Original Paper

MiR-132 Regulates Rem Expression in Cardiomyocytes During Long-Term β -Adrenoceptor Agonism

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Key Words

L-type calcium channel • miR-132 • miR-214 • Rem • microRNAs • Isoproterenol • α_{1c} Subunit
Cav1.2 channel • L-type Ca^{2+} currents • Ca^{2+} transients

Abstract

Aims: To characterize the effects of long-term β -adrenergic receptor stimulation on Rem protein and mRNA expression in rat heart and possible involvement of miR-132. **Methods:** Adult rats were treated with isoproterenol (ISO, 150 $\mu\text{g.kg.h}^{-1}$) for 2 d and Rem, miR-132, and α_{1c} (the principal subunit of Cav1.2 channels) were measured at protein and mRNA levels with western blot and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) experiments, respectively. Ca^{2+} currents and intracellular Ca^{2+} signals were evaluated in isolated cardiomyocytes. **Results:** Systemic administration of ISO led to decreases in Rem protein and mRNA levels (down to 49%). Furthermore, levels of the microRNAs (miRs) miR-132 and miR-214 were upregulated 5- and 9-fold, respectively. Transfection of miR-132, but not miR-214, into HEK293 cells reduced the expression of a luciferase reporter gene controlled by a conserved 3'-untranslated region (UTR) of Rem by half. Chronic ISO administration also led to a 25% decrease in the amplitude of peak L-type Ca^{2+} currents, a 40% decrease in α_{1c} subunit protein abundance at the membrane level, and a 60% decrease in expression of α_{1c} channel subunit mRNA. **Conclusions:** These results suggest that Rem expression is down-regulated posttranscriptionally by miR-132 in response to long-term activation of β -adrenergic signaling, but this down-regulation does not produce a larger Ca^{2+} influx through Cav1.2 channels.

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Introduction

Studies in many systems have shown that microRNAs (miRs), small non-coding RNAs, repress gene expression posttranscriptionally by binding to discrete miR regulatory elements (MREs) typically located in the 3'-untranslated region (UTR) of target mRNAs [1, 2]. Regulation of target mRNAs is dependent on the degree of complementarity between a miR and the MRE sequence being bound [1] as well as the number of MREs present [3]. miRs can repress expression of target genes by promoting mRNA degradation, inhibiting translation, or both [1, 4-6], with the relative contribution of each mechanism varying across cell types. miRs have been implicated in organogenesis and differentiation of cells [7], and in particular miR-132 has been implicated in differentiation of periodontal ligament cells, but no direct targets of this microRNA were defined [8].

In recent years, miRNA dysregulation has been implicated in a number of human diseases, including cancer, Alzheimer disease, psychiatric disorders, and cardiac hypertrophy [7]. Cardiac hypertrophy has been shown to depend on the presence of miR-132, which is upregulated in cardiomyocytes following exposure to hypertrophizing stimuli. The only target of miR-132 identified in heart muscle thus far is Forkhead box O3, a powerful anti-hypertrophic transcription factor that is suppressed by miR-132 [9].

Cav1.2 channels (L-type Ca^{2+} channels) are critically involved in excitation-contraction coupling and Ca^{2+} homeostasis in the heart and other tissues [10]. In cardiomyocytes, stimulation of β -adrenergic receptors promotes cAMP-mediated phosphorylation of Cav1.2 channels, resulting in a transient increase in Ca^{2+} influx and myocyte contraction (see reviews by Shabb, [11] and Van der Heyden et al., [12]). Although beneficial in the short term, if sustained, these changes lead to cardiac hypertrophy (see review by Nichtova [13]). Hence, chronic β -adrenergic signaling has been implicated in the development of cardiac hypertrophy, possibly through a pathway involving Cav1.2 channels, though the exact mechanism by which this may occur is unclear. In rats, development of cardiac hypertrophy becomes apparent within days of β -adrenergic receptor stimulation [14-16]. Indeed β -adrenergic receptor agonism with isoproterenol (ISO) has been shown to alter the expression of a variety of genes associated with the development of cardiomyocyte hypertrophy [17]. In human hypertensive patients, Greenwood et al. [18] found that left ventricular hypertrophy was associated with a high sympathetic discharge. Determining the signaling mechanisms that underlie pathological processes underlying cardiac hypertrophy may provide information necessary for preventing and treating the condition.

