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ANGIOPOIETIN-LIKE 8 (ANGPTL8) EXPRESSION IS REGULATED BY miR-143-3p IN HUMAN

HEPATOCYTES

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Abstract

Angiopietin-like protein 8 (ANGPTL8) is associated with reduced HDL-cholesterol levels and may contribute to the development of dyslipidemia. Factors regulating ANGPTL8 expression remain poorly understood. Here we analyzed the relationship between miRNA-143-3p and ANGPTL8 in liver cells. Using target prediction algorithms, we identified a putative binding site for miR-143-3p in the ANGPTL8 3' untranslated region (3'UTR). Exogenous miR-143-3p interacted with the ANGPTL8 3'UTR to downregulate its expression compared to scrambled sequence control. Transfection of HepG2 cells with miR-143-3p mimic or siRNA resulted in decreased or increased ANGPTL8 transcript and protein levels, respectively. Treatment of HepG2 cells with 30 mM glucose, 100 nM insulin, or 75 ng/mL lipopolysaccharide to mimic hyperglycemic, hyperinsulinemic, and proinflammatory conditions corresponded with increased miR-143-3p and ANGPTL8 levels. Inhibition of miR-143-3p amplified ANGPTL8 response to these treatments, suggesting that the miRNA acts to suppress ANGPTL8 expression under metabolically distorted conditions. These results, combined with growing evidence supporting a role for ANGPTL8 in the regulation of HDL-C metabolism, provide a better understanding of the molecular mechanisms underlying ANGPTL8 expression.

Keywords

microRNA; miRNA; miR-143-3p; betatrophin; angipietin-like protein 8; HDL-C; inflammation; hyperglycemia; atherosclerosis

1. Introduction

Dietary composition, cigarette smoking, sedentary lifestyle, and genetic factors are known to affect lipid levels and contribute to the development of dyslipidemia [1–3]. A number of studies have identified genetic variants with significant effects on lipoprotein concentrations or association with earlier onset of clinical vascular disease [4]. One variant, located in the angipietin-like 8 (*ANGPTL8*) gene, has been associated with unfavorable lipid profiles across a number of independent studies [5–7], including one in which we reported lower

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HDL-C levels in American Indian and Mexican American individuals carrying the variant allele [8]. ANGPTL8 (also known as betatrophin, lipasin, TD26, LOC55909, C19orf80, and RIFL) belongs to a family of secreted proteins with key roles in lipid trafficking and metabolism [5, 9–19]. ANGPTL8 is predominantly expressed in the liver and inhibits lipoprotein lipase [15, 18, 20], an enzyme that breaks down dietary lipids for uptake in peripheral tissues through mechanisms that may include masking of its catalytic site [21]. In humans, serum ANGPTL8 concentrations were lower in dyslipidemic patients compared to individuals with normal lipid levels and were associated with low HDL-C levels [22]. Together, these findings point toward a relationship between ANGPTL8 and HDL-C concentration.

Fasting, hyperinsulinemia, and hyperglycemia [5, 8, 15, 18] have been reported to influence levels of ANGPTL8, but little is known of the molecular mechanisms that mediate its expression. MicroRNAs (miRNAs), small noncoding RNA molecules that regulate gene expression, have emerged as important components contributing to the regulation of lipoprotein and lipid metabolism, as well as cholesterol homeostasis [23, 24]. As miRNAs are known to fine-tune expression of many genes, including ANGPTL3 [25], we postulated that ANGPTL8 might also be affected by miRNA-mediated regulation. Indeed, a recent study showed that miR-221–3p regulated ANGPTL8 expression in adipocytes [26]. In this study, we sought to identify miRNAs with functional consequences on ANGPTL8 expression. We used target prediction algorithms to identify miRNA-binding sites in the ANGPTL8 3' untranslated region (3'UTR). One candidate, miR-143–3p, was found to directly interact with the ANGPTL8 3'UTR, yield functional effects on ANGPTL8 expression in HepG2 cells, and respond to treatment with glucose, insulin, and lipopolysaccharide.

2. Methods

2.1. Prediction of potential ANGPTL8-interacting miRNAs

We used the miRWalk2.0 algorithm [27, 28] to identify miRNAs predicted to bind to the ANGPTL8 3'UTR sequence. The identified miRNA binding sites were then compared to the miRNA-target prediction program TargetScanHuman 7.1 [29].

