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MicroRNA-665 is involved in the regulation of the expression of the cardioprotective cannabinoid receptor CB2 in patients with severe heart failure

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ABSTRACT

The myocardial endocannabinoid system has been linked to stress response and cardioprotection. In chronic heart failure (CHF), protective CB2 receptors are markedly up-regulated while CB1 receptors are slightly down-regulated. We here provide evidence that myocardial CB receptors are subject to microRNA regulation. By a combined computational and experimental approach we show that CB1 receptors are regulated by miR-494, and CB2 receptors are targeted by miR-665. Moreover, we demonstrate that in CHF, miR-665 expression is significantly decreased while miR-494 is slightly increased, which is concordant with the previously reported alterations of CB receptors. These results suggest that in CHF, altered expression of specific miRNAs may contribute to a compensatory response of the diseased myocardium.

1. Introduction

Recent studies point to a significant role of the endocannabinoid system (ECS) in cardiovascular function and disease [1,2]. The ECS basically consists of two types of G-protein-coupled cannabinoid receptors, CB1 and CB2, expressed in various degrees in multiple tissues throughout the human body, and their endogenous ligands, mainly the endocannabinoids 2-arachidonoyl-glycerol and arachidonyl-ethanolamide [3]. In the heart, CB receptors and their ligands have been described to exert a protective role including decreasing tissue damage and arrhythmia after myocardial infarction [2]. The beneficial effects have mainly been attributed to the activation of CB2 receptors [1,4–6] whereas the role of the CB1 receptor long has been a matter of debate: In earlier studies a cardioprotective role was discussed [7–9]; recent studies, however, have proved negative effects of CB1 activation and beneficial effects of CB1 antagonism [10–13]. The at least partially opposing roles of the two receptor subtypes strongly suggest the importance of balance in the expression of the receptor subtypes in health and disease [2]. We previously demonstrated substantially altered CB1 and CB2 expression patterns in myocardium of patients with congestive heart failure (CHF) as compared to healthy controls, with markedly increased expression of the CB2 receptor and slightly decreased expression of the CB1 receptor [14]. The mechanisms regulating the expression of the CB receptor subtypes in human myocardium under normal and pathological conditions, however, have not been investigated yet.

MiRNAs are small noncoding RNAs, which posttranscriptionally repress gene expression by base-pairing to the 3′-untranslated region (3′-UTR) of their target genes. They have increasingly gained attention as regulators of gene expression in the recent past [15,16]. Alterations in the expression of microRNAs and their specific target genes have repeatedly been reported in cardiovascular and cardiac diseases including CHF [17]. In this work, we aimed to investigate whether microRNAs might regulate myocardial CB receptor expression and might potentially be involved in the altered expression of CB1 and CB2 receptors in CHF.

2. Material and methods

2.1. Cell culture and transfections

Human cardiomyocytes (Celprogen, San Pedro, CA) were maintained in Celprogen's Complete Growth Media in T75 flasks precoated with Celprogen's Extra-cellular Matrix. HEK-293 cells

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were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplied with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin/glutamine and 1% NEAA (non-essential amino acids). Cell transfections were accomplished by electroporation using the Neon™ Transfection System (Life Technologies, Thermo Fischer Scientific, Waltham, MA). Knock-down experiments were performed in human cardiomyocytes using siRNA (50 nM) directed against ribonuclease type III (DICER1) or non-targeting control (Dharmacon, Lafayette, CO). Cardiomyocytes were transiently transfected with 50 nM premiR-494 or premiR-665 molecules (Ambion, Austin, TX). HEK-293 cells were transiently cotransfected with 50 nM premiR-494 or premiR-665 molecules (Ambion, Austin, TX) and 1 μg psiCheck-2 reporter vector containing CB1 (CNR1) or CB2 (CNR2) 3′UTR variants; thirty hours after transfection, cells were harvested and firefly as well as renilla luciferase activities were measured using the Dual-Glo-Luciferase Assay System (Promega, Madison, WI).