There are numerous agents that influence Ca^{2+} influx. Among them, small GTPases are especially potent regulators of Cav1.2 channels (reviewed by Flynn and Zamponi [19]). Small GTPases are members of the RGD protein subfamily, which is a division of the Ras superfamily. There are four distinct members in this subfamily: Rad, Gem/Kir, Rem (also known as Rem1) and Rem2, which are differentially expressed in several tissues. Rad is expressed most prominently in skeletal muscle, expressed at moderate levels in lung and heart, and expressed at extremely low levels in kidney and brain [20]. Conversely, Gem is expressed at high levels in kidney, expressed to a lesser extent in lung and spleen, and has very low expression levels in heart and skeletal muscle tissues [21]. Rem2 is abundant in the brain, significantly expressed in the kidney, and barely detectable in heart and skeletal muscle [22]. Meanwhile, Rem is distinguished from other RGD proteins by its strong expression in heart, with relatively low abundance in skeletal muscle, kidney, and lung, and very low expression in brain [23]. Rem expression in heart cells is regulated by adrenergic receptor activation. Chronic β -adrenoceptor stimulation has been reported to alter *Rem* mRNA expression in mice [24] and Rem becomes phosphorylated in human cells after sustained application of α -adrenoceptor agonists [25]. Thus, given that elevated intracellular Ca^{2+} has been linked to cardiac hypertrophy [26], we would expect dysregulation of Rem expression to alter Cav1.2 channel function and Ca^{2+} homeostasis.

Thus, the primary aims of the present study were firstly to characterize the effects of long-term β -adrenergic receptor stimulation on Rem expression at the protein and

mRNA levels in the adult rat heart. Secondly, we examined the role of miRs in such effects. Specifically, the focus in our miR experiments was based on prior microarray results obtained by Carrillo and colleagues in experimental conditions similar to ours and related work in our laboratory. Carrillo et al. [16] found that 12 miRs were upregulated by ISO, and among those, we identified two, namely miR-132 and miR-214, as candidate regulators of Rem based on visual examination of the base-pairing of reported miRs with the 3'-UTR of rat *Rem* using RNAhybrid software [27]. Finally, the effects of ISO on Ca^{2+} current amplitude and on expression of the α_{1c} channel subunit of Cav1.2 channels were assessed. The present study focused on Rem because this RGK is preferentially expressed in heart and Rem inhibition of L-type channels has been studied in detail (reviewed by Flynn and Zamponi, [19]).

Materials and Methods

Animals

The experiments were performed on adult male Wistar rats (200–250 g) according to protocols approved by the Division Laboratory Animal Unit (CICUAL) at Cinvestav, Mexico, approval ID 1101010108 (protocol No. 0258-05), in compliance with state law, federal statute and CONACyT policy.

ISO treatment

Rats were anesthetized by intraperitoneal injection of a solution consisting of 10% xylazine chlorohydrate (4 mg/kg, Pisa Agropecuaria, Atitalaquia, HI, Mexico) and Ketalin (80 mg/kg, Pisa Agropecuaria, Atitalaquia, HI, Mexico). A small lateral incision was made on the back of each rat's neck, and an osmotic minipump (Model 2ML2, Alzet, Cupertino, CA, USA) was implanted. *dl*-isoproterenol-HCl (Sigma-Aldrich) was administered via minipump for 2 d at a rate of 150 $\mu\text{g/kg/h}$ in normal saline solution as vehicle. This dose of ISO is within the range used in previous rat studies [16, 17]. A similar solution lacking ISO was administered in the same manner to control animals. Following the minipump infusion period, rats were anesthetized again, as described above, and their hearts were excised and processed as described below. The effects of 6-h and 12-h ISO were investigated following a subcutaneous injection at a concentration of 5 mg/kg.

Isolation of ventricular myocytes

Ventricular myocytes were isolated as described previously [28]. Briefly, hearts were rapidly excised and perfused in a Langendorff apparatus with an aortic cannula delivering $37 \pm 0.5^\circ\text{C}$ buffer at 66 mmHg and were perfused for 5 min with Ca^{2+} -free Tyrode solution (containing in mM: 136 NaCl, 5.4 KCl, 1 MgCl_2 , 10 HEPES, and 11 glucose plus heparin [10 U/ml], Sigma-Aldrich), followed by ~30 min with Tyrode solution containing 20–40 μM CaCl_2 , 0.1% bovine serum albumin (BSA), collagenase (type II, 70 U/ml; Worthington, Lakewood, NJ, USA), and protease type XIV (0.5 mg/100 ml; Sigma-Aldrich). Dissected ventricles were minced and shaken at 45 rpm for 7 min in the same solution 2–3 times. Ventricular cells were filtered through a cell strainer (100 μm nylon, BD Falcon) and centrifuged at $700 \times g$ for 2 min. The pellet was re-suspended in Tyrode solution (containing in mM: 136 NaCl, 5.4 KCl, 2 MgCl_2 , 10 HEPES, 2 CaCl_2 and 11 glucose, plus 1% BSA). Myocytes were used immediately.

