Regulation of Cardiac miR-208a, an Inducer of Obesity, by Rapamycin and Nebivolol

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Objective: Resistance to obesity is observed in rodents and humans treated with rapamycin (Rap) or nebivolol (Neb). Because cardiac miR-208a promotes obesity, this study tested whether the modes of actions of Rap and Neb involve inhibition of miR-208a.

Methods: Mouse cardiomyocyte HL-1 cells and Zucker obese (ZO) rats were used to investigate regulation of cardiac miR-208a.

Results: Angiotensin II (Ang II) increased miR-208a expression in HL-1 cells. Pretreatment with an AT1 receptor (AT1R) antagonist, losartan (1 μM), antagonized this effect, whereas a phospholipase C inhibitor, U73122 (10 μM), and an NADPH oxidase inhibitor, apocynin (0.5 mM), did not. Ang II-induced increase in miR-208a was suppressed by Rap (10 nM), an inhibitor of nutrient sensor kinase mTORC1, and Neb (1 μM), a 3rd generation β-blocker that suppressed bioavailable AT1R binding of 125I-Ang II. Thus, suppression of AT1R expression by Neb, inhibition of AT1R activation by losartan, and inhibition of AT1R-induced activation of mTORC1 by Rap attenuated the Ang II-induced increase in miR-208a. In ZO rats, Rap treatment (750 μg kg⁻¹ day⁻¹; 12 weeks) reduced obesity despite similar food intake, suppressed cardiac miR-208a, and increased cardiac MED13, a suppressor of obesity.

Conclusions: Rap and Neb suppressed cardiac miR-208a. Suppression of miR-208a and increase in MED13 correlated with attenuated weight gain despite leptin resistance.

Introduction

The worldwide epidemic of obesity now affects more than 2 billion people, costing the world’s economy $2 trillion per year (1). Because obesity is a risk factor for cardiovascular disease and diabetes (2-4), it is critical to examine the causal mechanisms and interrelationships of these diseases. Elegant studies have established the significance of cardiac microRNA miR-208a in cardiac development and pathology (5-8).Encoded by intron 27 of the cardiac-specific α-myosin heavy chain (MHC) gene Myh6, miR-208a is a member of the miR-208 family of miRNAs. Up-regulation of miR-208a in response to stress suppresses transcriptional repression of β-cardiac muscle myosin heavy chain gene (Myh7) and promotes switching of expression from αMHC to βMHC, resulting in myocardial contractile dysfunction. A unique trait of miR-208a is that pharmacological inhibition of miR-208a by anti-miR-208a confers resistance to obesity (9). This effect of miR-208a is attributed to its ability to inhibit Mediator Complex 13 (MED13) synthesis, since overexpression of cardiac-specific MED13 confers resistance to obesity and controls systemic energy homeostasis (9). MED13 is also known as thyroid hormone-associated protein 1 (THRAP1) and is implicated in the anti-obesity effects of thyroid hormone signaling (9).

Although β-adrenergic receptor (AR) blockers are effective in treating cardiovascular diseases (10-12), some cause weight gain (13). However, reports from our lab and others indicate that treatment with the 3rd generation β-blocker nebivolol (Neb) inhibits weight gain (14,15). The exact mechanisms by which Neb inhibits weight gain are unclear. It is known that the serine/threonine kinase mammalian target of rapamycin complex 1 (mTORC1) integrates inputs from nutritional stimuli with the cellular growth machinery, and activation of mTORC1 is central to the onset of obesity (4,16,17).
We have reported that Neb attenuates activation of mTORC1 in heart tissues of Zucker obese (ZO) rats, an animal model for hyperinsulinemia, leptin resistance, and obesity (14). Therefore, inhibition of mTORC1 could be the mechanism by which Neb suppresses body weight gain in ZO rats. It is not known whether suppression of cardiac miR-208a, which can also induce resistance to weight gain, is involved in Neb’s mode of action.