2.2. RNA isolation and synthesis of cDNA

Total RNA was extracted from human cardiomyocytes using the RNAqueous® RNA Isolation Kit (Life Technologies, Thermo Fischer Scientific, Waltham, MA) followed by DNase treatment (Turbo DNA-free Kit, Ambion, Austin, TX). cDNA was synthesized from 1 μg of total RNA using the SuperScript III First Strand Synthesis System (Invitrogen, Thermo Fischer Scientific, Waltham, MA) and random hexamers.

2.3. Quantitative real-time PCR (qPCR)

Quantitative analyses of mRNA levels were performed on a Light Cycler 480 system (Roche Diagnostics, Penzberg, Germany) using Universal ProbeLibrary (UPL) probes and specific primers (Table 1). Efficiency corrected relative expression ratios were calculated, and target gene expression was normalized to the reference genes GAPDH and B2M in myocardium [18] and B2M and SDHA in human cardiomyocytes. Expression of mature miRNA-494 or miR-665 was quantified using TaqMan® microRNA Assays (Applied Biosystems/Life Technologies, Carlsbad, CA) and U47 as endogenous control.

2.4. Western blot analysis and immunodetection

30 μg of total protein extracts were electrophoresed in a 10% SDS–PAGE and electroblotted onto PVDF membranes. Non-specific binding sites on the membrane were blocked using 5% non-fat dry milk in PBS-Tween. CB1 (anti-cannabinoid receptor 1) and CB2 (anti-cannabinoid receptor 2) antibodies (Sigma–Aldrich, St. Louis, MO) were diluted in PBST supplemented with 1% non-fat dry milk (dilution factor: 1:1000). β-actin (antibody AC-15, Sigma–Aldrich; 1:50,000) served as a loading control. Immunoreactive bands were visualized using horseradish peroxidise-labeled goat anti-mouse or goat anti-rabbit antibodies, 20× LumiGLO® Reagent and 20× Peroxide (Cell Signaling Technology, Danvers, MA).

2.5. Vector construction

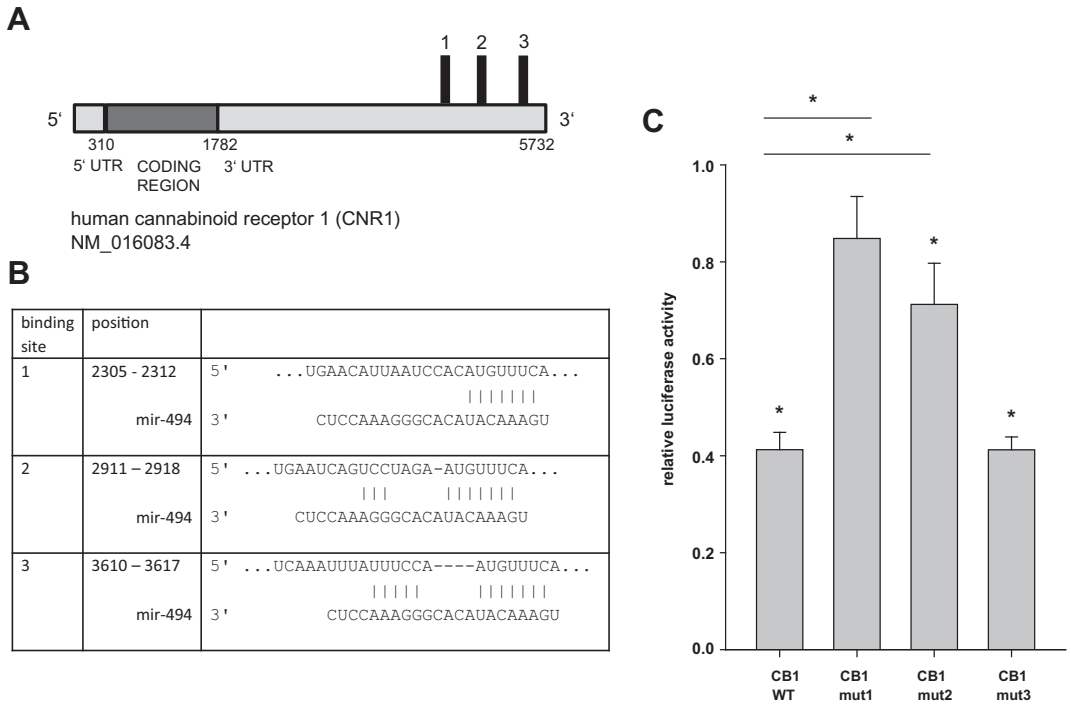
CB1 and CB2 reporter constructs were cloned into the multiple cloning site of the psiCheck-2 Dual-Luciferase Vector (Promega, Madison, WI). Briefly, the CB1 (CNR1) and CB2 (CNR2) 3′-UTR fragments containing the predicted target sites for miR-494 or miR-665 were amplified by PCR from 100 ng of human genomic DNA with the primers listed in Table 1 (synthesized by Metabion, Martinsried, Germany). Cycling conditions were as follows: 95 °C for 2 min denaturation; 30 cycles of 95 °C for 20 s, 60.4 °C for 20 s, 72 °C for 30 s; and a final extension at 72 °C for 3 min. PCR products were cloned into the *Pme*I and *Xho*I restriction sites of the psiCheck-2 plasmid. Site-directed mutagenesis of the putative miR-494 or miR-665 binding-sites within the CB1 or CB2 3′UTRs was performed using the QuikChange Lightning Mutagenesis Kit (Stratagene, Agilent Technologies, Santa Clara, CA) and the primers listed in Table 1. Sequence of all plasmids was verified by sequencing (MWG-Biotech, Ebersberg, Germany).