Western blot analysis

Immunoblots were performed as described previously [29]. In brief, frozen (-80°C) pulverized rat ventricles were subjected to Dounce homogenization in 10 volumes of homogenization medium (250 mM sucrose, 50 mM MOPS, 2 mM EGTA, pH 7.4) with the following protease inhibitors added: 1 mM benzamidine, 1 mM leupeptin, 0.1 mM aprotinin, 1 mM pepstatin A, 0.2 mM PMSF, and 0.4 mM pefabloc. The homogenates were centrifuged at $1000 \times g$ for 10 min. The supernatant was recovered and centrifuged at $5000 \times g$ for 10 min. The pellet was discarded and the supernatant was centrifuged at $100,000 \times g$ for 1 h. The final pellet, an enriched cell membrane fraction, was re-suspended in 100 μl homogenization medium. The final supernatant was a cytosolic fraction.

For analysis of the α_{1c} channel subunit and cadherin, a sample of the pellet (80 μg) was loaded on an 8% polyacrylamide gel. Meanwhile, for analysis of Rem and GAPDH, a sample of the supernatant (40

μg) was loaded on a 10% polyacrylamide gel and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It is believed that Rem signals from ventricle lysates originate selectively from cardiomyocytes because Rem is not expressed in fibroblasts [30]. Protein content was measured by the Bradford assay method [31] to confirm equal loading of gels.

Proteins were transferred to nitrocellulose membranes and Ponceau stained [32]. The transferred protein bands were probed in PBS solution with anti-Rem monoclonal antibody (1:1000; Abcam, Cambridge, England) or anti-GAPDH monoclonal antibody (1:10000; Sigma-Aldrich, St. Louis, MO, USA) for normalization. Anti- α_{1c} channel subunit polyclonal (1:100; Alomone Labs) and anti-pan cadherin monoclonal (1:1000; Abcam) antibodies were also used. After rinsing away excess primary antibodies with PBS plus tween (0.1%), the membranes were incubated with anti-rabbit (1:70,000) or anti-mouse (1:70,000) horseradish peroxidase-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA). Chemiluminescence was detected with Immobilon Western reagent (Millipore) and band densities were measured as previously described [28].

Measurement of Ca^{2+} transients

Intracellular Ca^{2+} levels were monitored with Fluo-3 AM (Molecular Probes, Eugene, OR, USA) as described previously [28]. This dye undergoes a marked shift in fluorescence upon Ca^{2+} binding. Adult cardiomyocytes were loaded with approximately 5 μM Fluo-3 AM in standard Tyrode solution for 40 min at room temperature. After loading, cells were incubated in dye-free solution for over 30 min to allow conversion of the dye to its Ca^{2+} -sensitive, free-acid form. Dye-loaded cells were placed on a laminin-coated coverslip at the bottom of a chamber on the stage of an Optiphot microscope (Nikon, Tokyo, Japan). Stained myocytes were illuminated episcopically with monochromatic light (485 nm). Emitted fluorescence was filtered with a high-pass barrier filter (cut-off wavelength 535 nm) and detected with a low-noise photodiode in a photovoltaic configuration. Analog signals were digitized and sampled every 60 μs for 40 s. Ca^{2+} signals were calculated as $\Delta F/F$, where F was the basal fluorescence of the myocyte, determined as the mean recorded value over the 300-ms interval prior to electrical stimulation and ΔF were values poststimulation. To induce Ca^{2+} transients during action potentials, myocytes were stimulated electrically with two extracellular platinum electrodes at a frequency of 0.3 Hz. To estimate the duration of the transients, we measured the half-width, which is the full duration of the signals at half-maximal amplitude. Because this study was designed to detect temporal and relative patterns of Ca^{2+} transients, absolute value measurements of intracellular Ca^{2+} concentration were not made.

RNA isolation and reverse transcription

Cellular RNA was isolated with the RNeasy Mini kit (Qiagen). Reverse transcription was performed with 500 ng of DNase-treated RNA in 20-μL reactions. Synthesis of cDNA was carried out with Superscript III reverse transcriptase (Invitrogen) and random hexamers (250 ng) following the manufacturer's instructions.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

The relative expression levels of Rem and α_{1c} mRNA were quantified using TaqMan assays (Applied Biosystems, Foster City, CA, USA). 18S ribosomal RNA (rRNA), which performs consistently for most applications [33] and is unaffected by ISO administration, was used as an internal control. Quantification was performed by the $2^{-\Delta\Delta Ct}$ method [34]. This procedure is valid if the amplification efficiencies of the target and reference genes are approximately equal, as was the case in our experiments.