Chronic rapamycin (Rap) treatment also inhibits weight-gain in rodent models of obesity (18-20). Originally identified as an anti-fungal compound, Rap is now widely used as an immunosuppressant and anticancer therapeutic (21,22). Rap is the canonical inhibitor of mTOR (mammalian target of rapamycin). Though the modes of action of Rap and Neb are different, both drugs confer resistance to obesity and attenuate mTORC1. We hypothesized that signaling by both Rap and Neb converges in a common mechanism that involves mTORC1 and miR-208a. To test this idea, we assessed the effects of angiotensin II (Ang II), Rap and Neb on mTORC1 activation and miR-208a expression using mouse cardiomyocyte HL-1 cells (23). We also tested whether chronic Rap treatment of leptin-resistant and hyperphagic ZO rats causes resistance to weight gain that is correlated with suppression of cardiac miR-208a and increased expression of cardiac MED13. Data presented here show for the first time that Rap and Neb inhibit AT1R-induced activation of mTORC1 and also inhibit up-regulation of miR-208a expression. Moreover, we report that chronic Rap treatment conferred resistance to weight gain in leptin resistant ZO rats, attenuated cardiac miR-208a expression and increased the obesity suppressor cardiac MED13.

**Methods**

**Cell culture and treatments**

Mouse atrial cardiomyocyte HL-1 cells were a gift from Dr. William Claycomb at Louisiana State University Medical Center and were cultured as described previously (18,23). Neb was a gift from Forest Laboratories (New York) and Rap was purchased from Cell Signaling Technology (Boston, MA). Human Ang II was purchased from Sigma-Aldrich. Losartan (AT1R inhibitor), PD123319 (AT2R inhibitor), apocynin (NADPH oxidase inhibitor), and U73122 (phospholipase C inhibitor) were purchased from TOCRIS Biosciences.

**Rap treatment of rats, body composition, food intake, and tissue collection**

All animal procedures used in this study were approved by the Harry S. Truman Veterans Memorial Hospital (HSTVMH) Subcommittee for Animal Safety and University of Missouri IACUC before commencing. All animals were cared for in accordance with the Guidelines for the Care and Use of Laboratory Animals (National Institutes of Health publication 85-23). Zucker obese (fa/fa) (ZO) and lean (ZL) rats (Charles River Laboratories) were used in this study. Rats were maintained on ad libitum food and water and housed singly at the HSTVMH animal housing facility under standard laboratory conditions (room temperature: 21-22°C; light and dark cycles: 12 h). Food intake was monitored by placing a pre-weighed amount of food in the cage and determining the weight of leftover food after 24 h. At 8 weeks of age, rapamycin pellets designed to deliver Rap at a concentration of 750 μg kg⁻¹ day⁻¹ for 21 days (from Innovative Research of America, Sarasota, FL) or placebo pellets were placed surgically under the skin behind the shoulder blades under brief isoflurane anesthesia and this procedure was repeated three times to achieve a 12-week treatment. Body composition was determined using the EchoMRI 4in1/1100 as described previously (18). Hearts were harvested at time of sacrifice as described before (18), flash frozen in liquid nitrogen, and stored at −80°C for future use.

**RNA isolation, quantitative RT-PCR, and immunoblotting**

The mirVana miRNA isolation kit (Ambion) was used for isolation of miRNA and mRNA. Quantitative RT-PCR (qRT-PCR) was performed as described previously (18). Taqman microRNA assay primers for miR-208a and small nuclear RNA (snRNA) (Taqman microRNA Assays) and rat MED13 and 18S RNA primers (Gene Expression Assays) were from Applied Biosystems. Experiments were performed in triplicate for each biological sample. Relative quantification (RQ) values were obtained by determining ΔΔCt values followed by determining ΔCt values and then RQ values via the equation 2(−ΔΔCt).

