

## CLINICAL STUDY

# The role of miR-146a on NF- $\kappa$ B expression level in human umbilical vein endothelial cells under hyperglycemic condition

Kamali K<sup>1</sup>, Salmani Korjan E<sup>1</sup>, Eftekhari E<sup>2</sup>, Malekzadeh K<sup>3</sup>, Ghadiri Soufi F<sup>3</sup>

Department of Physiology, Faculty of Medicine, Hormozgan University of Medical Sciences, Bandar Abbas, Iran. Dr.F.G.Soufi@gmail.com

**ABSTRACT**

Emerging studies have been shown that the expression of microRNA-146a (miR-146a, as a regulator of nuclear factor  $\kappa$ B (NF- $\kappa$ B)), is changed in diabetic patients and animals. This study was designed to evaluate the possible role of miR-146a in the pathogenesis of diabetes-related microvascular complications.

Concurrent with the creation of cellular hyperglycemia (25 mmol/L for 24 h), human umbilical vein endothelial cells (HUVECs) were transfected with 20 nmol/L of hsa-miR-146a antagomir or scramble using HiPerFect reagent (Qiagen). D-mannitol was used as osmotic control.

Hyperglycemia increased the NF- $\kappa$ B gene expression and protein activity (as an inflammation index) in cultured HUVECs. Moreover, the gene expression level of miR-146a, and its target proteins, tumor necrosis factor receptor-associated factor 6 (TRAF6) and interleukin-1 receptor-associated kinase 1 (IRAK1) were increased under hyperglycemic condition. The knockdown of miR-146a by transfection of miR-146a antagomir notably increased the NF- $\kappa$ B activity and decreased the NF- $\kappa$ B mRNA in hyperglycemic HUVECs. Furthermore, miR-146a antagomir significantly increased IRAK1 and TRAF6 mRNA levels under hyperglycemic condition.

These results demonstrate that the expression of miR-146a is upregulated in HUVECs during early phase of hyperglycemic condition possibly to regulate the NF- $\kappa$ B activity through inhibition of IRAK1 and TRAF6 (Fig. 4, Ref. 32). Text in PDF [www.elis.sk](http://www.elis.sk).

KEY WORDS: hyperglycemia, HUVECs, miRNA-146a, NF- $\kappa$ B, IRAK1, TRAF6.

**Introduction**

MicroRNAs (MiRNAs) are considered a class of highly conserved small single stranded non-coding RNAs that bind to the 3'-untranslated regions of their target messenger RNAs (mRNAs) and negatively regulate them by suppressing translation, mRNA degradation, or both (1, 2). More than 1000 miRNAs have been identified in human genome and it has been estimated that over 60 % of mammalian protein-encoding genes can be directly regulated by miRNAs (2). MiRNA-146a (MiR-146a), a member of miRNA family, has been confirmed as one of the key regulatory molecules in a wide range of physiological processes, including proliferation, differentiation, senescence and apoptosis of cells, as well as

immune and inflammatory responses by regulating nuclear factor kappa B (NF- $\kappa$ B) activation (3, 4).

NF- $\kappa$ B, as a master switch transcription factor, plays a critical role in progression and development of hyperglycemia-induced microangiopathy in diabetic patients by activating several downstream pro-inflammatory pathways, among them, activation of several genes encoding proinflammatory cytokines such as tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin 1 (IL-1) and 6 (IL-6) which activate Toll-like receptors (TLRs) and promote cells toward inflammation and eventually apoptosis (5, 6). It has been proposed that miR-146a prevents from NF- $\kappa$ B activation by downregulating the targeting gene tumor necrosis factor receptor-associated factor 6 (TRAF6) and interleukin-1 receptor-associated kinase 1 (IRAK1), two-key adaptor kinases originating from TLRs (7, 8).

There is now an increasing number of documents to suggest that miR-146a is involved in the pathogenesis of diabetes-related complications. While decreased expression of miR-146a has been reported in diabetic mouse wounds (9), diabetic rats aorta, kidney, heart and dorsal root ganglia (10–12), glycated albumin- and high glucose-stimulated endothelial cells (13), and in serum and peripheral blood mononuclear cells of patients with types 1 and 2 diabetes (14–16), increased miR-146a expression has also been documented in the kidney and sciatic nerve of diabetic rats (17–19), as well as in the kidney, plasma and limbal corneal epithelium compartment of diabetic patients (18, 20, 21). Given that the vascular endothelial cells and NF- $\kappa$ B play main roles in the pathogenesis of diabetic microangiopathy, we conducted this