Analysis of miR expression

miR-RNA was isolated with a miRNeasy Mini kit (Qiagen). The relative expression levels of rno-miR-132 and rno-miR-214 were quantified using TaqMan miR assays (Applied Biosystems, Foster City, CA, USA). miR expression was assessed relative to the small nucleolar RNA U87, as recommended by the manufacturer. Changes in expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [34].

Cloning of Rem 3'-UTR

The 3'-UTR of Rem (Rattus norvegicus RAS (RAD and GEM)-like GTP-binding 1, Acc. No. [NM_001025753.1]) was amplified from the cDNA of rat ventricles using primers: forward 3'-UTR: 5'-TTA

AGC TAG CCG CCA GCG TCT CTC TGG GGC-3' and reverse 3'UTR:5'-CCC CCC CCC CCG CTA GCT TTT TTT TTT TTT TTT TTT TTT TTT ATA ATT AAT TCA TTG ACT TTA TTG ATT GTC CAA GAC-3'. The amplified region was ligated at the *Xba* I site downstream of the luciferase-encoding region of the pGL3 vector (Promega GmbH, Mannheim, Germany). Cloning fidelity was verified by DNA sequencing.

Luciferase reporter assay

For luciferase reporter assays, HEK293 cells were seeded in 24-well plates (~40,000/well) and cultured in DMEM medium supplemented with 10% fetal bovine serum at 37 °C and 5% CO₂. Cells were transfected with 100 ng of pGL3 Rem 3'-UTR plasmid with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Co-transfections were performed with 60 nM of pre-miR-132 (PM10166, Ambion), pre-miR-214 (PM11376, Ambion), or pre-miRNA precursor control 1 (AM17110, Ambion) as a negative control. Luciferase activity assays were performed 48 h after transfection with the Steady-Glo Luciferase Assay System (Promega, Madison, WI, USA) and a Modulus Single Tube Reader (Turner Biosystems, Sunnyvale, CA, USA). Luciferase activity was normalized to sample protein concentration [31]. All reporter assays were performed in triplicate. The luciferase reporter assay is a typical procedure used to verify predicted miR/mRNA pairings and measure construct expression [35].

Electrophysiology

The whole-cell patch-clamp technique was used to record L-type Ca²⁺ currents in dissociated ventricular myocytes as described elsewhere [28]. Currents were recorded with an Axopatch 200-A amplifier (Axon Instruments) with the holding potential set to -80 mV. The pulse protocol involved a 65-ms pulse to -40 mV to inactivate Na⁺ currents followed by 20 consecutive 65-ms pulses to preselected potentials in +5 mV steps to activate L-type Ca²⁺ currents. To measure membrane capacitance and to subtract linear currents, the membrane was clamped to the holding potential and a +5 mV pulse lasting 62 ms was applied. Current records were digitized with a Digidata interface (Axon Instruments, Foster City, CA, USA) at a 16-bit resolution. The data were analyzed in pCLAMP 8.0 (Axon Instruments) and in-house software.

Peak Ca²⁺ channel current values were fitted to equation (1), which describes the current-voltage relationship of L-type currents.

$$I_m = G_{max} \cdot (V_m - V_{rev}) \{1 + \exp[(V - V_m)/k]\} \quad (1)$$

G_{max} is the maximum conductance, V_{rev} is the reversal potential, V_m is the membrane potential during depolarizing pulses, V is the potential at which G = 0.5 G_{max}, and k is a measure of the steepness of the curve. Numerical formulae were fitted to our experimental data using a non-linear, least-squares algorithm. The pipette solution contained (in mM): 100 cesium aspartate, 20 CsCl, 20 TEACl, 2 Mg-ATP, 1.8, MgCl₂, 0.05 EGTA and 5 HEPES, pH 7.2. The standard bath solution was Tyrode solution containing 1 mM Ca²⁺. Experiments were performed at room temperature (23°C).

Data analysis

Results data are expressed as means ± standard errors (SEs). Statistical analyses were performed with GraphPad Prism 4.0 (GraphPad Software) and Sigma Stat 2.0; unpaired t-tests, paired t-tests, and one-way analyses of variance (ANOVAs) were used as appropriate. A *P* < 0.05 was considered to be statistically significant.

Results

Hypertrophic effect of ISO

ISO treatment for 2 d had a hypertrophic effect on hearts under our experimental conditions. The mean ventricular weight to body weight ratio was 38% greater in ISO-treated rats (3.4 ± 0.2 mg/g, n = 14) than in controls 4.7 ± 0.3 (n = 13).

