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miRNA15a regulates insulin signal transduction in the retinal vasculature

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Abstract

We previously reported that tumor necrosis factor alpha (TNF α) could inhibit insulin signal transduction in retinal cells. We recently found that miR15a/16 also reduced TNF α 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.

Keywords

miR15a; retinal endothelial cells; insulin signaling

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1.0 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 (TNF α), 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 TNF α 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 TNF α can restore normal insulin signaling in the retina [6]. We also recently reported that miR15a can reduce TNF α levels in REC grown in high glucose [9], as well as retinal leukostasis. In an attempt to combine these findings, we searched targets can.org to determine if there was a common link between miR15a and TNF α . 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 TNF α through direct base pairing to increase target mRNA expression during serum starvation [17]. This increase in target mRNA expression seems to occur more commonly in quiescent cells versus cycling cells [19]. Since REC are 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.

2.0 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 (5mM) medium. Antibiotic, 10ug/mL gentamycin, and 0.25ug/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 20mM mannitol added as an osmotic control. (25mM glucose) supplemented with microvascular growth factors (MVGS). Antibiotic, 10ug/mL gentamycin, and 0.25ug/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 (30nM, 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: $2^{(-\Delta\Delta Ct)}$. $\Delta\Delta Ct$ values are $\Delta Ct_{exp} - \Delta Ct_{cont}$.

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.

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 (10mM 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 chemilluminescence 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.

2.5 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).

2.6 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. $P < 0.05$ was considered statistically significant.

3.0 Results

3.1 miR15a directly binds the insulin receptor

Insulin receptor is a target of 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. Figure 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 determine if resveratrol could increase miR15a expression in REC. Figure 2 confirms that treatment with resveratrol significantly increased miR15a expression in REC.

3.3 Resveratrol increased insulin receptor phosphorylation and Akt2 levels, while reducing IRS-1^{Ser307} and cleaved caspase 3

We have previously reported that insulin signaling is decreased in REC grown in high glucose [27]. The goal was to determine if resveratrol could reverse the high glucose-induced loss of insulin signaling in REC. REC grown in high glucose had reduced levels of insulin receptor and Akt phosphorylation (Figure 3A, C). Treatment with resveratrol significantly increased both insulin receptor and Akt phosphorylation in the cells grown in high glucose. Conversely, high glucose culturing conditions increased IRS-1^{Ser307} levels, as well as the cleavage of caspase 3 (Figure 3B, D). Resveratrol reduced the high glucose-induced changes in IRS-1 and caspase 3 in REC. Taken together, data suggest that resveratrol can regulate insulin signaling in REC grown in high glucose.

3.4 Resveratrol activates miR15a to regulate insulin signal transduction

Since RSV increased insulin signal transduction, we wanted to determine if this occurred through miR15a. Figure 4A shows that high glucose and the miR15a inhibitor both significantly decreased insulin receptor phosphorylation, while RSV increased insulin receptor phosphorylation. RSV was unable to increase insulin receptor phosphorylation when RSV was combined with the miR15a inhibitor, suggesting that miR15a is key to regulating insulin receptor phosphorylation. In contrast to the work on insulin receptor, high glucose and miR15a inhibitors increased IRS-1^{Ser307} (Figure 4B). RSV significantly decreased IRS-1 phosphorylation compared to HG, but this was blocked when RSV +miR15a inhibitor was used. Similar to insulin receptor, phosphorylation of Akt was decreased upon the loss of miR15a, and RSV could not rescue Akt phosphorylation when miR15a inhibitors were used (Figure 4C). Taken together, these data suggest that resveratrol increases miR15a expression to increase insulin receptor signaling in REC grown in high glucose.

3.5 Loss of miR15a reduces insulin receptor signaling in endothelial cell specific knockout mice

We have previously reported that miR15a/16 conditional knockout mice have decreased inflammatory marker levels [9]. Since we have shown that TNF α can regulate insulin signaling in other rat models [28, 29], we wanted to investigate whether insulin signaling was altered in the miR15a knockout mice. We used the miR15a/16 floxed and CreLox mice to explore whether miR15a regulated insulin signaling *in vivo*. Data show that loss of miR15a decreased insulin receptor (Figure 5A) and Akt phosphorylation (Figure 5C), while IRS-1^{Ser307} phosphorylation was increased (Figure 5B), which agrees with data in Figure 4 in REC treated with miR15a inhibitors.

3.6 Overexpression of miR15a increases insulin receptor actions

To support our findings on the actions of miR15a in mouse retina, we also used miR15a overexpressing mice [13]. We found that when miR15a was overexpressed in the retinal vasculature, there was an increase in total insulin levels (Figure 6A). Accordingly, there was also increased insulin receptor (Figure 6B) and Akt phosphorylation (Figure 6D), with decreased IRS-1^{Ser307} phosphorylation (Figure 6C).

4.0 Discussion

Defective insulin signaling is a hallmark of both type 1 and type 2 diabetes and can lead to complications in the retina, kidney, heart, and nervous system. We have previously reported that β -adrenergic receptors could restore normal insulin signal transduction in retinal endothelial and Müller cells [30, 31]. β -adrenergic receptor agonists also reduced key inflammatory mediators, such as TNF α , which are inhibitory to insulin signaling [30, 32]. Thus, when we found that miR15a significantly reduced TNF α levels in the retina [9], we questioned whether miR15a could regulate retinal insulin signaling.

