Cellular Signalling 44 (2018) 28–32

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

miRNA15a regulates insulin signal transduction in the retinal vasculature

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ARTICLE INFO

Keywords:
miR15a
Retinal endothelial cells
Insulin signaling

ABSTRACT

We previously reported that tumor necrosis factor alpha (TNFα) could inhibit insulin signal transduction in retinal cells. We recently found that miR15a/16 also reduced TNFα in retinal endothelial cells (REC) and in vascular specific miR15a/16 knockout mice. Since in silico programs suggested that miR15a could directly bind the insulin receptor, we wanted to determine whether miR15a altered insulin signal transduction. We used a luciferase-based binding assay to determine whether miR15a directly bound the insulin receptor. We then used Western blotting, ELISA, and qPCR to investigate whether miR15a altered insulin signaling proteins in REC and in both miR15a/16 endothelial cell knockout and overexpressing mice. We also treated some REC with resveratrol to determine if resveratrol could increase miR15a expression, since resveratrol is protective to the diabetic retina. We found that miR15a directly bound the 3'UTR of the insulin receptor. Treatment with resveratrol increased miR15a expression in REC grown in high glucose. While total insulin receptor levels were not altered, insulin signal transduction was reduced in REC grown in high glucose and was restored with treatment with resveratrol. miR15a knockout mice had reduced insulin receptor phosphorylation and Akt2 levels, with increased insulin receptor substrate 1 (IRS-1) phosphorylation on serine 307, a site known to inhibit insulin signaling. In contrast, overexpression of miR15a increased insulin signal transduction. Taken together, these data suggest that miR15a binds the insulin receptor and indirectly regulates insulin receptor actions. It also offers an additional mechanism by which resveratrol is protective to the diabetic retina.

1. Introduction

Diabetes will reach epidemic levels by 2030. A deeper understanding of the eye-specific effects of diabetes is needed in order to provide strategies for non-surgical treatments that could be effective at early stages to prevent or protect against disease progression. Impaired insulin signaling is a hallmark of both type 1 and type 2 diabetes. While a great deal of work has been done on insulin resistance in muscle, liver, and fat cells, much less is known for the retina. Work has shown increased levels of tumor necrosis factor alpha (TNFα), as well as reduced insulin receptor signaling in the retina, particularly in Müller cells, photoreceptors, and endothelial cells [1–6]. While it is clear that insulin resistance occurs in retinal cells, restoration of normal insulin signaling has remained elusive.

Insulin signaling is a very complex process and occurs primarily in adipocytes, liver, and skeletal muscle [7, 8]. Much work has focused on the mechanisms involved in insulin resistance in the insulin-responsive tissues, while less is known in other organs, such as the retina. We have reported that TNFα can increase insulin receptor substrate 1 (IRS-1)^{Ser307} phosphorylation in retinal endothelial cells (REC), which blocks insulin signal transduction [6]. In addition to insulin receptor and IRS-1, work also supports a role for Akt2 and the forkhead transcription factor (Foxo1A) in insulin resistance [7].

We have previously reported that inhibition of TNFα can restore normal insulin signaling in the retina [6]. We also recently reported that miR15a can reduce TNFα levels in REC grown in high glucose [9], as well as retinal leukostasis. In an attempt to combine these findings, we searched targetscan.org to determine if there was a common link between miR15a and TNFα. miR15a directly targets insulin receptor and IRS-1 [10]. While literature prior to 2014 does not report that miR15a targets the insulin receptor [11, 12], recent literature does support a role for miR15a in the diabetic retina [13]. They found that miR15a was decreased in REC, as well as in diabetic rat retina [13]. Work in type 2 diabetic humans also reported a significant decrease in miR15a, and showed an association of miR15a with type 2 diabetes or pre-diabetes [14]. Typically, miRNA repress translation [15]. However, during times of stress, specific miRNA have been shown to increase target mRNA expression [16–18]. Work on miR693–3 showed that it binds TNFα through direct base pairing to increase target mRNA expression during serum starvation [17]. This increase in target mRNA

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https://doi.org/10.1016/j.cellsig.2018.01.016
Received 12 December 2017; Received in revised form 10 January 2018; Accepted 11 January 2018
Available online 12 January 2018
0898-6568/

expression seems to occur more commonly in quiescent cells versus cycling cells [19]. Since REC is a fairly quiescent cell exposed to the stressor of high glucose, it is possible that miR15a can increase insulin receptor expression and signaling.

For therapeutic development, we attempted to use resveratrol (RSV) to increase miR15a levels, since work in leukemia cells showed a significant increase in miR15a and miR16 after RSV treatment [20]. We chose RSV for this retinal study, as RSV has been suggested as a therapy for diabetic retinopathy through decreased oxidative stress and NFkB levels [21, 22]. In an ocular ischemia/reperfusion model, RSV improved the electroretinogram (ERG) responses [23].

In this study, we used both conditional knockout and overexpressing mice for miR15a, as well as REC in culture, to investigate the actions of miR15a on insulin signal transduction in the retinal vasculature.

2. Materials and methods

2.1. Luciferase binding assay

Primary human retinal endothelial cells (REC) were acquired from Cell Systems Corporation (CSC, Kirkland, Washington). REC cells were grown in Cell Systems Medium supplemented with microvascular growth factors (MVGS). Cells were grown in normal glucose (5 mM) medium. Antibiotic, 10μg/mL gentamycin, and 0.25μg/mL amphotericin B (Invitrogen, Carlsbad, CA) were added into media. Cells were grown on attachment factor coated dishes and transferred into a 96 well plate at 80% confluence the day before transfection. Only cells prior to passage 6 were used.

