miRNA-1 regulates endothelin-1 in diabetes

Biao Feng a, Yanan Cao a,b, Shali Chen a, Michael Ruiz a, Subrata Chakrabarti a,b,*

a Department of Pathology, Western University, London, ON, Canada
b Mudanjiang Medical University, China

A B S T R A C T

Aims: MicroRNAs (miRNAs) play important roles in several biological processes. In this study, we investigated the role of miR-1, an endothelin-1 (ET-1) targeting miRNA, in endothelial cells (ECs) and tissues of diabetic animals. ET-1 is known to be of pathogenetic significance in several chronic diabetic complications.

Main methods: PCR array was used to identify alterations of miRNA expression in ECs exposed to glucose. miR-1 expression was validated by TaqMan real-time PCR assay. Human retinal ECs (HRECs) and human umbilical vein ECs (HUVECs) exposed to various glucose levels with or without miR-1 mimic transfection, and tissues from streptozotocin-induced diabetic animals after two months of follow-up, were examined for miR-1 expression, as well as ET-1 and fibronectin (FN) mRNA and protein levels.

Key findings: Array analyses showed glucose-induced alterations of 125 miRNAs (out of 381) in ECs exposed to 25 mM glucose compared to 5 mM glucose. Fifty-one miRNAs were upregulated and 74 were downregulated. 25 mM glucose decreased miR-1 expression and increased ET-1 mRNA and protein levels. miR-1 mimic transfection prevented HG-induced ET-1 upregulation. Furthermore, glucose induced upregulation of FN, which is mediated partly by ET-1, was also prevented by such transfection. Diabetic animals showed decreased miR-1 expression in the retina, heart and kidneys. In parallel, ET-1 mRNA expressions were increased in these tissues of diabetic animals, in association with upregulation of FN.

Significance: These results indicate a novel glucose-induced mechanism of tissue damage, in which miR-1 regulates ET-1 expressions in diabetes. Identifying such mechanisms may lead to RNA based treatment for diabetic complications.

© 2014 Elsevier Inc. All rights reserved.

Introduction

MicroRNAs (miRNAs) are highly conserved, ~22 nt long RNA molecules encoded in the genomes of plants and animals that negatively regulate gene expression (Bartel, 2005; Cuellar and McManus, 2005). Investigations have demonstrated the importance of miRNA-mediated regulation of gene expression in several disease processes, such as diabetes, cancer and neurodegeneration (Feng et al., 2013; Hashimoto et al., 2013; Thomson et al., 2011). miRNAs have emerged as a major mechanism in regulating basal and stress-induced alterations of gene expression. They are important in controlling cellular differentiation, proliferation, and apoptosis, as well as in providing feedback loops for various signal transduction pathways (Cuellar and McManus, 2005), miRNAs interact with the 3′ untranslated region (3′ UTR) of their target mRNA and negatively regulate gene expression post-transcriptionally (Bartel, 2009). Mature miRNA sequences are highly conserved among species and a single gene may be regulated by many different miRNAs (Soon and Kiaris, 2013). Conversely, one miRNA may regulate a large number of target genes (Soon and Kiaris, 2013; Rege et al., 2013).

Recent data from our and other laboratories have demonstrated the role of miRNA alterations in diabetic complications (McArthur et al., 2011; Feng et al., 2011; Putta et al., 2012; Feng and Chakrabarti, 2012; Natarajan et al., 2012). We have also shown that other epigenetic changes, such as histone acetylation, also interact with miRNAs in mediated transcriptional regulation in diabetes (McArthur et al., 2011). Data from our laboratory has demonstrated the role of several miRNAs in chronic diabetic complications (McArthur et al., 2011; Feng et al., 2011). To this extent, we have shown that miR-200b regulates VEGF and controls blood vessel permeability and angiogenesis in diabetic retinopathy (McArthur et al., 2011). We have also shown that miR-146a regulates increased extracellular matrix protein production in diabetic retinopathy and nephropathy (Feng et al., 2011). Finally, we and others have demonstrated alterations of miR-133a in cardiomyocyte hypertrophy in diabetes (Feng et al., 2010; Yildirim et al., 2013; Chavali et al., 2012).

