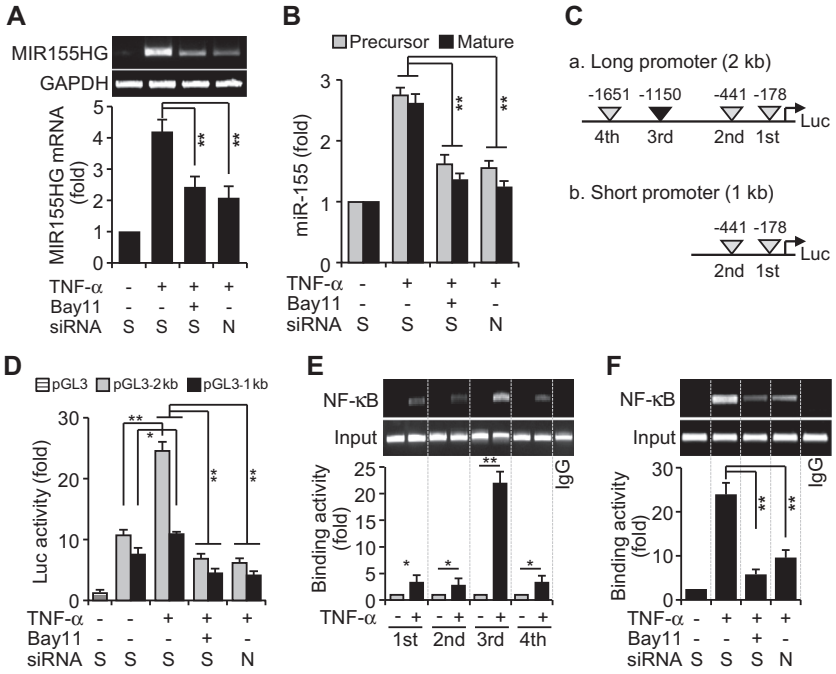

Fig. 4. NF-κB inhibitor inhibits TNF-α-mediated MIR155HG expression. (A and B) HUVECs transfected with NF-κB p65 (N) or scrambled siRNA (S) were treated with TNF-α or in combination with Bay11-7082. The levels of MIR155HG mRNA and precursor and mature miR-155 were determined by PCR and qRT-PCR. (C) Luc-reporter constructs containing four and two putative NF-κB-binding sites (long and short promoters) of MIR155HG promoter. (D) Cells were transfected with both reporter constructs, followed by stimulation with TNF-α for 24 h. Luciferase activity was determined using a luciferase assay kit. (E) Binding activity of NF-κB to its four putative binding sites of MIR155HG promoter was determined by ChIP analysis. (F) Binding activity of NF-κB to its third binding site of the promoter was analyzed by Chip assay. Data shown in graphs are the mean ± SD ($n = 3$). $^{*}p < 0.05$ and $^{**}p < 0.01$.

assay also showed strong binding of the NF-κB complex to the third κB-binding site at −1150 nt, compared with other sites (Fig. 4E). Moreover, NF-κB binding activity to the third site was effectively blocked by Bay11-7089 and NF-κB p65 knockdown (Fig. 4F). These results suggest that NF-κB is critically involved in the expression of miR-155, which targets the 3′UTR of eNOS.

4. Discussion

Endothelium-derived NO is considered an important determinant of vascular tone and inflammation, angiogenesis, and smooth muscle cell proliferation [1]. Physiological levels of NO produced eNOS in ECs maintain vascular homeostasis. However, inappropriate levels of NO production are directly implicated in the pathogenesis of a wide range of cardiovascular diseases, such as hypertension and atherosclerosis. Thus, optimizing NO production by regulating eNOS activity and expression is important for treating patients suffering from cardiovascular diseases.

The activity of constitutively expressed eNOS is regulated by several ways of post-translational modification. eNOS has a calmodulin-binding domain that is essential for the formation of its active dimer. As such, the activity of eNOS is sensitive to intracellular Ca^{2+} concentration [5]. eNOS catalytic activity is also regulated by phosphorylation at several Ser and Thr sites. For example, eNOS phosphorylation at Ser-1179 by Akt increases NO production [7], while basal phosphorylation of eNOS at Thr-495 reduced its catalytic activity [21]. Catalytic activity of eNOS is also finely turned-up by protein–protein interactions and trafficking between plasma membrane and intracellular membranes [22]. Although eNOS was originally considered a constitutive gene, accumulating data indicates its expression is regulated by a variety of stimuli. eNOS expression is transcriptionally increased in ECs exposed to physical factors, such as shear stress, [9] and hormones, such as estrogen [11]. On the other hand, LPS and TNF-α inhibit eNOS expression through destabilization of its mRNA, leading to decreased endothelial function [11,12]. These observations indicate that eNOS expression can be regulated at both transcriptional and post-transcriptional levels, modulating the promoter activity and mRNA stability of eNOS.