2.2. Cell culture

HepG2 (ATCC; Manassas, VA) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) according to the recommended guidelines. Cells were grown in 24-well culture dishes (VWR International; Radnor, PA) containing 1.0 mL cell culture medium and placed at 37°C in a Hera Cell 5% CO₂ incubator (Thermo Fisher Scientific; Waltham, MA). Culture medium was replaced the first day after seeding, and then every 48 hours thereafter.

2.3. Dual Luciferase Reporter assay

pmirGLO Dual-Luciferase miRNA Target Expression vectors (Promega Corp; Madison, WI) containing either the miR-143–3p target sequence in the ANGPTL8 3'UTR or scrambled sequence were constructed and sequence-verified (Emory Integrated Genomics Core;

Atlanta, GA). Approximately 10,000 HepG2 cells/well were seeded on a 96-well plate. After incubation at 37°C for 24 hours, cells were co-transfected with 100 ng of 3'UTR luciferase reporter vector and 100 nM miR-143-3p silencing RNA (siRNA), mimic, or scrambled sequence control (Dharmacon; Lafayette, CO) using 0.3 µl of Lipofectamine 2000 (Thermo Fisher Scientific; Waltham, MA) per well. Luciferase activity was measured 24 hours following transfection using the Dual-Glo Luciferase Assay System (Promega Corp; Madison, WI). For each sample, the ratio of firefly luciferase activity to renilla luciferase activity was determined, which was then used to estimate fold-change difference of experimental conditions compared to the scrambled sequence control. Experiments were performed using six biological replicates.

2.4. Cell transfection and total RNA extraction

Approximately 300,000 HepG2 cells/well were seeded on a 6-well plate and incubated at 37°C until 60% confluent. Cells were transfected with 7.5 µL Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific) containing 100 nM mirVana miR-143-3p mimic or siRNA, and a scrambled sequence control (Thermo Fisher). Total RNA for the miR-143-3p mimic and siRNA treatments was extracted at 48 and 72 hours post-transfection, respectively, using the RNeasy kit (Qiagen; Germantown, MD) according to the manufacturer's protocol. RNA concentration was determined by absorbance at 260 nm using the NanoDrop 2000 spectrophotometer (Thermo Scientific; Wilmington, DE). Verification of miR-143-3p over-expression and knockdown was determined using qPCR as described below.

2.5. Quantitative real-time PCR (qPCR) analysis

ANGPTL8 transcript levels were measured using TaqMan primers and the TaqMan RNA-to-Ct 1-Step kit (Thermo Fisher Scientific) in conjunction with the QuantStudio 6 Flex Real-Time PCR system (Thermo Fisher Scientific). Data were normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH). For miR-143-3p, total RNA was first converted to cDNA using the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific), and transcript levels were measured using the TaqMan MicroRNA Assay. Data were normalized against U6 snRNA. For both ANGPTL8 and miR-143-3p, cycle threshold levels were generated using QuantStudio Real-Time PCR Software 1.0 (Thermo Fisher Scientific). The $-Ct$ method was used to determine fold-change of expression. All experiments were performed in triplicate.

2.6. Total protein extraction and quantification and enzyme-linked immunosorbent assay (ELISA) analysis

Culture media from HepG2 cells was collected into 1.5 ml tubes and centrifuged at 5000 x *g* for 5 minutes at 4°C to remove particulates. The supernatant was concentrated using Amicon tubes (EMD Millipore; Billerica, MA) according to the manufacturer's protocol. Total protein concentrations for each sample were determined using the bicinchoninic acid assay (Thermo Fisher Scientific). Secreted levels of ANGPTL8 were measured using a commercial sandwich ELISA kit according to the manufacturer's protocol (EIAab; Wuhan, China). ANGPTL8 levels were determined as a percentage of total soluble protein (%TSP).

2.7. Cell treatments

Prior to all treatments, HepG2 cells were serum-starved overnight. The following treatments were performed using serum-free media: insulin (final concentration =100 nM), high glucose (final concentration =30 mM), 3-O-methyl-D-glucopyranose (final concentration =30 mM) as an osmotic control, and lipopolysaccharide (LPS: final concentration =75 ng/mL) for two hours. To investigate the role of miR-143-3p in treatment effects, HepG2 cells were serum-starved overnight, treated with miR-143-3p for 24 hours, and then subjected to individual treatments. After two hours, RNA was extracted and ANGPTL8 transcript levels were measured as described.