Chronic diabetes leads to both structural and functional damage in the vasculature and causes secondary complications, including retinopathy, nephropathy, cardiomyopathy, and peripheral vascular disease. Endothelial cells (ECs) are the primary targets of glucose-induced cellular damage in chronic diabetic complications due to...
their innate ability to uptake glucose independent of insulin activity (Giacco and Brownlee, 2010; Madonna and De Caterina, 2011). Hyperglycemia induced oxidative stress is known to activate multiple signaling pathways which converge onto the cell nucleus, leading to increased expression of multiple genes causing EC dysfunction (Kunsch and Medford, 1999). EC dysfunction may lead to increased permeability and increased extracellular matrix (ECM) production. In particular, ECM alterations and basement membrane (BM) thickening are structural hallmarks of diabetic complications. An important mediator of these ECM alterations is endothelin-1 (ET-1). We and others have demonstrated upregulation of ET-1 in ECs and in organs affected by chronic diabetic complications (Khan and Chakrabarti, 2007; Chen et al., 2007; Sagar et al., 2013; Ergul, 2011). Furthermore, we have previously demonstrated that miR-320 regulates glucose induced ET-1 expression in indirectly through ERK1/2 signaling in ECs (Feng and Chakrabarti, 2012).

In the present study we investigated the role of miRNA regulation on ET-1, as well as its downstream target fibronectin (FN), in diabetic complications. We chose to focus on a specific miRNA, miR-1, which was found reduced in an array analysis following glucose exposure. miR-1 has widespread biological effects as it regulates multiple important transcripts. miR-1 has shown to be of importance in cardiac hypertrophy (Karakikes et al., 2013). Recently it has also been demonstrated that miR-1 is important in cardiomyocyte apoptosis in diabetes (Shan et al., 2010). In addition, it has been shown to be of importance in multiple malignancies (Hudson et al., 2012; Li et al., 2012). Based on bioinformatic analysis, miR-1 is predicted to regulate ET-1 (www. microrna.org). However, it is unclear if this potential regulatory relationship is relevant in respect to diabetic complications.

We focused on miR-1 in the regulation of ET-1 in glucose induced EC damage. As mentioned, ECs are primary targets for damage in diabetic complications. Thus, we used both microvascular Human Retinal Endothelial Cells (HRECs) and macrovascular Human Umbilical Vein Endothelial Cells (HUVECs) for this study. We have previously demonstrated glucose-induced alterations of gene expression in these cells (McArthur et al., 2011; Feng et al., 2011; Feng and Chakrabarti, 2012). We further expanded our analysis to the tissues from the diabetic animals. We hypothesize that downregulation of miR-1 in response to glucose induction mediates ET-1 upregulation, which then has wide-ranging downstream effects on ECM genes, such as FN, thus facilitating ECM accumulation in diabetic complications.

Materials and methods

Animals

All animals were cared for according to the Guiding Principle in the Care and Use of Animals. All experiments were approved by the University of Western Ontario Council on Animal Care Committee. Male C57BL/6 mice (20–25 g) were obtained from the Charles River Colony (Wilmington, MA, USA) and were randomly divided into control and diabetic groups. Methods of diabetes induction using streptozotocin (STZ) and monitoring have previously been described (McArthur et al., 2011). Total RNA (2 μg) was used for cDNA synthesis with cDNA reverse transcription kit (Applied Biosystems Inc., CA, USA) and was performed using a Roche LightCycler 96 as previously described (Putta et al., 2012; Feng and Chakrabarti, 2012) (Roche Diagnostics Canada, QC, Canada). The primer sequences have been listed in Table 1. β-Actin mRNA was used as an internal control for normalization.

Cell culture and treatments

All reagents were purchased from Sigma (Oakville, Ontario, Canada) unless specified. Human retinal microvascular endothelial cells (HRECs) were obtained from Olaf Pharmaceuticals (Worchester, MA). HUVECs were obtained from Bio-Whittaker (San Diego, CA). ECs were grown in endothelial cell basal medium 2 (EBM-2, Lonza, Walkersville, MD) supplemented with 5% fetal bovine serum, endothelial cell growth supplement (Bio-Whittaker), and 100 μg/ml penicillin/streptomycin. Cells were plated at a density of 1 × 10^5 cells/ml. They were treated with various levels of d-glucose. No insulin was present in any media. All cells were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C incubation and were passaged in 6 well plates (Corning, Acton, MA). Cells were treated with various levels of d-glucose or 25 mmol l-glucose (osmotic control) for 48 h.