Long-term ISO administration decreases Rem expression

Western blot analysis demonstrated that Rem protein expression was significantly decreased in ventricle lysates isolated from 2 d ISO-treated rats compared with that in lysates from vehicle-infused controls, while GAPDH levels were unaffected by the drug treatment

Fig. 1. Effects of chronic ISO on Rem protein and mRNA levels. *A–B*: Immunoblots from control (left) and 2-d ISO-treated (right) ventricle cytosolic samples representing Rem (A) and GAPDH (B) protein levels. *C*: Quantitative analysis of Rem protein band density from control (open bar) and ISO-treated (filled bar) rats. Values were normalized to GAPDH band densities ($n = 6$, $*p < .01$). *D*: Quantitative analysis of Rem mRNA expression in ventricle lysates from control (open bar) and ISO-treated (filled bar) rats ($n = 4$, $*p < .01$). All values are group means $\pm$ SEs.

Fig. 2. Posttranscriptional regulation of Rem expression by miRs. *A*: miR-132 and miR-214 seed sites within the Rem 3'-UTR. Note that nucleotides 2 through 8 exhibit perfect complementarity in both cases. Bases paired by Watson-Crick bonds are depicted with a vertical black line, and G:U pairs are depicted by a double-dotted vertical line. *B*: Quantitative analysis of miR levels determined by qRT-PCR and $2^{-\Delta\Delta CT}$ in ventricle samples from ISO-treated vs. control animals. U87 was used for normalization ($n = 6$ per group, $*p < .01$). *C*: Quantitative analysis of luciferase reporter gene activity in HEK-293 cells expressing pGL3 vectors with Rem 3'-UTR in combination with a negative control, or with indicated miRs ($n = 10-12$ per group, $*p < .01$). All values are group means $\pm$ SEs.

(Fig. 1A–C). Long-term ISO administration decreased Rem protein levels by approximately 40%. Furthermore, the qRT-PCR experiments indicated that the ISO treatment decreased Rem mRNA levels significantly, with the ISO group having levels that were ~50% of control group levels (Fig. 1D).

Posttranscriptional miR regulation of Rem mRNA

qRT-PCR experiments showed that both miR-132 and miR-214 were upregulated by ISO within 48 h, with miR-132 having a noteworthy more than 6-fold increase (Fig. 2B). The binding sites for these two miRs within the 3'-UTR region of the rat Rem gene are shown in Figure 2A. Luciferase reporter gene assays in cells expressing the 3'UTR region of Rem showed that miR-132 repressed luciferase activity significantly, by 60%, but miR-214 did not have a significant effect on luciferase activity (Fig. 2C).

Fig. 3. ISO effects on *Rem* mRNA and miR-132. **A:** Relationship between ISO administration time and the relative expression of *Rem* mRNA. ($n = 4$ per group). **B:** The effect of chronic ISO on the relative expression of miR-132 ($n = 3$ per group). Level of expression at $t = 0$ is shown with a dotted line in both graphs. All values are group means $\pm$ SEs ($*p < .05$).

Fig. 4. Effects of chronic ISO on Rem protein levels. **A:** Immunoblots from control (left) and 12-h or 24-h ISO-treated ventricle cytosolic samples (right). Blots represent Rem and GAPDH protein levels. **B:** Quantitative analysis of Rem protein band density as a function of treatment duration. Values were normalized to GAPDH band densities ($n = 3$, $*p < .05$). Level of expression at $t = 0$ is shown with a dotted line. All values are group means $\pm$ SEs.

Early regulation of Rem by ISO

A closer examination of the effects of ISO infusion on *Rem* mRNA expression over time revealed a biphasic time course, peaking at the 12-h time point when an almost 3-fold increase in *Rem* mRNA levels; at all other times *Rem* mRNA expression was actually decreased relative to baseline levels (Fig. 3A). In contrast, miR-132 levels increased monotonically following ISO treatment (no biphasic peaks), with miR-132 levels reaching a plateau that was more than 10-fold baseline levels (Fig. 3B).

Western blot analysis revealed that the time course of Rem protein expression was also biphasic. Ventricle lysates isolated from rats treated with ISO for 12 h or 24 h produced Rem bands of increased and decreased density, respectively, relative to controls (Fig. 4A and B). Note that the Rem protein expression increases observed in the 12-h treatment group were quite substantial, reaching up to 40% of basal levels.

Effects of long-term ISO on Cav1.2 channels

In patch-clamp experiments, 2 d ISO treatment did not increase the amplitude of Ca^{2+} currents as we would have expected given the inhibitory actions of Rem on Ca^{2+} channel activity (Fig. 5A). L-type Ca^{2+} currents under control conditions reached a peak of approximately -5 pA.pF^{-1} , 10 ms after pulse onset. Under control conditions, peak currents were observed at 0 mV. The current was not sustained, but rather was inactivated during the pulse via a Ca^{2+} -dependent inactivation process. Currents from ISO-treated rats were smaller and decayed more slowly than those observed in controls. Long-term administration of ISO produced a slight decrease in current density values corresponding to an 8% decrease in G_{max} . A +4 mV shift in the current-voltage relation was also observed.