2.8. Statistical analysis

Data were analyzed as means $\pm$ standard deviation (SD) from three independent assays using Microsoft Excel. The Student's t-test (two-tailed) was used to assess differences between conditions. A *P*-value <0.05 was considered statistically significant.

3. Results

3.1. miR-143-3p functionally interacts with the ANGPTL8 3'UTR

We analyzed the ANGPTL8 3'UTR to identify putative miRNA binding sites. We observed a predicted binding site for miR-143-3p, which has been previously implicated in the regulation of circulating cholesterol levels [30] and obesity-induced insulin resistance [31]. The predicted miR-143-3p binding site aligns to sequence position 866–872 in the ANGPTL8 3'UTR (Figure 1A). To determine whether miR-143-3p regulates ANGPTL8 expression, we assessed the interaction between the miRNA and the ANGPTL8 3'UTR using a dual luciferase reporter assay. Co-transfection of a construct containing the ANGPTL8 3'UTR and miR-143-3p siRNA resulted in a significant increase in relative luciferase activity compared to scrambled sequence controls (Figure 1B). In the presence of miR-143-3p mimic, we observed a significant decrease in relative luciferase activity compared to control conditions. These results indicate that miR-143-3p regulates expression of the luciferase construct containing the ANGPTL8 3'UTR.

3.2. miR-143-3p regulates ANGPTL8 expression in HepG2 cells

We next transfected HepG2 cells with miR-143-3p mimic or siRNA and measured levels of ANGPTL8 transcript and protein. In the presence of miR-143-3p mimic, ANGPTL8 transcript (Figure 2A) and protein (Figure 2B) levels decreased 2.1-fold and 20% (*p*<0.05), respectively. Treatment with miR-143-3p siRNA resulted in a 1.3-fold and 40% increase in ANGPTL8 transcript (Figure 2C) and protein (Figure 2D) levels, respectively (all *p*<0.05).

3.3. Hyperglycemia, insulin and LPS regulate miR-143-3p in HepG2 cells

Previous studies have shown that glucose, insulin, and LPS significantly upregulate ANGPTL8 expression in liver cells [8, 15, 32]. To determine whether these treatments also affect miR-143-3p expression, we treated HepG2 cells with glucose, insulin, and LPS and measured miRNA levels. As shown in Figure 3A-C, levels of miR-143-3p increased in hepatocytes by 3.0-fold, 1.7-fold, and 2.7-fold (all *p*<0.05), in response to treatment with

glucose, insulin, and LPS, respectively. To determine whether previously reported changes in ANGPTL8 expression in response to glucose, insulin, and LPS are mediated by miR-143-3p, we treated cells as described in the presence or absence of miR-143-3p siRNA. Consistent with earlier findings, we found that treatment with glucose, insulin, and LPS increased ANGPTL8 levels (2.1-fold, 1.7-fold, and 1.2-fold, respectively; $p < 0.05$); in the presence of miR-143-3p siRNA, ANGPTL8 levels further increased to 4.1-fold, 3.8-fold, and 2.9-fold under these conditions (Figure 4A-C).

4. Discussion

The major findings of this study were 1) miR-143-3p regulated expression of a luciferase construct containing the ANGPTL8 3'UTR; 2) addition of exogenous miR-143-3p resulted in decreased levels of ANGPTL8 transcript and protein, while knockdown of miR-143-3p expression corresponded with increased ANGPTL8 levels; and 3) levels of miR-143-3p increased in response to treatment with high glucose, insulin, and LPS in HepG2 cells. We also confirmed earlier results showing regulation of ANGPTL8 levels by glucose, insulin, and LPS in hepatocytes, and our findings indicated that miR-143-3p might contribute to these effects. To our knowledge, this study provides the first evidence demonstrating that miR-143-3p regulates ANGPTL8 expression via a regulatory element in the 3'UTR of the gene.

ANGPTL8 gained prominence in the literature as a regulator of β -cell expansion in mice [33], a finding that failed to be replicated in subsequent studies [34–36]. However, a role for ANGPTL8 in lipid trafficking and metabolism has been confirmed across numerous independent investigations. ANGPTL8 has been shown to regulate serum triglyceride levels [5, 15, 18] by inhibiting lipoprotein lipase, leading to reduced triglyceride clearance [18, 37–39]. Overexpression of ANGPTL8 corresponds with elevated triglyceride levels [5, 18], while knockdown of ANGPTL8 leads to reduction of triglyceride concentrations associated with decreased LDL-C secretion and increased activity of lipoprotein lipase in mice [20, 35].