Table 1
PCR-primer.

3′UTR CB1 FW	5′-CTC GAG GCC AGT CTT TTG TCC TGC AT-3′ (*Xho*I)
3′UTR CB1 RV	5′-GTT TAA ACG GTT GCA ACG ATG TTA CCA G-3′ (*Pme*I)
3′UTR CB2 FW	5′-GTT TAA ACA ATC ACT CCG TGG CCA GAT-3′ (*Pme*I)
3′UTR CB2 RV	5′-CTC GAG GGC AAG TCA ACA CCT TAA TCC-3′ (*Xho*I)
3′UTR CB1 mut1 FW	5′-GCA TCA TCT TGA ACA TTA ATC CAC ATG GCT CAG AGC TCA CCA GG-3′
3′UTR CB1 mut1 RV	5′-CCT GGT GAG CTC TGA GCC ATG TGG ATT AAT GTT CAA GAT GAT GC-3′
3′UTR CB1 mut2 FW	5′-GAT GAA TCA GTC CTA GAA TGG CTC ATT TGC ACA AGT AGG GCT GC-3′
3′UTR CB1 mut2 RV	5′-GCA GCC CTA CTT GTG CAA ATG AGC CAT TCT AGG ACT GAT TCA TC-3′
3′UTR CB1 mut3 FW	5′-GGA TTC AAA TTT ATT TCC AAT GGC TCA AGC GGG AAA CAT GAC TC-3′
3′UTR CB1 mut3 RV	5′-GAG TCA TGT TTC CCG CTT GAG CCA TTG GAA ATA AAT TTG AAT CC-3′
3′UTR CB2 mut1 FW	5′-AGA AAT CAG TTC ACT CAA TGG AAG AGA GAG AGG GGT C-3′
3′UTR CB2 mut1 RV	5′-CCC TCT CTC TCT TCC ATT GAG TGA ACT GAT TTC TGA C-3′
3′UTR CB2 mut2 FW	5′-GCT GAT GAG TGT TGG GAC TGA CTA ATG GAA GAC AGC C-3′
3′UTR CB2 mut2 RV	5′-GGC TGT CTT CCA TTA GTC AGT CCC AAC ACT CAT CAG C-3′
3′UTR CB2 mut3 FW	5′-GCC AAA GCG AGC CTC ATG GCC CAG CAA TGA GG-3′
3′UTR CB2 mut3 RV	5′-CCT CAT TGC TGG GCC ATG AGG CTC GCT TTG GC-3′
3′UTR CB2 mut4 FW	5′-GCC TAA TTG TCA AGG CCT CAA TGG CTC TGG AGC TAT GAA A-3′
3′UTR CB2 mut4 RV	5′-TTT CAT AGC TCC AGA GCC ATT GAG GCC TTG ACA ATT AGG C-3′
pPCR primer	
SDHA #132 FW	5′-GAG GCA GGG TTT AAT ACA GCA-3′
SDHA #132 RV	5′-CCA GTT GTC CTC CTC CAT GT-3′
B2 M #42 FW	5′-TTC TGG CCT GGA GGC TAT C-3′
B2 M #42 RV	5′-TCA GGA AAT TTG ACT TTC CAT TC-3′
GAPDH #60 FW	5′-AGC CAC ATC GCT CAG ACA C-3′
GAPDH #60 RV	5′-GCC CAA TAC GAC CAA ATC C-3′
DICER1 #47 FW	5′-AGC AAC ACA GAG ATC TCA AAC ATT-3′
DICER1 #47 RV	5′-GCA AAG CAG GGC TTT TCA T-3′
CB1 #43 FW	5′-GCT CTC GAG ATA CCC AAG CA-3′
CB1 #43 RV	5′-GCC TTA GAG CGT GAA CCG TA-3′
CB2 #24 FW	5′-GGG AGA GGA CAG AAA ACA ACT G-3′
CB2 #24 RV	5′-GAG CTT GTC TAG AAG GCT TTG G-3′