Fig. 5. Effects of chronic ISO administration on L-type Ca^{2+} currents and Ca^{2+} signals. **A:** The inset shows representative recordings of L-type Ca^{2+} currents at +5 mV steps under control conditions and after 48-h ISO administration. The graph shows mean ($\pm$ SE) I - V relationship of peak L-type currents. To allow comparisons among experiments, membrane current values were normalized to unit capacitance (C_m). $C_m = 232.9 \pm 7.5$ ($n = 44$) pF in controls and $C_m = 307.7 \pm 8.0$ pF ($n = 19$) in ISO pretreated rats. Peak currents are shown for myocytes isolated from controls ($\bullet$; $n = 44$) and from ISO-pretreated rats ($\blacktriangle$; $n = 19$) at the indicated potentials ($*p < .05$ between -20 mV and 0 mV). The smooth lines are best fit curves for equation (1) with $G_{\text{max}} = 0.12 \eta\text{S}\cdot\mu\text{F}^{-1}$ for controls and $G_{\text{max}} = 0.11 \eta\text{S}\cdot\mu\text{F}^{-1}$ for the treatment group. **B:** The inset shows representative recordings of fluo-3 fluorescent transients. The bar graphs show mean $\pm$ SE $\Delta F/F$ peak values (left) and half-width values (right). Values for myocytes isolated from ISO-pretreated rats ($n = 76$) differed significantly from those obtained from control myocytes ($n = 205$) for half-width only ($*p < .05$).

Fig. 6. Changes in α_{1c} subunit mRNA and protein levels following ISO administration. **A:** Relationship between ISO administration time and α_{1c} subunit mRNA relative expression. Dotted line: level of expression at $t = 0$. ($n = 3$). **B:** Independent immunoblots from control (left) and 48-h ISO-treated (right) ventricle membrane samples representing the α_{1c} channel subunit (upper panel) and pan-cadherin (lower panel). **C:** Relationship between ISO administration time and relative protein expression of the α_{1c} subunit. Values were normalized to pan-cadherin band densities. ($n = 3-9$). Dotted line: level of expression at $t = 0$. All values are group means $\pm$ SEs. $*p < .05$.

No significant difference was detected in the peak amplitude values of intracellular Ca^{2+} signals during action potentials, as indexed by fluo-3 fluorescence signals (Fig. 5B), though the signals decayed more rapidly and had smaller half-width values with ISO treatment than without. Hence, the small decreases in current amplitude produced by the ISO treatment had no observable impact on the release of intracellular Ca^{2+} .

The unexpected decrease in L-type Ca^{2+} currents observed after long-term exposure to ISO suggested that chronic β -agonism may down-regulate Cav1.2 channels. Therefore, we tested the hypothesis that ISO may decrease expression of α_{1c} , the principal subunit of the Cav1.2 channel. We observed significant decreases in both the mRNA and protein levels of the α_{1c} subunit. The mRNA levels of the α_{1c} subunit were found to be decreased by 60% after a 12-h ISO administration period and remained at this low level throughout the remainder of the observation period (Fig. 6A). The decrease in the mRNA transcript levels preceded that of the protein. Thus, western blot experiments showed that the abundance of the α_{1c} protein subunit did not change after 12-h ISO administration, but was decreased significantly at later times. Following ISO treatment for 2 d, α_{1c} subunit abundance fell by 60% compared to control levels (Fig. 6B,C). The expression of pan-Cadherin (normalization protein) was unaffected by the drug treatment.

Discussion

In the present study, we demonstrated for the first time that Rem is down-regulated in a posttranscriptional manner by sustained β -adrenergic receptor activation induced by ISO. qRT-PCR analysis confirmed upregulation of miR-214 and miR-132, miRs that bind the 3'-UTR region of *Rem*, in the hearts of ISO-treated rats. However, only miR-132 exhibited robust regulation in response to ISO, and co-transfection of miR-132, but not of miR-214, repressed expression of a reporter gene regulated by the 3'-UTR of *Rem*, suggesting that miR-132 may regulate the expression of Rem posttranscriptionally *in vivo*.

qRT-PCR experiments demonstrated that down-regulation of Rem protein was accompanied by a significant decrease in *Rem* mRNA levels. This finding is not incompatible with regulation of Rem protein expression at a posttranscriptional level because, although miRs can repress target translation with little change in mRNA levels, mRNA cleavage by miRs has been observed to lead to changes in mRNA abundance [4, 36]. *Rem* mRNA showed a distinct peak at 12 h into a 48-h ISO treatment period, when an almost three-fold increase was observed, followed by a decrease in mRNA abundance compared to control levels. Schroder et al. [24] described a two-fold increase of *Rem* mRNA 24 h after ISO infusion in mice, but other ISO periods were not explored. Our data suggest that there is dual regulation of *Rem* mRNA abundance. Initially, upregulation prevails, but subsequent accumulation of miR-132 appears to lead to down-regulation of Rem at the messenger and protein levels.