<sup>1</sup>Department of Physiology, Faculty of Medicine, Hormozgan University of Medical Sciences, Bandar Abbas, Iran, <sup>2</sup>Department of Biochemistry, Faculty of Medicine, Hormozgan University of Medical Sciences, Bandar Abbas, Iran, and <sup>3</sup>Molecular Medicine Research Center, Hormozgan University of Medical Sciences, Bandar Abbas, Iran

**Address for correspondence:** F.Ghadiri Soufi, MSc, PhD, Molecular Medicine Research Center, Hormozgan University of Medical Sciences, Bandar Abbas, Iran.  
Phone/Fax: +98.763.3338965

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study to explore whether miR-146a (as an NF- $\kappa$ B regulating factor) and its adapter proteins (TRAF6 and IRAK1) are affected by hyperglycemia in the human umbilical vein endothelial cells (HUVECs). To evaluate this hypothesis, we measured the gene expression level of miR-146a, IRAK1, TRAF6, and NF- $\kappa$ B in HUVECs under normoglycemic and hyperglycemic conditions. Moreover, in order to study the role of miR-146a, we transfected miR-146a antagomir in cultured HUVECs and measured the target gene expression levels as well as the NF- $\kappa$ B activity.

## Materials and methods

### Cell culture

HUVECs were purchased from the National Cell Bank of Iran (NCBI, Pasteur Institute, Tehran) and cultured in endothelial cell growth medium, EGM-2 Bullet kit (Lonza, Basel, Switzerland), according to the manufacturer's recommendations. Subculture was performed in a 75-T flask using trypsin solution (Gibco, Grand Island, NY, USA) at 70–80% confluence every 3–4 days. Twenty-four hours before transfection, the cells were passaged in 6-well plates, at the density of  $3 \times 10^5$  cells/well in  $2.3 \times 10^3$   $\mu$ l medium and incubated at standard culture condition for 24 h. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

### Transfection of miR-146a antagomir

On the day of transfection, cell culture medium was replaced, and to achieve the final concentration of 25 mM, 20 mM D-glucose was added to the medium of hyperglycemic groups. D-mannitol was used as osmotic control (5 mM D-glucose and 20 mM D-mannitol) (22). Concurrent with the creation of cellular hyperglycemia, the HUVECs were transfected in parallel with 20 nmol/L of hsa-miR-146a antagomir or scramble (Qiagen, Crawley, UK) using HiPerFect reagent (Qiagen), according to the manufacturer's protocol. All experiments were carried out for 24 h. Transfection efficiency was determined by real-time RT-PCR.

### Real time RT-PCR analysis

Twenty-four hours after transfection, total RNA was isolated using miRNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. RNA quantity and quality were determined using Nanodrop (Thermo scientific) and agarose gel electrophoresis (Bio-Rad), respectively. Total RNA (1  $\mu$ g) was used for cDNA synthesis using miScript II RT cDNA synthesis Kit (Qiagen) according to the manufacturer's instructions. Each cDNA was used as a template for separate assay for miR-146a and mRNAs (NF- $\kappa$ B, IRAK1 and TRAF6) quantitative real-time RT-PCR by using miScript SYBR Green PCR Kit (Qiagen). All reactions were performed in duplicate on a Corbett Rotor-Gene RG-6000 (Australia). The amount of PCR was normalized to that for housekeeping gene  $\beta$  actin for mRNA samples and U6 for miR-146a using relevant primers (Qiagen). The  $2^{-\Delta\Delta C_t}$  method was used for relative quantification of individual mRNAs and miR-146a expression. The results have been expressed as fold change differences compared to the relevant controls.

### NF- $\kappa$ B activity

The NF- $\kappa$ B/p65 activation rate was determined by measuring the phosphorylated NF- $\kappa$ B p65 levels with ELISA (Cayman chem.), according to the manufacturer's instructions. Briefly, after homogenization of HUVECs according to the instructions of nuclear extraction kit (Cayman chem.), the supernatant containing the nuclear fraction was used for quantification of NF- $\kappa$ B activity. The sample concentration was measured spectrophotometrically at 450 nm. Also, Cayman protein determination kit (Item No: 704,002) was used for detection of protein concentrations in nuclear extracts.