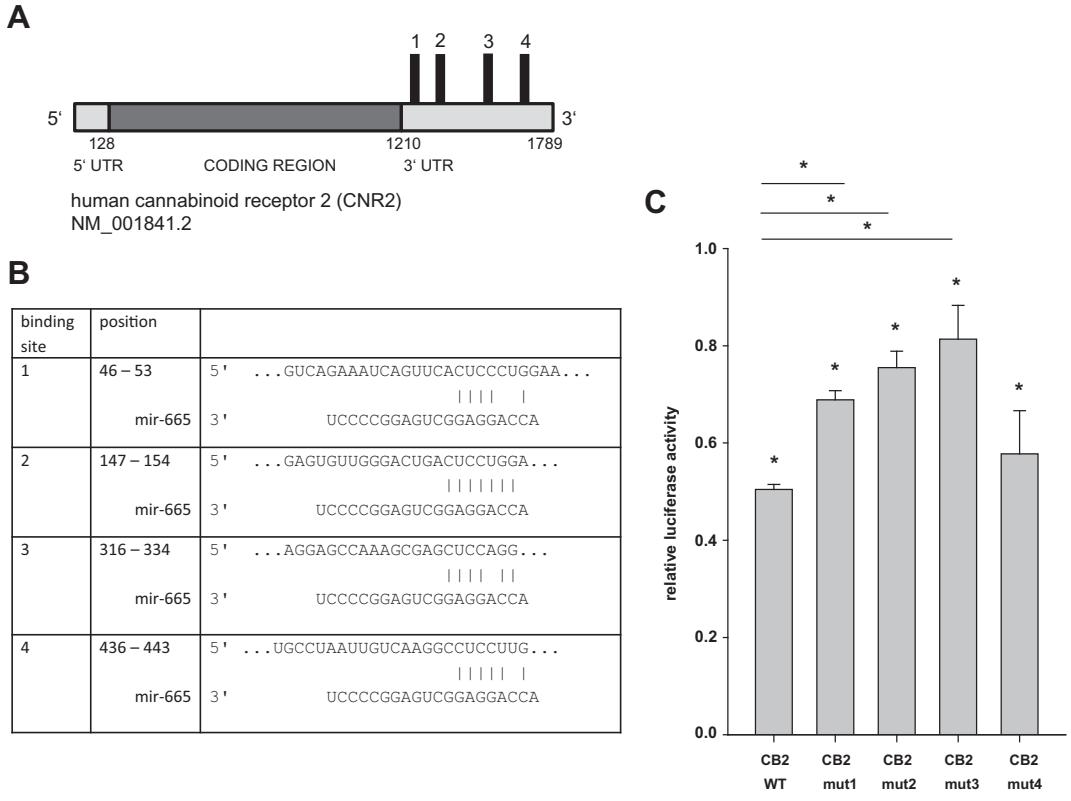
2.6. Tissue samples

Myocardial biopsy specimens were obtained from 12 explanted hearts during cardiac transplantations for end-stage CHF as previously reported [14] (demographics: Supplementary Material, Table 1). Control tissue samples were obtained from patients without signs of CHF who underwent open heart surgery using excess tissue from needle punctures of the left ventricle in routine surgical deairing maneuvers. RNA was extracted using RNAlater (Ambion, Austin, TX). The study was approved by the local ethical committee and all subjects provided informed consent. Additionally, commercially available total RNA derived from normal left ventricle (Ambion, Austin, TX) was used.



binding site	position	
1	2305 - 2312	5' ...UGAACAUUAAUCCACAUGUUUCA...
	mir-494	3' CUCCAAAGGGCACAUACAAAGU
2	2911 – 2918	5' ...UGAAUCAGUCCUAGA-AUGUUUCA...
	mir-494	3' CUCCAAAGGGCACAUACAAAGU
3	3610 – 3617	5' ...UCAAAUUUAUUUCCA----AUGUUUCA...
	mir-494	3' CUCCAAAGGGCACAUACAAAGU

Fig. 1. Mir-494 targets CB1. (A) Graphical depiction of the CB1 (CNR1) transcript. Locations of the predicted miR-494 binding sites are illustrated. (B) Alignment of miR-494 seed sequences to