Cell lysates of HL-1 cells were prepared and Western blotting was performed as described previously (18). Experiments were performed in at least in triplicate for each biological sample. All antibodies except anti-β-MHC antibody were from Cell Signaling Technology Inc. (Boston, MA). The mouse monoclonal anti-β-MHC antibody which is highly specific for Myh7 product was from Sigma, (St. Louis, MO; Mouse monoclonal anti-myosin (skeletal, slow) antibody, M8421). The blots were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline-Tween 20 (TBST) for one hr. After blocking, PVDF membranes were probed with primary antibodies (1:1000 dilution of each antibody) for mTOR, phospho-mTOR (Ser2448), p70S6K, phospho-p70S6K (Thr389), RP56, phospho-RPS6 (Ser235/236). Jak2, phospho-Jak2 (Tyr1007/1008), STAT1, phospho-STAT1(Tyr701), or β-MHC, in 5% BSA in TBST overnight and washed with TBST prior to the addition of horseradish peroxidase-conjugated secondary antibody (1:20,000). After 1-h incubation at room temperature with secondary antibodies and washing with TBST, chemiluminescent substrate (Supersignal West Femto Maximum Sensitivity Substrate kit; Thermo Scientific) was used to visualize antibody binding. Images were captured using a Bio-Rad ChemiDoc XRS image-analysis system. Quantitation of phosphorylated protein band density normalized to the density of total protein or protein band density normalized to the density of β-actin band for each sample, was performed using Quantity One software (Bio-Rad Laboratories, Berkeley, CA). Data are reported as the normalized protein band density in arbitrary units.

**Immunofluorescence**

Immunofluorescence was used to determine the changes in the expression of β-MHC in HL-1 cells in response to different treatments. Briefly, HL-1 cells were grown on cover slips as described previously (18). All treatments were performed in triplicates. After treatments with Ang II (100 nM; 12 h) or Neb (1 μM; 12 h) cover-slips were washed with HEPES (Sigma), fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.5% Triton-X-100, washed with HEPES-T (1 mL Tween-20/L), and blocked with 1% bovine serum albumin (BSA) (Jackson Immuno Research), 10% goat serum (Sigma) and 0.1% Tween 20 (Fisher Scientific). Cells were incubated with anti-β-MHC antibody (Sigma)
(1:100 dilution) overnight at 4°C and repeatedly washed with HEPES. The coverslips were then incubated with Alexa Fluor 488 goat anti-rabbit (Invitrogen Inc.) (1:200 dilution) for 1 h at room temperature. Coverslips were washed with HEPES and mounted with Fluoroshield with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) and visualized using a Leica DMI 4000B inverted confocal microscope using Leica Application Suite software. Imaging was done at 60x magnification using oil immersion.

Radioligand binding studies

HL-1 cardiomyocytes were incubated in serum-free Claycomb medium with or without Neb (1 μM) or losartan (1 μM) or PD123319 (5 μM) for 12 h. Each treatment was performed at least in triplicate. Cells were collected by trypsinization (0.05% trypsin), and resuspended in ice-cold Claycomb medium. The number of cells per sample was determined using Millipore Scepter and cell numbers in all samples were adjusted to have 10^6 cells mL^-1. Next, 200 μL of cell suspension for each treatment group (untreated or Neb-, losartan-, and PD123319-treated) was washed with HEPES and mounted with Fluoroshield with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) and visualized using a Leica DMI 4000B inverted confocal microscope using Leica Application Suite software. Imaging was done at 60x magnification using oil immersion.

Figure 1 Ang II activates, while Rap and Neb suppress, Ang II activated mTORC1 signaling and miR-208a expression in mouse cardiomyocyte HL-1 cells. (A) qRT-PCR data expressed as RQ values show that Ang II (100 nM; 12 h) increases miR-208a while the AT1R blocker losartan suppressed Ang II-induced increases in miR-208a expression. Neither the phospholipase C inhibitor U73122 (10 μM) nor the NADPH oxidase inhibitor apocynin (0.5 mM) could effectively suppress Ang II-induced miR-208a expression. (B) qRT-PCR data expressed as RQ values show that addition of Neb (1 μM) or Rap (10 nM) to the incubation medium during the overnight treatment suppresses the Ang II-induced increase in miR-208a expression. n ≥ 3 treatments for each treatment group except for Ang II. Because Ang II treatment was repeated with each data set shown in Figure 1A and B and in Figure 3, biological replicates for Ang II was ≥ 10. qRT-PCR was performed in triplicate for RNA isolated from each biological sample. Values are means ± SEM. *P < 0.05 for Ang II treated vs. untreated (Con). #P < 0.05 for Ang II treated vs. Neb, Rap, or losartan treated. Representative images of autoradiograms showing elevated levels of (C) pSer2448 mTOR, (D) pThr389 S6K1, and (E) pSer235/236 RPS6 in HL-1 cells incubated with Ang II (100 nM; 12 h) and suppression of this effect by co-treatment with Neb (10 nM), Rap (1 μM), or the AT1R blocker losartan (1 μM), but not by AT2R blocker PD123319 (5 μM). Images are from same gel with intervening lanes excluded for clarity. Graphs show results of densitometric analysis of the intensity of the phosphorylated protein bands after adjusting for the intensity of total protein bands (tMTOR, tS6K1, tRPS6). Values are means ± SEM. N ≥ 3 for each treatment group. **P < 0.05 for untreated (Con) vs. Ang II treated or PD + Ang II treated. #P < 0.05 for Ang II treated vs. Neb, Rap, or losartan treated.
was added to achieve a final concentration of 1 nM. After a 2-h incubation on ice, cells were collected by centrifugation, washed three times with ice-cold PBS and the radioactivity in the pellet was monitored using Perkin Elmer-Wallac Wizard 1480 gamma counter. Specific binding for the cells from each group was determined by subtracting the counts obtained from cell pellets that were incubated in the preincubation medium containing non-radioactive Ang II (1 μM).