Our results are consistent with previous studies relating miR-132 to β -adrenergic signaling. Vo et al. [37] reported evidence indicating that miR-132 is a target of the transcription factor CREB (cAMP response element) and that the miR-132 response is rapid and long-lasting. Although long-term regulation of Rem via β -adrenergic stimulation had not been previously explored, regulation of Rem by α -adrenergic receptor agonists has been described in neonatal rat cardiomyocytes [25]. In Jhun and colleagues' preparation, sustained stimulation for 2 h by the α_1 -adrenergic receptor agonist phenylephrine led to activation of protein kinase D1 and phosphorylation of Rem, resulting in increased L-type current density values and over-expression of Cav1.2 channels in transverse tubular system membranes. The increase in channel expression was attributed to the release of channels from a putative reserve, though possible changes at the mRNA level were not explored [25]. The present results suggest that regulation of Rem by activation of β -adrenergic receptors involves distinct mechanisms from those associated with α -adrenergic stimulation.

Sustained adrenergic activation leads to cardiovascular disorders such as pathological hypertrophy. Hypertrophy develops rapidly in rats. Indeed, Zierhut and Zimmer [15] documented a 40% increase in the ventricle/body ratio after a 3-day norepinephrine treatment. Carrillo et al. [16] obtained similar results with long-term administration of ISO. Recently, Ucar et al. [9] demonstrated in primary neonatal cardiomyocytes that several hypertrophic stimuli upregulate the expression of miR-132 and that this miR is necessary to drive the hypertrophic growth of cardiomyocytes. The large increase in miR-132 expression

observed in our study with sustained β -adrenergic activation and cardiac hypertrophy are consistent with this view. Furthermore, miR-132 overexpression developed within hours of ISO administration, reaching a plateau at approximately 12 h. This relatively fast time course is consistent with the development of hypertrophy.

It is likely that other factors beyond miR-132, such as Ca^{2+} -dependent signaling [10], play a role in hypertrophy. Because Cav1.2 channels are regulated by Rem, down-regulation of Rem by miR-132 would be expected to have an impact on Ca^{2+} homeostasis [19]. Rem is a potent inhibitor of Cav1.2 channel activity and although the inhibitory mechanisms are still being elucidated, the actions of Rem on Cav1.2 currents are thought to be mediated via inhibition of surface expression or inhibition of channel function [19, 38]. Therefore, down-regulation of Rem would be expected to lead to larger Cav1.2 currents. Indeed, Magyar et al. [39] described increased Cav1.2 channel current density values in Rem knock-out mice. In the present study, we evaluated Cav1.2 channel function both indirectly and directly. Our indirect assessment, which entailed the measurement of action potential-associated Ca^{2+} signals, revealed no changes in the values of peak signals, though we did observe a decrease in half-width values suggestive of a more efficient uptake of Ca^{2+} by SR. This observation is consistent with the work of Anwar et al. [40], who described an enhancement of SERCA2A expression after chronic ISO stimulation in rat cardiomyocytes in culture. Meanwhile, our direct measurements of Cav1.2 channel currents indicated that although Rem expression was down-regulated by chronic ISO administration, Cav1.2 channel current density values were *decreased* moderately, rather than increased. Consistent with our results, Zhang et al. [41] found that sustained β -adrenergic stimulation of cultured adult guinea pig cardiomyocytes for 2 d led to a reduction in the maximum amplitude of Cav1.2 channel currents. Others have observed minimal changes in Cav1.2 channel current density values following long-term ISO administration. For example, daily injection of ISO for 7 d led to hypertrophy in rats, but Cav1.2 channel currents were minimally affected [42]. Likewise, in other hypertrophy rat models, Cav1.2 channel current density values remained unchanged [43, 44]. These observations do not rule out a relevant role for Cav1.2 channels in hypertrophy entirely because the small population of these channels housed in caveolae have been shown to contribute very little to whole-cell L-type Ca^{2+} currents though they play a fundamental role in the nuclear translocation of NFAT (nuclear factor of activated T cells) [45], a well characterized mediator of hypertrophy [46]. Determining whether down-regulation of Rem by chronic ISO leads to an increase in L-type Ca^{2+} currents in this subpopulation of channels is an important future direction of research.