In humans, ANGPTL8 levels have been positively correlated with obesity, insulin resistance, and type 2 diabetes (T2D) in many, but not all, studies [40, 41]. ANGPTL8 levels have also been reported to be increased in patients with hypertension [42] and surgically induced weight loss [43]. In contrast, circulating ANGPTL8 concentrations were decreased in patients with dyslipidemia, defined as either low HDL-C or high TG levels [22]. In these individuals, differences in ANGPTL8 levels were more marked in those with dyslipidemia defined by low HDL-C concentrations, as opposed to hypertriglyceridemia, suggesting that ANGPTL8 may be a stronger determinant of HDL-C-related dyslipidemia. Concordant with these findings, an arginine-to-tryptophan substitution at position 59 in the ANGPTL8 sequence has been consistently associated with low HDL-C levels, but with no apparent effects on triglyceride concentrations [5–8]. The association of the variant allele with reduced HDL-C levels suggests that reduced ANGPTL8 activity may lead to increased HDL-C concentration. Interestingly, in metabolic syndrome patients with the lowest levels of ANGPTL8, the greatest increases in HDL-C levels in response to a hypocaloric diet were observed [44].

The involvement of miRNAs as central regulators of lipoprotein metabolism and cholesterol homeostasis is now well established [23] and miR-143 has been previously implicated in related phenotypes. For example, genetic ablation of the miR-143/145 cluster in LDLR^{-/-} mice caused a marked decrease in circulating cholesterol, but not triglyceride, levels compared to LDLR^{-/-} animals [30]. In these animals, plasma levels of very-low density lipoprotein (VLDL) and low-density lipoprotein (LDL) cholesterol were also decreased compared to the LDLR^{-/-} mice. Overexpression of miR-143 in mouse models of obesity was associated with impaired insulin secretion and glucose homeostasis, while animals with decreased miR-143 levels were protected from obesity-induced insulin resistance [31]. A recent study reported that overexpression of miR-143-3p resulted in increased insulin-stimulated lipogenesis in human in vitro differentiated adipocytes [45].

Our findings that miR-143-3p expression is regulated in liver cells by hyperglycemia and insulin are concordant with its involvement in impaired glucose homeostasis and insulin signaling [31, 45]. Previous studies have shown that miR-143 levels are higher in vascular smooth muscles cells (SMCs) from diabetic individuals compared to healthy controls, but in vitro treatment of SMCs with high glucose or insulin produced no significant effect on miRNA expression [46]. Similarly, miR-143-3p upregulation in the presence of LPS in the current study is consistent with findings of increased circulating leukocyte levels of the miRNA in healthy male volunteers following LPS infusion [47]. In that study, miR-143 levels were also associated with downregulation of pro-inflammatory genes. We observed that treatment with miR-143-3p siRNA increased ANGPTL8 transcript levels in response to high glucose, insulin, and LPS, suggesting that the miR-143-3p-ANGPTL8 interaction may participate in pathways by which chronic inflammation produces changes in lipid metabolism and contributes to the development of dyslipidemia [48]. Together, these findings point to a potential mechanism by which miR-143-3p contributes to the effects of hyperglycemia, insulin, and LPS on atherogenic dyslipidemia, possibly through regulation of ANGPTL8 expression, and further work to elucidate this interaction is warranted.

Previous work recently demonstrated that ANGPTL8 expression was regulated by miR-221-3p in adipocytes [26]. In that study, the authors also identified a binding site for miR-143-3p in the ANGPTL8 3'UTR; however, they did not observe expression changes in primary adipocytes in response to macrophage-conditioned media, suggesting that this miRNA may not be regulated by inflammation in fat cells. It is possible that tissue-specific ANGPTL8 expression patterns in response to glucose, insulin, and inflammation are mediated, in part, by miRNAs. Additional work will be necessary to explore this possibility.

Despite the significance of this study in delineating miR-143-3p-mediated regulation of ANGPTL8 expression, we acknowledge that the focus on a single miRNA is a limitation of this work, given that many miRNAs are predicted to interact with ANGPTL8. Likewise, we only looked for miRNAs with predicted complementarity to the 3'UTR, although other regions of the ANGPTL8 transcript are expected to be targets of miRNA-mediated regulation. Clearly, additional studies of miRNA-mediated regulation of this gene are warranted, given its role in HDL metabolism and atherogenic dyslipidemia.