After 24 h in serum-free, cells were transfected with miRiDIAN microRNA mimic miR-1 (100 nM) (DHARMACON Inc., Chicago, IL, USA) using transfection reagent Lipofectamine 2000 (Invitrogen, ON, Canada). Negative control miRNA (100 nM) was used for control transfection.

miRNA validation by real time RT-PCR

Reverse transcription was performed after isolating of miRNA (Life Technologies Inc., ON, Canada). TaqMan™ miR-1 assay (Ambion Inc., Austin, TX, USA) was used with quantitative real-time PCR (qRT-PCR) to analyze the expression of miR-1, in accordance with the manufacturer’s instructions. Normalization was performed to U6 snRNA to account for differences in reverse-transcription efficiencies and amount of template in the reaction mixtures.

miRNA analysis of miRNA expression

miRNA expression profiling was performed following the manufacturer’s protocol (Life Technologies, ON, Canada). In brief, 1 μg of isolated total miRNA was added with Megaplex™ RT primer, dNTPs, MultiScribe™ reverse transcriptase, RNase inhibitor, buffer to perform Megaplex reverse transcription. Following reverse transcription, the real-time PCR reaction mixture was prepared by mixing the Megaplex RT product with TaqMan universal PCR master mix, No AmpErase UNG (2 ×). The PCR reaction mixture was dispensed into each port of the TaqMan miRNA array card to run the array using the 384 well TaqMan low density array default thermal-cycling conditions in the 7900HT system (Life Technologies, ON, Canada). The results were shown using relative quantification (ΔΔCt).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5′ → 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET-1</td>
<td>5′ AGACGTTCACAGACGGTTAT 3′</td>
</tr>
<tr>
<td></td>
<td>5′ CGAACGTTCCTCACAATCTT 3′</td>
</tr>
<tr>
<td></td>
<td>6FAM-TGCCATACACGGAC-BHQ1</td>
</tr>
<tr>
<td>FN-1</td>
<td>5′ GTAATATACCAAGTGAGACG 3′</td>
</tr>
<tr>
<td></td>
<td>5′ CCCACATCTTGCGATGACGTA 3′</td>
</tr>
<tr>
<td></td>
<td>5′ CATCCTGACCCGTGGCTCGGC 3′</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5′ CTCCTATGCCAACACACCTGC 3′</td>
</tr>
</tbody>
</table>

Table 1

Oligonucleotide sequences for RT-PCR.
ELISA for ET-1 and FN expression

Supernatants were collected from cultured ECs. ELISA for FN was performed using a commercially available kit (Millipore, CA, USA) according to the manufacturer’s instructions. Similarly ET-1 was measured using specific ELISA kit (Enzo Life Sciences, Inc., NY, USA) following the manufacturer’s instructions.

Statistical analysis

All experimental data are expressed as means ± SE and were analyzed by ANOVA or Student t-test as appropriate. A p value of 0.05 or less was considered significant.

Results

miR-1 is downregulated in ECs exposed to elevated glucose levels in association with increased ET-1 and FN

Since hyperglycemia initiates damage in ECs, we focused our investigation on these cells. To this extent we screened two endothelial cell types, HRECs, a microvascular EC, and HUVECs, a macrovascular EC. To examine glucose related change in ET-1 expression, we investigated ET-1 mRNA expression in the HUVECs. We examined ET-1 mRNA levels at various glucose conditions. A dose dependent ET-1 mRNA upregulation was seen with various glucose levels, peaking at 25 mM glucose. No change was seen in 25 mM L-glucose (osmotic control). Then data are keeping with previous studies from ours and multiple other laboratories (McArthur et al., 2011; Feng et al., 2011; Feng and Chakrabarti, 2012; McGinn et al., 2003; Peng et al., 2013; Tien et al., 2013). Hence, in subsequent experiments we used 5 mM glucose simulating normoglycemia and 25 mM glucose (simulating hyperglycemia). Similar concentrations of glucose are also common in such studies by several laboratories (McArthur et al., 2011; Feng et al., 2011; Feng and Chakrabarti, 2012; McGinn et al., 2003; Peng et al., 2013; Tien et al., 2013). 25 mM glucose further caused upregulation of FN, a downstream target of ET-1 (Fig. 1). We then examined weather such mRNA changes are associated with alterations of protein levels. ELISA analysis demonstrated glucose induced upregulation of protein levels of ET-1 and FN (Fig. 1). Similarly 25 mM glucose induced changes were presented in HRECs. In parallel, we examined the alterations in miRNA expression between ECs exposed to 5 mM glucose and 25 mM glucose using a miRNA PCR-array. We observed alterations of 125 miRNAs (out of 381) in ECs exposed to 25 mM glucose (HG) compared to 5 mM glucose. Fifty-one miRNAs were upregulated and 74 were downregulated (Suppl. Table 1).