The regulation of mRNA stability has emerged as an important mechanism for regulating target gene expression. Although mechanisms that control mRNA stability of different genes have unique features, it appears that in each case specific RNA sequences are required for the recognition of regulatory protein factors [23]. These specific sequences are mostly identified within 3′UTRs, which interact with *trans*-acting protein factors, leading to regulation of mRNA half-lives. Alonso et al. has shown that suppression of eNOS mRNA levels by TNF-α is elicited by increasing the binding activity of endothelial cytosolic proteins to an unknown *cis* element contained within its 3′UTR [24]. Recent evidence also shows that specific sequences of eNOS 3′UTR are complementary to several miRNAs, which mediate negative regulation of eNOS expression via a decrease in its mRNA stability. Of the many miRNAs induced by TNF-α, miR-155 decreases eNOS expression and NO production by binding to its 3′UTR and exerts vasoconstriction in human arteries [25]. We found that pro-inflammatory stimuli, such as LPS, TNF-α, and IL-1β, decreased eNOS expression by destabilizing its mRNA via induction of miR-155. Since eNOS-derived NO plays an important role in the suppression of vascular inflammation [1], our data suggests that TNF-α-responsive miR-155 may induce vascular inflammation via suppression of endothelial NO production.

LPS, TNF-α, and IL-1β used in this study are potent activators of NF-κB, which play a pivotal role in the expression of inflammation-associated genes. Activation of NF-κB is involved in the pathogenesis of a variety of vascular inflammatory diseases. Thus,

functional inhibition of the NF-κB pathway is beneficial to the treatment of inflammation-associated vascular diseases. Since the promoters of human and murine iNOS genes, but not eNOS, contain NF-κB binding sites [3,4], the inflammatory stimuli increase NO production by NF-κB-mediated iNOS induction in various types of cells, including macrophages and hepatocytes. Although eNOS is not transcriptionally regulated by NF-κB activation, immune activators decrease endothelial NO production by destabilizing eNOS mRNA [11,12], suggesting that NF-κB may be involved in the negative regulation of eNOS expression and NO production. However, the molecular mechanism by which NF-κB downregulates eNOS expression has not been elucidated. We here found that the immune stimulants reduce endothelial NO production by decreasing eNOS mRNA stability via miR-155 expression. Moreover, our results showed that NF-κB inhibitors restored TNF-α-mediated suppression of eNOS expression by inhibiting expression of MIR155HG that encodes miR-155. The human MIR155HG promoter contains four putative NF-κB binding sites at −1651, −1150, −441, and −178 [20,26]. Gatto et al. has initially showed that the third and fourth proximal NF-κB binding sites are responsive for MIR155HG-dependent miR-155 expression [26]. However, a recent study demonstrated that the first and second proximal sites are more important to NF-κB-responsive expression of human MIR155HG, leading to elevation of miR-155 biogenesis [20]. We found that the third NF-κB site at −1150 nt is the most important for MIR155HG-dependent miR-155 biogenesis. These findings indicate that the third NF-κB-binding site is critically involved in the negative regulation of eNOS expression in ECs via miR-155 biogenesis.

NF-κB activation and diminished eNOS expression are found in atherosclerotic plaques from patients [2,13]. Therefore, NF-κB inhibitor and eNOS gene delivery are suggested to represent a unique and potential approach to help control atherosclerosis. Under these pathological conditions, a major role of eNOS-derived NO is associated with protection from endothelial dysfunction and deduction of inflammatory gene expression, suggesting that endothelial NO exerts anti-inflammatory protective function [1]. Our results show that activation of NF-κB suppresses eNOS expression and NO production. These results indicate that the inflammatory effects of NF-κB are linked to transcriptional induction of inflammatory gene expression and post-transcriptional suppression of eNOS expression. The suppressive effect on eNOS expression is due to NF-κB-dependent MIR155HG-miR-155 biogenesis. Although miR-155 can bind to the 3′UTR of eNOS from human, chimpanzee, and rhesus, but not other species including bovine, as analyzed by TargetScan, TNF-α and LPS downregulated eNOS expression in bovine ECs [11,12]. This evidence suggests that other NF-κB-responsive miRNAs that are complementary to 3′UTR of eNOS mRNA are involved in the regulation of eNOS expression bovine ECs. Thus, species-specific types of miRNAs may involve the regulation of eNOS expression.

Taken together, our study demonstrates that inflammatory stimulants, including TNF-α, suppressed eNOS expression in HUVECs via NF-κB-mediated upregulation of miR-155, which decreases eNOS mRNA stability by binding to its 3′UTR. Although our findings indicate that NF-κB is a negative regulator of eNOS expression and the NO/cGMP pathway via miR-155 biogenesis in HUVECs, we could not completely exclude the possibility that other NF-κB-responsive miRNAs can be involved in suppressing eNOS expression. Therefore, inhibition of NF-κB may be a new therapeutic intervention to improve endothelial dysfunction, an important risk factor of atherosclerosis and hypertension, by functional restoration of the eNOS/NO/cGMP pathway via expressional inhibition of miR-155 expression.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.079.

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