A reduction in the α_{1c} subunit's abundance would be expected to lead to smaller currents because the subunit contains a pore through which Ca^{2+} ions permeate [47]. Hence, the decreased whole-cell Cav1.2 channel current amplitude observed in this study is likely attributable to the post-ISO decrement in α_{1c} channel subunit levels. Decreased expression of the α_{1c} channel subunit was demonstrated in two sets of experiments. Firstly, reduced α_{1c} subunit mRNA levels were observed following ISO administration, particularly at the 12-h post-ISO time point. Secondly, western blot analysis showed reduced α_{1c} protein subunit abundance. Given that the half-life of the α_{1c} subunit is approximately 25 h [48], a significant overall reduction at the protein level would be expected following 1-2 d of β -adrenergic receptor stimulation, as we did indeed observe. We measured α_{1c} subunit expression in lysates enriched with the membrane fraction and with cadherin as a normalizer, and observed a significant reduction in levels of the α_{1c} subunit of the L-type Ca^{2+} channel while cadherin levels remained unchanged. Cadherin is a major membrane protein that localizes in specialized cell membrane domains [49], which makes it an ideal plasma membrane marker for cadherin-expressing cells such as cardiac myocytes [50]. While no changes in cadherin were seen by the ISO treatment the levels of the α_{1c} subunit of the L-type Ca^{2+} channel were significantly reduced.

Down-regulation of the α_{1c} subunit could represent a protective mechanism against excessive Ca^{2+} influx during action potentials. In fact, transgenic overexpression of α_{1c} subunits in mouse hearts led to increased L-type current density values and the mice

developed severe cardiomyopathy indicating that changes in channel density were sufficient to induce disease [26].

In recent years, RGK proteins have emerged as important regulators of Cav1.2 channels and play relevant roles in cells in which Ca^{2+} acts as a second messenger. The differential expression of the four RGKs is probably related to each RGK's unique role. Rem2, which is most abundantly expressed in brain [22], plays a role in synapse formation and maintenance [51]. Meanwhile, Rem (i.e., Rem1), which is expressed most prominently in heart, modulates the activity of Cav1.2 channels in cardiac myocytes [39], crucial elements in excitation-contraction coupling. Thus, overexpression of Rem dramatically reduces the amplitude of Cav1.2 currents in adult cardiomyocytes [52]. Rad, the other RGK member that is also significantly expressed in heart [20], regulates cardiac Cav1.2 channel activity and its overexpression also leads to smaller Cav1.2 currents [53]. Experiments with knock-out mice designed to investigate the role of Rem on Ca^{2+} homeostasis have revealed larger cardiac Cav1.2 currents and smaller transient Ca^{2+} elevations during a twitch. However, Rem deletion results in no obvious cardiac phenotype [39]. Likewise, deletion of Rad leads to larger Cav1.2 currents with no changes in heart weight, body weight or in the heart to body weight ratio, but unlike deletion of Rem, cardiomyocytes from Rad null mice have larger Ca^{2+} transients [54, 55]. The susceptibility to cardiac hypertrophy and stress has not been investigated in Rem null mice but ablation of Rad leads to exacerbated cardiac hypertrophy in the thoracic transverse aorta constriction model [56] and to severe cardiac fibrosis [57]. Furthermore, Rad null mice exhibit prolonged QT intervals and diverse arrhythmias [58]. Unlike Rad, Rem expression in cardiomyocytes and other striated muscle cells is maintained throughout myogenic development, suggesting that it may play a role at all stages [30].

Given the diversity of the physiological roles of RGKs, it is not surprising that overall homology among RGKs is relatively low. The homology between Rem and the other RGKs is around 47–58%. Given their heterogeneity, it would be expected that the four RGKs would have different regulatory mechanisms. In this regard, it is interesting to note that the miR-132-hybridizing nucleotide sequence of the 3'UTR region of Rem identified in the present work is not present in Rad or in the other RGKs. However, this does not rule out the possibility that the expression of Rad, Rem2 or Gem/Kir may be also regulated by miRs and given the importance of Rad on Ca^{2+} homeostasis in heart, this is an interesting future research topic.

In conclusion, the present work demonstrated that long-term β -adrenoreceptor agonism results in down-regulation of Rem in rat ventricles at the mRNA and protein levels. Our findings further indicate that this down-regulation does not lead to a whole-cell increase in Ca^{2+} influx through Cav1.2 channels.

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Disclosure Statement

The authors have no conflict of interest to disclose.

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