We purchased the 3'UTR of the insulin receptor tagged with luciferase from Genecopoeia (Rockville, MD). We transfected the 3'UTR of the insulin receptor into REC. Additional cells were co-transfected with the miR15a mimic using duofect (Dharmacon). Some cells were transfected with the luciferase receptor with a negative control, while other cells received only the empty vector. Samples were processed for luciferase activity using the LightSwitch Assay kit (SwitchGear Genomics, Inc.), according to manufacturer's instructions. Luciferase activity was measured using a SpectraMax L luminometer.

2.2. Retinal endothelial cell culture (REC)

Primary human retinal endothelial cells (REC) were acquired from Cell Systems Corporation (CSC, Kirkland, Washington). REC cells were grown in Cell Systems Medium (normal glucose) or high glucose medium. Some cells were grown in normal glucose medium with 20 mM mannitol added as an osmotic control. (25 mM glucose) supplemented with microvascular growth factors (MVGS). Antibiotic, 10μg/mL gentamycin, and 0.25μg/mL amphotericin B (Invitrogen, Carlsbad, CA) were added into the media. Cells were grown on attachment factor coated dishes. Only cells prior to passage 6 were used. Some cells were treated with resveratrol (50uM, [24]) to activate miR15a in the REC.

Additional cells were transfected with miR15a inhibitors (30 nM, Invitrogen) or a negative control prior to treatment with resveratrol.

2.3. qPCR

Total RNA was isolated and purified using the Trizol method and the purity and quantity of RNA were measured using Synergy HTX multi-mode reader (BioTek; Winooski, VT). For polyA tailing reverse-transcriptase PCR, 5 μg of total RNA was treated with DNase I for 15 min at room temperature (Promega; Madison, WI) and then added polyA using (polyA) polymerase (NEB; Ipswich, MA) at 37 °C for 1 h. The final reaction mixtures were extracted with phenol/chloroform, precipitated with isopropanol, and re-dissolved in 25 μl diethylpyrocarbonate (DEPC)-treated water using previously published methods [9, 25]. The SYBR-Green-based real-time PCR was performed using the CFX Connect PCR system (BioRad; Hercules, CA). The relative expression of miRNA was calculated based on the formula: $2^{(-\text{Delta Delta Ct})}$. Delta–Delta Ct values are Delta Ct $_{\text{exp.}}$–Delta Ct $_{\text{cont.}}$.

2.4. Mice

All animal procedures were reviewed and approved by the Institute Animal Care and Use Committees of the Wayne State University School of Medicine (Protocol #A 11-08-14) and conform to NIH guidelines. These experiments were done using endothelial cell specific miR15a knockout mice [9] and miR15a overexpressing mice [26] at 3 months of age.

2.5. Western blotting

Whole retinal lysates or REC samples were rinsed with cold PBS, collected in lysis buffer containing protease and phosphatase inhibitors, and scraped into tubes. Equal amounts of protein were separated on precast tris-glycine gels (Invitrogen, Carlsbad, CA), and then blotted onto a nitrocellulose membrane. After blocking in TBST (10 mM Tris–HCl buffer, pH 8.0, 150 mM NaCl, 0.1% Tween 20) and 5% (w/v) BSA, the membrane was treated with appropriate primary antibodies followed by incubation with secondary antibodies labeled with horseradish peroxidase. Primary antibodies used were phosphorylated insulin receptor (Tyr 1150/1151), total insulin receptor, phosphorylated Akt (Ser473), Akt, phosphorylated IRS-1 (Ser307), total IRS-1 (Cell Signaling Corp, Danvers, MA), and beta actin (Santa Cruz, Santa Cruz, CA). Antigen–antibody complexes were detected using a chemiluminescence reagent kit (Thermo Scientific, Pittsburgh, PA). Mean densitometry of immunoreactive bands was assessed using a C500 (Azure Biosystems), and results were expressed in densitometric units and compared to control groups for each individual experiment. For the phosphorylated antibodies, the ratio of phosphorylated to total protein levels are presented.

2.6. ELISA

A cleaved caspase 3 ELISA was done according to manufacturer's instructions (Cell Signaling Corp, Danvers, MA). An insulin ELISA was done according to manufacturer's instructions (Sigma, St. Louis, MO).

2.7. Statistical analyses

Non-parametric Kruskal-Wallis with Dunn's post-hoc tests were used for the cell culture data. One-way ANOVA with Student Newman Keul's post-hoc test was used for animal work. $P < .05$ was considered statistically significant.

3. Results

3.1. miR15a directly binds the insulin receptor

Insulin receptor is a target or miR15a based upon targetscan.org. We verified this in silico finding using REC grown in normal glucose transfected with the 3'UTR of the insulin receptor and miR15a mimics. Fig. 1 shows that luciferase activity is reduced when the 3'UTR of the insulin receptor is bound to miR15a. There is no change in luciferase activity when the reporter alone is used, reporter without miR15a, or an empty vector. This data indicate that miR15a does directly bind to the insulin receptor.

3.2. Resveratrol increases miR15a expression

Literature from cancer work suggested that resveratrol increased miR15a levels in leukemia models [20]. Resveratrol has been suggested as a therapy for diabetic retinopathy [21, 22]. Therefore, we wanted to