the CB1 3′UTR as predicted by bioinformatics. (C) Luciferase assay demonstrating the response of wild-type CB1 3′UTR (WT) and of selectively mutated controls (mut1–mut3, lacking miRNA binding sites 1–3) after transfection of premiR-494. Results are expressed as hRluc/Fluc activity relative to scrambled control (mean ± SD; $n = 3$; $^{*}p < 0.05$).



binding site	position	
1	46 – 53	5' ...GUCAGAAAUCAGUUCACUCCCUGGAA...
	mir-665	3' UCCCCGGAGUCGGAGGACCA
2	147 – 154	5' ...GAGUGUUGGGACUGACUCCUGGA...
	mir-665	3' UCCCCGGAGUCGGAGGACCA
3	316 – 334	5' ...AGGAGCCAAAGCGAGCUCCAGG...
	mir-665	3' UCCCCGGAGUCGGAGGACCA
4	436 – 443	5' ...UGCCUAAUUGUCAAGGCCUCCUUG...
	mir-665	3' UCCCCGGAGUCGGAGGACCA

Fig. 2. Mir-665 targets CB2. (A) Graphical depiction of the CB2 (CNR2) transcript. Locations of the predicted miR-665 binding sites are illustrated. (B) Alignment of miR-665 seed sequences to the CB2 3′UTR as predicted by bioinformatics. (C) Luciferase activity as measured after cotransfection of premiR-665 and wild-type of CB2 3′UTR (WT) and controls with selectively mutated binding sites (mut1–mut4), respectively (hRluc/Fluc activity relative to non-targeting control; mean ± SD; $n = 3$; $^{*}p < 0.05$).