Specific binding obtained for the untreated sample under these conditions was taken as 100%. The % specific binding of 125I-Ang II to HL-1 cells after each treatment in comparison with the untreated sample was calculated.

**Statistics**

The SPSS 20 software package was used for statistical analysis. Results were expressed as means ± SEM (standard error of mean). Differences among groups were tested by using One-Way ANOVA followed by Tukey’s test or Student’s t test, as appropriate, and two-tailed P values are reported. A P value of ≤0.05 was considered statistically significant.

**Results**

**Effects of angiotensin II, Rap, and Neb on miR-208a expression in HL-1 cardiomyocytes**

We investigated whether Ang II would increase, and Neb and Rap would suppress Ang II-induced miR-208a expression in HL-1 cardiomyocytes. Ang II treatment (100 nM: 12 h) of serum starved HL-1 cardiomyocytes increased miR-208a expression by approximately fivefold (Figure 1A). Pretreatment with AT1R blocker losartan (1 μM) suppressed this effect; however, pretreatment with the NADPH oxidase inhibitor apocynin (0.5 mM) and phospholipase C (PLC)
inhibitor U73122 (10 μM) did not significantly attenuate the Ang II-induced increase in miR-208a expression (Figure 1A). Importantly, Rap (10 nM) and Neb (1 μM) suppressed the Ang II-induced increase in miR-208a expression (Figure 1B). These observations suggest that the Ang II-induced increase in miR-208a expression is mediated via a mechanism regulated by Rap and Neb. Because Rap inhibits mTORC1, we tested whether Ang II activated mTORC1, and whether Rap and Neb suppressed this effect in HL-1 cells. We examined phosphorylation (p) status of Ser2448 of mTOR, Thr389 of p70 S6 kinase (S6K1), the substrate of mTORC1, and Ser235/236 of Ribosomal Protein S6 (RPS6), the substrate of S6K1. Ang II (100 nM:12 h) increased stimulatory phosphorylation of mTOR (pSer2448), S6K1 (pThr389) and RPS6 (p Ser235/236), and this effect was suppressed by pretreatment with Rap (10 nM), Neb (1 μM), and losartan (1 μM), but not by the AT2R inhibitor PD123319 (1 μM) (Figure 1C-E). Thus, Ang II activates mTORC1 via AT1R, while both Rap and Neb suppress this effect.

Increased miR-208a is reported to increase β-myosin heavy chain (βMHC) protein levels (5-8). To further confirm that Ang II and Neb treatments have opposing effects on βMHC expression, we treated HL-1 cardiomyocytes with Ang II (100nM:12hr), Neb (1μM:12hrs), and Ang II plus Neb (100 nM and 1 μM, respectively: 12 h). Western blotting analysis showed that Ang II increased the expression of βMHC in HL-1 cells and Neb suppressed the Ang II-induced increase (Figure 2A). Immunofluorescence analysis further confirmed Neb’s inhibitory effect on βMHC expression (Figure 2B). Western blotting analysis also showed that Rap (10nM:12hrs) suppressed the Ang II-induced increase in βMHC expression (Figure 2C).