Using Targetscan.org, we found that miR15a should directly bind the insulin receptor 3'UTR. We performed a luciferase-based assay and confirmed that miR15a does directly bind the insulin receptor in REC. We used miR15a/16 endothelial cell-specific knockout mice and miR15a transgenic mice to measure insulin receptor actions. Interestingly, loss of miR15a/16 decreased insulin receptor and Akt phosphorylation, which was associated with increased IRS-1^{Ser307} and cleaved caspase 3 levels. Typically, miRNA will repress translation of the mRNA. We did not see a change in total insulin receptor levels, but we found that miR15a increased insulin receptor phosphorylation in the retinal vasculature. While unexpected, work in other cell types has shown that some miRNA can increase actions of their binding targets [17, 19]. Since we did not see a change in total insulin receptor levels, the actions of miR15a on insulin receptor signaling are likely indirect. Future work will dissect the exact mechanisms by which miR15a regulates insulin receptor activation.

Since it is difficult to activate miRNA for drug development, we used resveratrol (RSV) to increase miR15a expression based upon work in leukemia cells showing an effect of resveratrol on miR15a [20]. We also chose to use RSV, as it has been shown to play a role in diabetic retinopathy. Work in streptozotocin-treated rats and mice have reduced retinal leakage, reduced VEGF levels, decreased NF κ B levels, and decreased apoptosis after RSV treatment [21, 22]. RSV improves the ERG of diabetic rats, as well as increase levels of glutamine synthase in Müller cells grown in high glucose [33]. We found treatment with RSV significantly increased miR15a expression. It also increased insulin receptor and Akt phosphorylation in REC grown in high glucose. These actions of RSV occurred through activation of miR15a, since RSV could not rescue insulin receptor signaling when miR15a inhibitors were used. Future studies will address the specific mechanisms by which RSV can regulate miR15a; however, our work does provide another potential explanation for the effectiveness of RSV treatment of diabetic animals [33, 34].

Taken together, our data support that *in silico* programs showing that the insulin receptor is a directly target of miR15a. While we did not find changes in total insulin receptor levels in

the mice, we did find that miR15a regulated insulin receptor phosphorylation and downstream signaling. Additionally, RSV significantly increased miR15a expression, which was key to RSV actions on insulin signal transduction in REC.

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Highlights

- miR15a binds the insulin receptor
- loss of miR15a in the retinal vasculature decreased insulin signaling
- resveratrol increased miR15a expression

Figure 1.

Luciferase activity of retinal endothelial cells treated with the 3'UTR for insulin receptor (reporter), the reporter and miR15a mimic (reporter+miR15a), the reporter with a negative control mimic (reporter+neg), or the empty vector. * $P < 0.05$ vs. reporter only. N=4.

Figure 2.

miR15a expression levels in retinal endothelial cells grown in normal glucose (NG), normal glucose plus 20mM mannitol as osmotic control (NG+Mannitol), or high glucose (HG). Some cells in NG and HG were treated with resveratrol (RSV). * $P < 0.05$ vs. NG, # $P < 0.05$ vs. HG. $N = 5$ for all groups.

Figure 3.

Western blotting for the ratio of phosphorylated insulin receptor to total insulin receptor (A), phosphorylated IRS-1 on serine 307 to total IRS-1 (B), phosphorylated Akt to total Akt (C) in retinal endothelial cells grown in normal glucose (NG), normal glucose plus 20mM mannitol as osmotic control (NG+Mannitol), or high glucose (HG). Some cells in NG and HG were treated with resveratrol (RSV). Panel D is ELISA results for the cleavage of caspase 3 in the same groups of cells. *P<0.05 vs. NG, #P<0.05 vs. HG. N=5 for all groups.

Figure 4.

Western blotting for the ratio of phosphorylated insulin receptor (A), phosphorylated IRS-1 on serine 307 to total IRS-1 (B), and phosphorylated Akt to total Akt (C) in retinal endothelial cells grown in normal glucose (NG), normal glucose plus 20mM mannitol as osmotic control (NG+Mannitol), or high glucose (HG). Some cells in HG were treated with resveratrol (RSV), a miR15a inhibitor, a negative inhibitor as a control or miR15a inhibitor and RSV. *P<0.05 vs. NG, #P<0.05 vs. HG. N=5 for all groups.

Figure 5.

Western blotting for ratio of phosphorylated insulin receptor to total insulin receptor (A), phosphorylated IRS-1 on serine 307 to total IRS-1 (B), and phosphorylated Akt to total Akt (C) in whole retinal lysates from miR15a floxed mice or cdh5-miR15a-CreLox mice.

*P<0.05 vs. miR15a floxed. N=5 for all mice.

Figure 6.

An ELISA for total insulin levels (A) and Western blotting for the ratio of phosphorylated insulin receptor to total insulin receptor (B), phosphorylated IRS-1 on serine 307 to total IRS-1 (C), and phosphorylated Akt to total Akt (D) in whole retinal lysates from control littermates or miR15a overexpressing mice. * $P < 0.05$ vs. control littermates. $N = 5$ for all mice.