Several open sourced softwares (www.TargetScan.org, www.microrna.org, www.ebi.ac.uk) were used for miRNA target predictions. miR-1, identified to be significantly downregulated in the ECs exposed to 25 mM glucose, was found to be associated with ET-1. We verified the downregulation of miR-1 with qRT-PCR in both HRECs and HUVECs (Figs. 1, 2). No change in miR-1 level was observed when the cells were challenged with 25 mM L-glucose (osmotic control).

miR-1 regulates glucose induced upregulation of ET-1 and ECM protein in the ECs

To establish a cause–effect relationship, we chose to focus on HRECs as an in vitro model system, as microvascular endothelial damage affecting the retina is of particular clinical significance. We and others have shown that ECs exposed to high levels of glucose (simulating hyperglycemia) recapitulate molecular and functional features of diabetic vascular pathologies, including upregulation of ET-1 and FN (McArthur et al., 2011; McGinn et al., 2011; Giacco and Brownlee, 2010; Madonna and De Caterina, 2011; Kunsch and Medford, 1999).

In parallel to decreased miR-1 level upon exposure to 25 mM glucose conditions, mRNA levels of ET-1 were increased. Such increases were prevented by miR-1 mimic transfection (Fig. 2). Transfection efficiency, assessed by measuring miR-1 expression with qRT-PCR,
showed >8 fold increase in intracellular miR-1 expression compared to scrambled miRNA transfection. We did similar experiments with ECM matrix protein FN as we have shown FN is a downstream target of ET-1 in hyperglycemia. Glucose induced FN mRNA upregulation in the HRECs was significantly prevented by miR-1 mimic transfection (Fig. 2).

As microRNAs are post-transcriptional modifiers, we further examined protein levels of FN and ET-1 using ELISA. In parallel to the mRNA data, miR-1 transfection prevented glucose-induced upregulation of FN and ET-1. No effects were seen when cells were transfected with scrambled mimic (Fig. 2).

miR-1 is downregulated in the tissues of diabetic animals: in association with increased ET-1

We then wanted to see whether such changes are indeed of significance in a clinically relevant model of diabetic complications. To this extent, STZ-induced diabetic mice showed hyperglycemia (blood glucose of diabetics 23.0 ± 3.6 mmol/L vs controls 7.4 ± 1.1 mmol/L, p < 0.01) and reduced body weight (body weight of diabetics 29.4 ± 2.1 g vs controls 23.5 ± 1.3 g, p < 0.001). We examined retinal, renal cortical and cardiac tissues from STZ diabetic mice, two months after onset of diabetes. All of these tissues are targets of chronic diabetic complications and augmented ET-1 induced increased ECM protein FN production has been previously demonstrated in these organs (Khan and Chakrabarti, 2007; McGinn et al., 2003). Diabetic animals showed hyperglycemia, glucosuria and reduced body weight gain (data not shown). Analyses of miR-1 levels demonstrated significantly decreased levels in retinal, cardiac and renal tissues of these animals (Fig. 3). Such reductions were associated with increased levels of ET-1 and FN (Fig. 3).

Discussion

In this research, we have described a novel mechanism of ET-1 regulation in diabetes. Using both in vivo and in vitro systems, we demonstrated that that miR-1 is downregulated in ECs following exposure to high glucose and in the target organs affected by chronic diabetic complications. Furthermore, this process leads to glucose-induced upregulation of ET-1, a key mechanism by which extracellular matrix protein FN is regulated in diabetes. Following initial identification of glucose induced down-regulation of miR-1 by a PCR based array, in association with upregulation of ET-1 and FN, we validated such changes in two different endothelial cell systems. We further established functional significance of these changes at the mRNA and protein levels using miR-1 mimic transfection and normalized glucose-induced upregulation of ET-1. Our data indicates that miR-1 regulates glucose-induced ET-1 upregulation in the ECs and that such process mediates ECM protein FN production. It has to be noted that FN is not a direct target of miR-1 (www.targetscan.org), but is induced following ET-1 induction by high glucose.