2.7. Bioinformatic and statistical analyses

Putative miRNAs targeting the 3′UTR of the CB1 and CB2 receptor genes were identified using the miRNA target prediction tools TargetScan and PITA, implementing both prediction scores and agreement between the different programs [19,20]. All data were analyzed using SigmaPlot 11.0 software (Systat Software, Chicago, IL). If not stated otherwise, data are expressed as mean ± SD. All experiments were performed at least in triplicates. Analyses were performed with the Student's *t*-test or the nonparametric Mann–Whitney Rank Sum Test, as appropriate, with $p < 0.05$ considered as statistically significant.

3. Results

3.1. CB1 and CB2 receptor expression is regulated by microRNAs

We first investigated whether the expression of CB-receptors in human myocardium might generally be under the regulatory control of miRNAs. A siRNA-based knockdown of DICER1, the critical enzyme of cellular miRNA processing, was applied to generate a general functional knock-down of miRNAs. Upon siRNA-mediated knockdown of DICER1, CB1 mRNA as well as CB2 mRNA levels significantly increased by 44.2% ± 5.2% for CB1 and 38.7 ± 4.6% for CB2 in human cardiomyocytes, indicating a significant role of miRNAs in the posttranscriptional regulation of CB1 and CB2 receptor expression (Supplementary data, Fig. 1).

3.2. CB1 receptor expression is regulated by mir-494

In silico target prediction identified three specific binding sites of miR-494 with high probability within the 3′-UTR of the CB1 (CNR1) transcript (Fig. 1A and B). To provide an experimental proof of a direct interaction between miR-494 and the CB1 3′-UTR, we performed luciferase reporter assays on a psiCheck-2 plasmid containing a *Renilla* luciferase gene upstream of the CB1 3′UTR. HEK293 cells were transiently co-transfected with the reporter vector construct and premiR-494, which reduced luciferase activity by 58.7 ± 3.5% as compared to scrambled control ($n = 3$; $p < 0.001$, Fig. 1C). Site-directed mutagenesis of the miR-494 binding sites 1 and 2 within the CB1 3′UTR restored diminished luciferase activity by 43.4 ± 8.6% (mut1; $p < 0.005$) or 30.0 ± 8.5% (mut2; $p < 0.005$), respectively, indicating specific effects of miR-494 via binding to these sites, whereas an interaction of miR-494 with binding-site 3 in the 3′UTR of CB1 could not be proven.

To validate the impact of miR-494 on the expression of CB1, we assessed CB1 mRNA and protein levels after transfection of human cardiomyocytes with premiR-494. As shown in Fig. 3A, transient overexpression of miR-494 resulted in a significant decrease of CB1 mRNA (0.58 ± 0.08; $n = 3$; $p = 0.013$) as well as CB1 protein as compared to control (Fig. 3A and B).

3.3. CB2 receptor expression is regulated by mir-665

By combining TargetScan and PITA prediction, miR-665 was identified *in silico* to potentially target CB2 (Fig. 3A and B). Specific effects of miR-665 on CB2 (CNR2) mRNA were evaluated by luciferase reporter assays on a psiCheck-2 plasmid containing the Renilla luciferase gene upstream of the CB2 3′UTR. Transient cotransfection with reporter vector constructs and premiR-665 decreased luciferase activity by 49.6 ± 1% ($n = 3$; $p = 0.002$, Fig. 3D). Sequential dinucleotide exchanges within the proposed seed match sequences within the CB2 3′UTR restored luciferase reduction by 18.4 ± 1.8% (mut1; $p < 0.005$), 25.0 ± 3.3% (mut2; $p < 0.005$) and 30.9 ± 6.9% (mut3; $p < 0.005$), respectively, whereas