### Statistical analysis

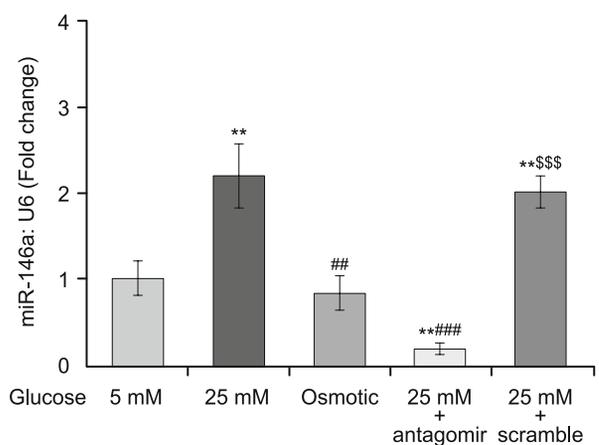
Data have been expressed as mean  $\pm$  SD, and covariance was analyzed (ANCOVA) using the SPSS 21.0 software (IBM, Armonk, NY, USA). The Tukey post hoc test was used to determine the differences between groups with significant p-values.  $p < 0.05$  was considered statistically significant.

## Results

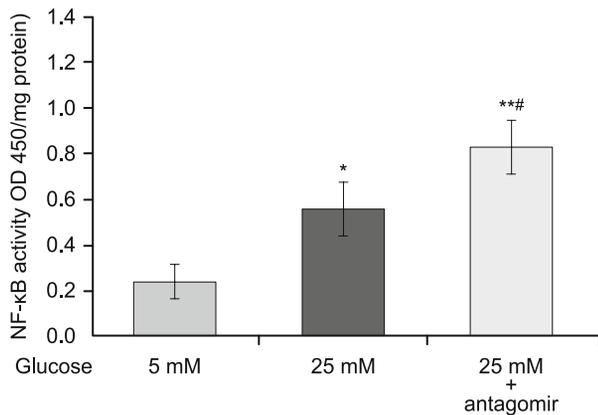
### Hyperglycemia causes upregulation of miR-146a in the endothelial cells.

Figure 1 represents the efficiency of transfection protocol and the effect of hyperglycemia on miR-146a expression levels in HUVECs after 24 h of incubation. The real-time PCR analysis demonstrated that the miR-146a expression was increased after incubation with 25 mM glucose (but not by 25 mM of osmotic control) when compared with 5 mM glucose control group ( $p = 0.002$ ).

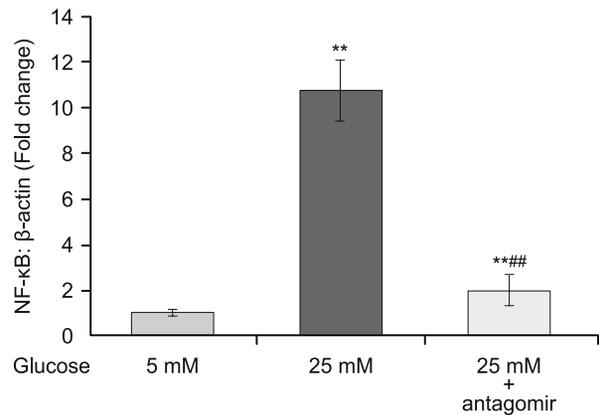
Transfection efficiency depicted that the miR-146a expression level was blocked by knockdown of miR-146a in hyperglycemic



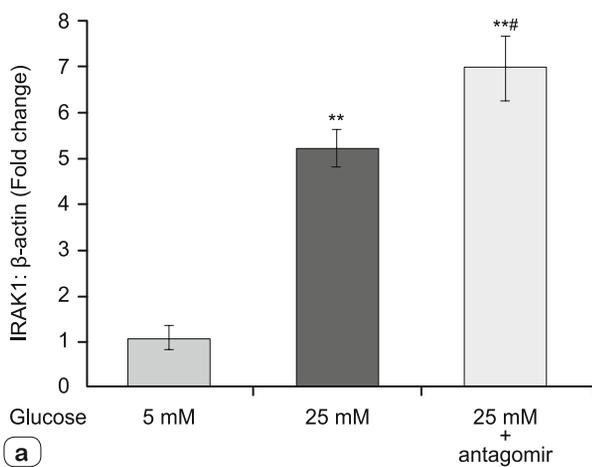
**Fig. 1.** The expression of miR-146a in HUVECs exposed to 5 mM and 25 mM D-glucose and osmotic control (5 mM D-glucose + 20 mM D-mannitol). Bars represent the Mean  $\pm$  SD; \*, \*\* and \*\*\* represent  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$  vs 5 mM D-glucose respectively; #, ## and ### represent  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$  vs 25 mM D-glucose, respectively; \$\$\$ represent  $p < 0.001$  vs 25 mM D-glucose + antagomir.