We used two well-characterized ECs to identify the in vitro biologic significance. These are well-studied cell systems in vascular biology, including the study of diabetic complications (Onat et al., 2011). To study functional significance we used HRECs, which are of significance in the context of a major chronic diabetic complication, i.e., diabetic retinopathy. In parallel, we demonstrated importance of this process in the retina, kidneys and heart of diabetic animals, in a well-established model
of chronic diabetic complications (Tesch and Allen, 2007; Shen and Bornfeldt, 2007).

Although miR-1 alteration has recently been described in other biological and disease processes, this is the first report of miR-1 mediated ET-1 regulation in the context of chronic diabetic complications. Recently it has been shown that miR-1 inhibits proliferation of hepatocellular carcinoma cells through modulating ET-1 (Li et al., 2012). In the context of chronic diabetic complications, miR-1 downregulation has been shown to contribute to diabetes induced oxidative stress through regulation of junctin (Yildirim et al., 2013). Also, miR-1 has been thought to be a mediator of non-diabetic cardiac hypertrophy (Fichtlscherer et al., 2011). Circulating miR-1 levels have been correlated with occurrence of myocardial infarction (Ai et al., 2010).

The current study demonstrates potential therapeutic implication of miR-1 in chronic diabetic complications, as it is altered in multiple organs affected by chronic diabetic complications. It is possible that manipulation of miR-1 may work through alteration of ET-1 as well as several other molecules as described above. Most target mRNA predictions for miRNAs stem from computational analysis examining sequence complementarity (Vlachos and Hatzigeorgiou, 2013). It is well accepted that a single miRNA (e.g. miR-1) has multiple targets. This may be an advantage in therapy, as multiple genes can be corrected. However, at the same time, these interactions may pose therapeutic challenges via off-target effects. As seen in this study, the levels of miR-1 alteration do not parallel ET-1 level (Figs. 1, 2). Exact reason for this discrepancy is not known. As noted above that multiple miRNAs may control a single transcript. Hence it is possible that simultaneously other miRs are controlling ET-1 expression, producing additional effects. In keeping with this notion, we have previously showed that miR-320 also regulates glucose induced ET-1 upregulation (Feng and Chakrabarti, 2012).

As functions of various miRNAs and their role in biological processes become clearer, several of them appear to play significant roles in chronic diabetic complications. We have previously demonstrated the role of miR-133a in diabetic cardiomyopathy (Feng et al., 2010). Other investigators have shown the role of miR-192 in diabetic nephropathy (Putta et al., 2012; Kato et al., 2007). We have also recently demonstrated downregulation of miR-200b and miR-146a in several chronic diabetic complications (McArthur et al., 2011; Feng et al., 2011). The mechanisms which hyperglycemia causes cellular damage in the context of chronic diabetic complications are indeed complex and are not fully understood. However, it is accepted that glucose-induced oxidative stress plays an important role (Giacco and Brownlee, 2010; Madonna and De Caterina, 2011; Kaur et al., 2006). Such oxidative stress causes DNA damage and modifies transcription machinery through the activation of the redox-sensitive transcription factors (Matsumoto et al., 2008). Increased oxidative stress causes and altered expression of a number of genes, including ET-1 (McGinn et al., 2003; Matsumoto et al., 2008). All such transcripts may however be post transcriptionally regulated by miRNAs. Hence, miRNAs lend themselves to be therapeutic agents. However, the challenge for miRNA research is to define the function of specific miRNAs in various tissues and in the context of specific disease states.

In summary, we have identified a specific miRNA, i.e., miR-1, that has importance in regulating ET-1 in chronic diabetic complications. A possible mechanistic summary diagram has been presented in Fig. 4. Understanding such novel pathways will help to better understanding

![Diagram](image_url)
of the pathogenesis of chronic diabetic complications and will pave the way towards the development of novel adjuvant treatments.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.lfs.2013.12.199.

Conflict of interest statement

No interests disclosed.

Acknowledgment

Supported by grants from the Canadian Diabetes Association and Heart and Stroke Foundation of Ontario (SC).

References