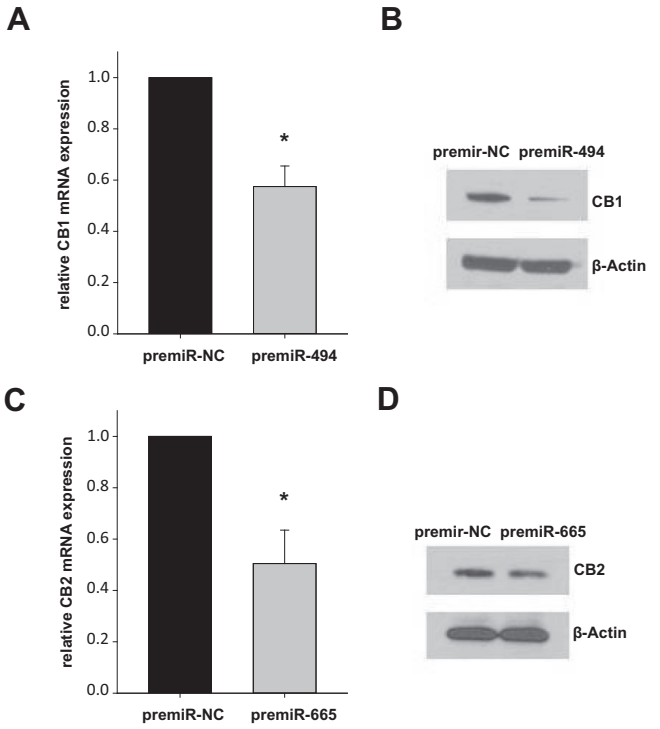


Fig. 3. CB1 and CB2 receptor expression is regulated by mir-494 and mir-665, respectively, (A) Human cardiomyocytes were transiently transfected with premiR-494 or with scrambled control (premiR-NC), and relative CB1 mRNA levels were analyzed by qPCR (mean ± SD; $n = 3$; $^*p < 0.05$). (B) In the same samples, CB1 protein expression was determined by Western blotting. One experiment representative of three is shown. (C) Human cardiomyocytes were transiently transfected with premiR-665 or with scrambled control (premiR-NC), and relative CB2 mRNA levels were analyzed by qPCR (mean ± SD; $n = 3$; $^*p < 0.05$). (D) In the same samples, CB2 protein expression was determined by Western blotting. One experiment representative of three is shown.

a selectively mutated binding site 4 exerted no effect. Thus our results indicate a direct effect of miR-665 resulting from specific binding to three binding-sites within the CB2 3′-UTR (Fig. 2C).

To confirm a specific effect of premir-665 on CB2 expression in vivo we transfected human cardiomyocytes with premir-665, and subsequently assessed CB2 mRNA and protein expression. As depicted in Fig. 3C and D, CB2 transcripts as well as CB2 protein were significantly decreased (mRNA: 0.51 ± 0.13; $n = 3$; $p = 0.05$).

3.4. Expression miR-494 and mir-665 in human in end-stage CHF myocardium

As assessed by qPCR, expression levels of mir-494 did not differ significantly between CHF and normal myocardium, however, a trend towards an up-regulation of miR-494 was seen (Fig. 4A). In these patients' samples we had previously found slightly decreased CB1 receptor expression [14].

In contrast, we found a significantly decreased expression of miR-665 (0.79 ± 0.20 vs. 1.16 ± 0.10; $p < 0.005$) in myocardium samples of patients with end-stage heart failure as compared to normal controls (Fig. 4B). In the same samples, an 11.6-fold increase in CB2 receptor expression had previously been determined [14]. These results suggest that in CHF, miR-665 reduction contributes to a compensatory up-regulation of cardioprotective CB2 receptors.

4. Discussion

Recent studies strongly propose that the myocardial ECS acts as a "stress response" system supporting the organism's attempt to