In summary, our results provide evidence that miR-143-3p functionally interacts with ANGPTL8. We also demonstrate that transfection of HepG2 cells with miR-143-3p siRNA leads to reductions in ANGPTL8 transcript and protein levels, and that the miRNA may dampen effects of hyperglycemia, insulin, and LPS on ANGPTL8 expression. These results, combined with growing evidence supporting a role for ANGPTL8 in the regulation of HDL-C metabolism, provide a better understanding of the molecular mechanisms underlying ANGPTL8 expression.

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Abbreviations

ANGPTL8	Angiopoietin-like protein 8
HDL-C	High density lipoprotein cholesterol
3'UTR	3' untranslated region
siRNA	Silencing RNA
LPS	Lipopolysaccharide
CAD	Coronary artery disease
LDL-C	Low-density lipoprotein cholesterol
miRNA	MicroRNA
ANGPTL3	Angiopoietin-like protein 3
FBS	Fetal bovine serum
qPCR	Quantitative real-time PCR
ELISA	Enzyme-linked immunosorbent assay
SD	Standard deviation
T2D	Type 2 diabetes
LDLR	Low-density lipoprotein receptor
SMC	Smooth muscles cell

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Highlights

- miR-143-3p functionally interacts with the ANGPTL8 3' untranslated region.
- miR-143-3p regulates expression of ANGPTL8 transcript and protein levels.
- Glucose, insulin and lipopolysaccharide increase miR-143-3p levels in liver cells.
- miR-143-3p contributes to effects of glucose, insulin, and lipopolysaccharide on ANGPTL8 expression.

Figure 1.

A) Predicted alignment of miR-143-3p with the ANGPTL8 3' UTR. The miR-143-3p target site was predicted to align to position 866–872 in the ANGPTL8 3' UTR. TargetScanHuman 7.1 and miRWalk2.0 were used to identify predicted miRNA binding sites in this region. Vertical lines indicate paired sequence alignment between miRNA sequence and ANGPTL8. **B) miR-143-3p directly interacts with the ANGPTL8 3' UTR.** HepG2 cells were co-transfected with 3' UTR luciferase reporter vector and miR-143-3p siRNA, mimic, or scrambled sequence control miRNA (sc-miR) or scrambled sequence control luciferase vector (sc-con), and luciferase activity was measured 24 hours post-transfection. For each sample, the ratio of firefly luciferase activity to renilla luciferase activity was determined, which was then used to estimate fold-change difference of experimental conditions compared to the scrambled sequence control. Experiments were performed in six biological replicates. Data is presented as mean $\pm$ SD. * $p < 0.05$.

Figure 2.

A) miR-143-3p overexpression is associated with decreased levels of ANGPTL8 transcript and B) protein. HepG2 cells were transfected with miR-143-3p mimic for 48 hours, following which, total RNA and protein were extracted as described in the METHODS section. A) Relative quantification of ANGPTL8 transcript was assessed using RT-qPCR. Protein concentration was quantified using ELISA and data are shown as percent total soluble protein (%TSP). All experiments were performed in triplicate. Data are presented as mean ± SD. *p < 0.05. **C) Transfection with miR-143-3p siRNA is associated with increased levels of ANGPTL8 transcript and D) protein.** HepG2 cells were transfected with miR-143-3p siRNA for 72 hours, after which, total RNA and protein were extracted as described in the METHODS section. Relative quantification of ANGPTL8 transcript was assessed using RT-qPCR. Protein concentration was quantified using ELISA and data are shown as percent total soluble protein (%TSP). All experiments were performed in triplicate. Data are presented as mean ± SD. *p < 0.05.

Figure 3.

Levels of miR-143-3p increase in response to hyperglycemia, insulin, and LPS

treatment. HepG2 cells were treated with A) 30 mM glucose, B) 100 nM insulin, and C) 75 ng/mL LPS, after which, total RNA was extracted as described in the METHODS section. Relative quantification of miR-143-3p was assessed using RT-qPCR. All experiments were performed in triplicate. Data are presented as mean $\pm$ SD. * $p < 0.05$.

Figure 4.
Effects of miR-143-3p silencing on high glucose, insulin, and LPS-induced upregulation of ANGPTL8 transcript. HepG2 cells were treated with A) 30 mM glucose, B) 100 nM insulin and C) 75ng/mL LPS in the presence or absence of miR-143-3p siRNA. Relative quantification of ANGPTL8 transcript was assessed using RT-qPCR. All experiments were performed in triplicate. Data are presented as mean $\pm$ SD. * $p < 0.05$.