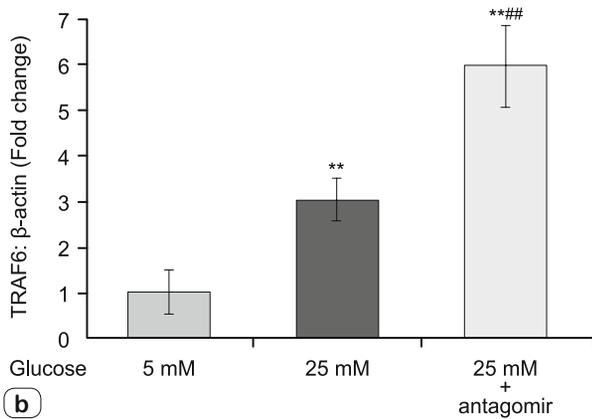
**Fig. 2.** ELISA measurements of NF-κB activity in HUVECs under normal (5 mM D-glucose) and hyperglycemic (25 mM D-glucose) conditions. Bars represent the Mean ± SD; Symbols are similar to those in Figure 1.



**Fig. 4.** The mRNA expression levels of NF-κB in HUVECs under normal (5 mM D-glucose) and hyperglycemic (25 mM D-glucose) conditions. Bars represent the Mean ± SD; Symbols are similar to those in Figure 1.



**(a)**



**(b)**

**Fig. 3.** Real-time quantitative RT-PCR analysis of IRAK1 (a) and TRAF6 (b) mRNAs in HUVECs undergoing conditions: normal (5 mM D-glucose), hyperglycemic (25 mM D-glucose) and hyperglycemic plus miR-146a antagomir (20 nM). Bars represent the Mean ± SD; Symbols are similar to those in Figure 1.

HUVECs when compared with 5 mM, and 25 mM glucose groups ( $p = 0.003$  and  $p = 0.0001$ , respectively). Such effect was not seen by scramble transfection.

*miR-146a modulates hyperglycemia-induced NF-κB activation in HUVECs*

Figure 2 shows that the NF-κB activity in HUVECs incubated with 25 mM of glucose was significantly higher than those incubated with 5 mM of glucose ( $p = 0.02$ ). Moreover, the NF-κB activity in hyperglycemic HUVECs transfected by miR-146a antagomir was markedly higher than untransfected cells ( $p = 0.043$ ). These results suggest an inhibitory role of miR-146a on the NF-κB activity in HUVECs.

*miR-146a reduces IRAK1 and TRAF6 mRNA expression levels in HUVECs*

Based on the hypothesis that miR-146a exerts its role through inhibition or degradation of TRAF6 and IRAK1, we measured the mRNA expression level of these two adaptor proteins using quantitative real time-PCR. Figure 3 indicates that HUVECs exposed to 25 mM glucose expressed a significant higher level of mRNA for IRAK1 and TRAF6 than those exposed to 5 mM glucose (Figs 3a and 3b, respectively). Knockdown of miR-146a significantly increased IRAK1 and TRAF6 mRNA expression levels in hyperglycemic HUVECs (Fig. 3a,b).

*miR-146a upregulates hyperglycemia-induced NF-κB mRNA in endothelial cells.*

Quantitative real time-PCR analysis of hyperglycemic HUVECs (25 mM D-glucose) confirmed a 10-fold increase in NF-κB mRNA expression compared with the 5 mM D-glucose cells (Fig. 4) ( $p = 0.007$ ). Figure 4 also shows that transfection of miR-146a antagomir significantly prevented from upregulation of NF-κB mRNA in hyperglycemic HUVECs ( $p = 0.009$ ); however, it was yet higher than in the control cells ( $p = 0.023$ ).

## Discussion

Recent studies suggest that miR-146a can serve as a potential biomarker and therapeutic targets for diabetic complications (18, 23). Although many studies have shown that miR-146a is altered in this disease, there is no consensus about the type of change (increase or decrease), and molecular underlying mechanisms. The present study was designed to evaluate the effect of 24-h hyperglycemia on miR-146a, NF- $\kappa$ B, TRAF6 and IRAK1 expression in HUVECs. Also, in order to evaluate the role of miR-146a, we transfected miR-146a antagomir in cultured HUVECs and measured the target gene expression levels as well as NF- $\kappa$ B activity.

The main finding of the current study was a marked upregulation of miR-146a in vascular endothelial cells under hyperglycemic condition. Furthermore, knockdown of miR-146a in these cells caused a significant increase in NF- $\kappa$ B activity and prevented from hyperglycemia-induced NF- $\kappa$ B mRNA expression.