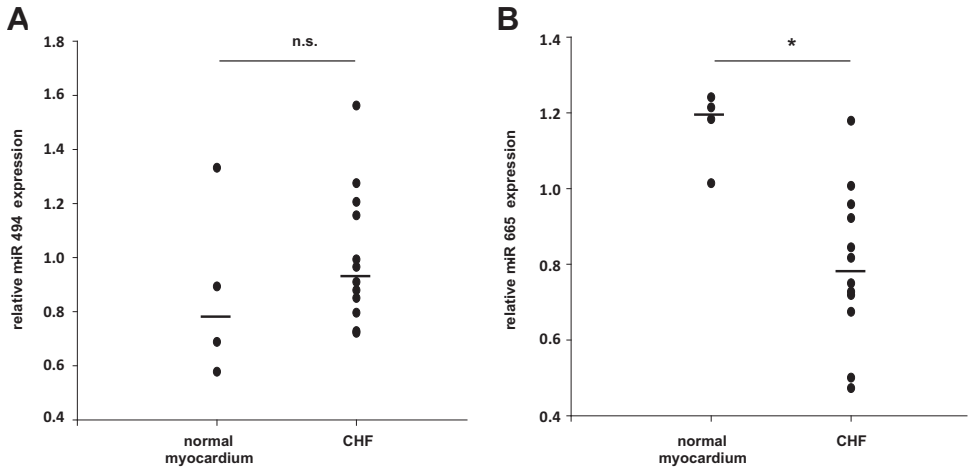


Fig. 4. MiR-494 expression in CHF patients and non-CHF-controls. Real-time PCR analysis of miR-494 (A) and miR-665 (B) expression in ventricles of CHF patients ($n = 12$) as compared to normal myocardium of healthy subjects ($n = 4$). Data were calculated relative to GAPDH and B2M, single values and median are shown; $^{*}p < 0.05$.

regain homeostasis in response to stressful events [1–3]. Altered expression levels of CB1 and CB2 receptor subtypes in CHF, as described by Weis et al. [14], thus may be regarded as an adaptive mechanism ameliorating the pathological changes of the disease. In particular the marked up-regulation of CB2 receptors might significantly contribute to an endogenous cardioprotective response in severe heart failure [14]. The mechanisms underlying the differential CB1 and CB2 expression in healthy and diseased myocardium, however, have not been investigated yet.

In the current study we provide evidence that myocardial endocannabinoid receptors are under the regulatory control of microRNAs. MiRNAs emerged as a novel class of non-coding endogenous RNAs with wide-spread implications for gene regulation at the post-transcriptional level in eukaryotic cells [15,16]. In the heart, altered expression levels of miRNAs have been implicated in pathological conditions including fibrosis [21–23], hypertrophia [24,25], arrhythmia [26], and neoangiogenesis [27,28]. Furthermore, recent studies demonstrated that different types of heart disease, such as myocarditis [29], myocardial infarction [21,30], and CHF [31,32] are associated with distinct changes of miRNAs.

Knock-down of DICER, leading to an impairment of microRNA processing, resulted in increased expression of CB1 and CB2 receptors, which provided a first proof that both endocannabinoid receptor subtypes are regulated by microRNAs. This finding was confirmed and defined in detail by a combined computational and experimental approach, which provided evidence that miR-494 specifically regulates the expression of CB1 receptors, whereas the expression of CB2 receptors is under the regulatory control of miR-665.

In myocardial tissues of healthy subjects we detected considerable expression levels of both miRs. In cardiac tissue samples of end-stage CHF, we observed a significant decrease in expression of miR-665, while expression of miR-494 slightly increased, however, without reaching statistical significance. Although limited by the small number of control samples, which is due to ethical and practical constraints, these results strongly support the hypothesis that alterations of CB receptor expression in CHF may indeed be influenced by miR-665 and miR-494: In CHF, a marked up-regulation of CB2 receptor expression has been reported [14], which may-at least partly-be enabled by a decrease of its regulating miR-665. For CB1 receptor expression, a slight decrease has been described, which is in concordance with a slight increase of its regulating miR-494.

In conclusion, our results highlight a potential role of myocardial miR-665 and miR-494 in mediating cardioprotective effects via controlling the expression of CB1 and CB2 receptors.

It is tempting to speculate that myocardial changes occurring in CHF may elicit specific changes in the cardiac miRNA transcriptome thereby promoting compensatory changes of myocardial receptor expression profiles, and further studies are needed to investigate these issues.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.008.

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