**Mode of action of Neb in miR-208a regulation**

Because Neb suppressed Ang II-induced increases in miR-208a we investigated which Neb-mediated signaling mechanism(s) contributed to this effect. In addition to being a β1-AR blocker, Neb is known to act as an agonist of β3-AR and also to activate endothelial nitric oxide synthase (eNOS) and AMP kinase (27-31). Therefore, it is conceivable that Neb may inhibit miR-208a expression via mechanisms independent of Ang II-induced AT1R activation, such as activation of β3-AR, NOS, or AMP kinase. However, treatment with β3-AR blocker SR59230 (10 μM), AMP kinase inhibitor Compound C (10 μM) or NOS inhibitor L-NAME (10 μM) did not significantly attenuate the Ang II-induced increases in miR-208a expression. Therefore, Neb inhibited Ang II-induced increase in miR-208a in HL-1 cardiomyocytes (Figure 3A). These observations suggest that Neb inhibits Ang II-induced increases in miR-208a by a mechanism that does not involve Neb-induced activation of the β3-AR-AMK-eNOS pathway.

To further understand the mechanism of Neb-mediated suppression of Ang II-induced miR-208a expression, we investigated whether Neb suppressed bioavailable AT1R levels in HL-1 cardiomyocytes that bind 125I-Ang II. When HL-1 cells were pretreated with either Neb (1 μM: 12 h) or losartan (1 μM:12 h) before a ligand binding experiment was performed, both treatments caused equivalent reductions in 125I-Ang II binding to HL-1 cells (Figure 3B). However, when cells were not pretreated with Neb or losartan overnight and these drugs were added only 30 min prior to the addition of 125I-Ang II for the radioligand binding assay, Neb did not suppress 125I-Ang II binding, whereas losartan did. These results suggest that losartan competitively inhibited 125I-Ang II binding because it inhibited 125I-Ang II binding after both preincubation time intervals, but that Neb did not compete for 125I-Ang II binding to HL-1 cells because it did not inhibit 125I-Ang II binding when added 30 min before the binding assay. Therefore, Neb reduced 125I-Ang II binding by decreasing bioavailable AT1 receptors.

**Effect of Rap treatment on weight gain, fat, lean muscle, and expression levels of cardiac miR-208a and MED13**

To determine whether Rap-mediated suppression of cardiac miR-208a occurs in an obese rodent model, we investigated the effect of chronic Rap treatment (750 μg·kg⁻¹·day⁻¹ delivered by subcutaneous implantation of Rap pellets) for 12 weeks on ZO rats. ZL rats were used as healthy lean controls and ZL and ZO rats receiving placebo pellets served as controls to distinguish the specific effects of Rap treatment on both ZL and ZO rats. Body weights of ZO rats were significantly different from that of ZL rats (Figure 4A). ZO rats began to exhibit a reduction in body weight gain after 2 weeks of Rap treatment and their body weight was statistically significantly less than untreated ZO rats 10-12 weeks (Figure 4A). Rap treatment did not significantly affect body weight in ZL rats (Figure 4A).
Weight loss in Rap-treated ZO rats was not due to a reduction in food intake (Figure 4B). EchoMRI analysis showed that Rap treatment significantly lowered fat content in ZO rats (Figure 4C) and increased their lean muscle mass (Figure 4D). Rap treatment did not significantly change the fat content or lean muscle mass of ZL rats. Because cardiac miR-208a is associated with obesity, we determined expression levels of cardiac miR-208a in ZO and ZL rats at the age of 12 and 20 weeks (Figure 5A). qRT-PCR showed that ZO rats had significantly higher levels of cardiac miR-208a than ZL rats at the age of 12 and 20 weeks (Figure 5A). Next we tested whether Rap treatment could suppress miR-208a expression. As shown in Figure 5B, Rap treatment suppressed cardiac miR-208a in ZO rats. Therefore, a possible mechanism by which Rap confers resistance to obesity is via suppression of cardiac miR-208a. Previous studies have shown that transgenic overexpression of cardiac MED13 confers resistance to obesity (9). Indeed, qRT-PCR analysis showed that Rap treatment increased cardiac MED13 mRNA expression levels in ZO rats (Figure 5C). This is the first report showing concurrent Rap-induced reduction of cardiac miR-208a, increase of cardiac MED13, and reduction of weight gain in an animal model of obesity.