It has been demonstrated that hyperglycemia can activate NF- $\kappa$ B by a range of intracellular signaling pathways such as overproduction of reactive oxygen/nitrogen species, advanced glycation end products, hexosamines and polyols, as well as protein kinase C isoforms (24). NF- $\kappa$ B in turn, enhances the expression of several genes that encode proinflammatory cytokines such as TNF- $\alpha$  and IL-6 inducing TLRs and promoting inflammation and cell death (5). It widely has been documented that IRAK1 and TRAF6, two-key adaptor kinases downstream of TLR superfamily play a significant role in NF- $\kappa$ B activation through phosphorylation and proteasome-mediated degradation of I $\kappa$ B protein (25-27). The results of the present study are in line with the above mentioned studies in which the NF- $\kappa$ B activity and mRNA expression level increased in HUVECs after 24-h hyperglycemic condition. Moreover, hyperglycemia increased IRAK1 and TRAF6 gene expression levels in these cells.

NF- $\kappa$ B is a member of structurally related eukaryotic transcription factor family which is found in almost all cell types and plays a critical role in responses to inflammatory signaling not only through TLRs, but also through TNF and IL-1 receptors (6, 25). In addition, this transcription factor regulates the expression of a large number of genes that are important for regulation of inflammation, apoptosis, and those linked to the complications of diabetes (28, 29).

NF- $\kappa$ B has been proposed to regulate its own activation partly through separate negative feedback loops by transactivation of several microRNAs such as miR-155, miR-34, miR-21, and miR-146a (4, 30). In this context, it is believed that NF- $\kappa$ B activation promotes transactivation of miR-146a (through binding to the NF- $\kappa$ B binding site in the promoter region) that, upon processing and maturation, enters to cytoplasm and prevents from translation of IRAK1 and TRAF6 mRNAs to proteins (12, 29, 31). These two adapter molecules activate I $\kappa$ B kinases (Ikks) which in turn phosphorylate I $\kappa$ B to release NF- $\kappa$ B from its inhibition (27). Indeed, it has been suggested that miR-146a negatively regulates the NF- $\kappa$ B activation by reducing IRAK1 and TRAF6 proteins (12). However, this negative feedback loop has not been demonstrated by some investigations (8, 17, 32).

Overexpression of miR-146a in hyperglycemic HUVECs in our study probably has resulted from the NF- $\kappa$ B activation for negative feedback purpose. Interestingly, this overexpression was not followed by reduction of IRAK1 and TRAF6 mRNAs. One explanation for this observed result may be the possibility that IRAK1 and TRAF6 are under other levels of control, such as TLRs activators like pro-inflammatory cytokines (17, 29). Decreased expression of miR-146a has been previously reported in diabetic mouse wounds (9), diabetic rats aorta, kidney, heart and dorsal root ganglia (10-12), glycated albumin- and high glucose-stimulated endothelial cells (13), and in serum and peripheral blood mononuclear cells of diabetic patients with types 1 and 2 diabetes (14-16). On the other hand, increased miR-146a expression has also been documented in the kidney and sciatic nerve of diabetic rats (17-19), as well as in the kidney, plasma and limbal corneal epithelium compartment of diabetic patients (18, 20, 21). Currently, the reason for different behavior of miR-146a in different tissues is unclear. But it may depend on tissue type, blood and tissue cytokines concentrations, timing, and duration of inflammation.

The present study also demonstrated that while miR-146a and NF- $\kappa$ B are upregulated in HUVECs under hyperglycemic condition, the knockdown of miR-146a increased the NF- $\kappa$ B activity probably through enhancement of IRAK1 and TRAF6 in these cells. Surprisingly, miR-146a antagomir caused a significant decrease in NF- $\kappa$ B expression level in HUVECs. The cause of this result is unclear for us now but it may be a compensatory response to the inhibitory effect of miR-146a under hyperglycemic condition. Further studies are required to confirm this result and to clarify the underlying mechanisms.

In conclusion, given that the activation of NF- $\kappa$ B is a key step in the progression of diabetic complications (6, 29), the results of present study may suggest that, miR-146a upregulation may play an anti-inflammatory effect in early phases of hyperglycemia. But, presumably a defect in the regulation of IRAK1 and TRAF6 can weaken the miR-146a regulatory negative feedback loop and provide a situation for sustained activation of NF- $\kappa$ B and its targets to promote cells toward abnormalities during hyperglycemic condition.

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