Discussion

MicroRNA miR-208a serves as a link between cardiac pathology and obesity since mice treated with anti-miR-208a do not gain weight in response to DIO (9). Given the role of miR-208a in the induction of obesity, it is reasonable to hypothesize that drugs that confer resistance to weight gain in rodent models that are hyperphagic due to deficient signaling of the satiety hormone leptin may do so by suppressing cardiac miR-208a expression. The modes of
action of Rap and Neb differ; Rap directly blocks mTORC1 whereas
Neb binds β1-AR and does not directly interact with mTORC1 (15-
17,28,29). However, both Neb and Rap induce weight loss in rodent
models and humans (14,15,19,20). Rap-induced inhibition of weight
gain is attributed to its ability to inhibit the nutrient sensor kinase
mTORC1 signaling. Neb is an interesting 3rd generation β1-AR
blocker that has pleotropic effects including activation of β3-AR
and subsequent activation of AMP kinase that may lead to activation
of eNOS (Figure 6). Moreover, Neb may also function as a GRK/β-
arrestin biased agonist of β1-AR (32). We previously observed that
Neb suppresses mTORC1 in ZO rat heart (14); however, the path-
way by which this occurs is still unknown.

Our observation that Neb and Rap suppress mTORC1 activation and
miR-208a expression induced by Ang II in HL-1 cardiomyocytes sug-
gests that both drugs use a common mechanism as part of their mode of
action that involves mTORC1 and its ability to promote miR-208a forma-
tion. Rap is known to suppress stimulatory phosphorylation of Ser2448
of mTOR (33). Data presented here show that Neb also suppresses stimulatory
phosphorylation of Ser2448 of mTOR. Therefore, signaling activated by
Rap and Neb in cardiomyocytes converges at inhibition of stimulatory
phosphorylation of Ser2448 of mTOR and subsequent down-stream sig-
naling (stimulatory phosphorylation of S6K1 at Thr389 and RPS6 at
Ser235/236). We found that losartan, an AT1R blocker, could inhibit Ang
II-induced mTOR phosphorylation and up-regulation of miR-208a
expression whereas PD123319, an AT2R antagonist, did not inhibit
mTOR phosphorylation and down-stream signaling. Interestingly, both
Rap and Neb reduced miR-208a RNA expression more than losartan.
This suggests that AT1 receptor signaling is not the sole activator of
mTOR in the HL-1 cells. Because mTORC1 inhibition decreases miR-
208a formation, it is likely that AT1R-mTORC1 signaling regulates
miR-208a (Figure 6). Antagonism of the β3-AR, inhibition of AMP
kinase, and NOS inhibition did not reverse Neb-mediated suppression of
the Ang II-induced increase in miR-208a in HL-1 cells, affirming the
lack of β3-AR involvement in Neb’s effect on inhibition of Ang II-
induced miR-208a expression. Interestingly, overnight treatment with
Neb suppressed 125I-Ang II binding to HL-1 cells. However, a 30-min
pretreatment with Neb did not suppress 125I-Ang II binding to HL-1 cells
whereas losartan did. These observations suggest that Neb does not
directly compete with 125I-Ang II for binding sites; rather, the prolonged
treatment with Neb substantially reduces bioavailable AT1R levels.
Whether this involves inhibition of translation or transcription of the
AT1R gene, or an impairment of trafficking of the AT1R to the mem-
brane, remains to be determined.

Importantly, results presented here show for the first time that in the
ZO rat model for obesity, Rap, an inhibitor of cardiac miR-208a,
increases cardiac MED13 level and confers resistance to obesity
without reducing food intake. Previous studies have shown that inhi-
bition of cardiac miR-208a by pharmacological intervention with
anti-miR-208a oligonucleotide can confer resistance to obesity (9).
Our observation that the cardiac mTORC1-miR-208a-MED13
signaling axis that modulates obesity can be regulated by the widely used drugs Neb and Rap expands the potential clinical utility of these drugs. However, given the role of mTORC1 and miR-208a in cardiomyocyte development and cardiac stress response, any approach to suppress this signaling axis must be taken with great caution (18,34-37).

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