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Docosahexaenoic acid reverses angiotensin II-induced RECK suppression and cardiac fibroblast migration

Jalahalli M. Siddesha [a,b], Anthony J. Valente [c], Tadashi Yoshida [b], Siva S.V.P. Sakamuri [b], Patrice Delafontaine [b], Hideo Iba [d], Makoto Noda [e], Bysani Chandrasekar [a,b,*]

[a] Research Service, Southeast Louisiana Veterans Health Care System, New Orleans, LA 70161, United States
[b] Heart and Vascular Institute, Tulane University School of Medicine, New Orleans, LA 70112, United States
[c] Department of Medicine, University of Texas Health Science Center and South Texas Veterans Health Care System, San Antonio, TX 78229, United States
[d] Department of Microbiology and Immunology, University of Tokyo, Tokyo 108-8639, Japan
[e] Department of Molecular Oncology, Kyoto University Graduate School of Medicine, Sakyo-ku, Kyoto 606-8501, Japan

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ABSTRACT

The omega-3 polyunsaturated fatty acids (ω−3 fatty acids) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been reported to inhibit or delay the progression of cardiovascular diseases, including myocardial fibrosis. Recently we reported that angiotensin II (Ang II) promotes cardiac fibroblast (CF) migration by suppressing the MMP regulator reversion-inducing-cysteine-rich protein with Kazal motifs (RECK), through a mechanism dependent on AT1, ERK, and Sp1. Here we investigated the role of miR-21 in Ang II-mediated RECK suppression, and determined whether the ω−3 fatty acids reverse these effects. Ang II induced miR-21 expression in primary mouse cardiac fibroblasts (CFs) *via* ERK-dependent AP-1 and STAT3 activation, and while a miR-21 inhibitor reversed Ang II-induced RECK suppression, a miR-21 mimic inhibited both RECK expression and Ang II-induced CF migration. Moreover, Ang II suppressed the pro-apoptotic PTEN, and the ERK negative regulator Sprouty homologue 1 (SPRY1), but induced the metalloendopeptidase MMP2, all in a manner that was miR-21-dependent. Further, forced expression of PTEN inhibited Akt phosphorylation, Sp1 activation, and MMP2 induction. Notably, while both EPA and DHA reversed Ang II-mediated RECK suppression, DHA appeared to be more effective, and reversed Ang II-induced miR-21 expression, RECK suppression, MMP2 induction, and CF migration. These results indicate that Ang II-induced CF migration is differentially regulated by miR-21-mediated MMP induction and RECK suppression, and that DHA has the potential to upregulate RECK, and therefore may exert potential beneficial effects in cardiac fibrosis.

1. Introduction

Cardiac fibrosis, characterized by the excessive deposition of extracellular matrix (ECM) in the heart, contributes causally to the pathogenesis of most cardiac diseases, and is a major clinical problem [1,2]. For example, dilated cardiomyopathy is characterized by left atrial fibrosis, myocardial infarction by interstitial fibrosis, and pressure overload hypertrophy by both interstitial and perivascular fibrosis. The diastolic dysfunction seen during heart failure is attributed to cardiac fibrosis. While fibrotic processes are critical for proper wound healing and post-infarct scar formation, unrestrained fibrosis results in impaired cardiac function, and progression to heart failure. Cardiac fibroblasts (CFs), the predominant cell type in the heart, are the principal cells responsible for ECM homeostasis. Under physiological conditions, ECM turnover is maintained by a balance in activity between matrix-degrading metalloproteinases (MMPs) or matrixins, and their endogenous inhibitors, principally the TIMPs (tissue inhibitors of MMPs) [3]. Disruption of this delicate balance towards excess MMP activity leads to excessive ECM destruction, subsequent proliferation and migration of the CF, and increased deposition of new ECM, resulting in fibrosis, myocardial dysfunction, and ultimately heart failure.

Nearly 28 MMPs have been cloned and characterized in humans, and the four known TIMPs (*i.e.*, TIMPs 1–4) inhibit all the MMPs [3–5]. Since therapeutic strategies that target MMPs using various broad-spectrum inhibitors resulted in musculoskeletal complications [6,7] and necessitated withdrawal, identifying and characterizing newer MMP inhibitors is critical. In 1998, Noda and colleagues identified a membrane protein in NIH 3T3 cells transduced with the proto-oncogene v-Ki-RAS, and named it **re**version-inducing-**c**ysteine-rich protein with **K**azal motifs (RECK) based on its structure and role in the flat transformation of cells [8]. Importantly, RECK was shown to anchor to the plasma membrane *via* a C-terminal interaction with glycosylphosphatidylinositol (GPI), and inhibit release and activation of the metalloendopeptidases MMP9 and MMP2 MMPs 9 and 2 [8,9]. This unique membrane anchoring distinguishes it from other endogenous MMP inhibitors. Of note, *RECK*,

* Corresponding author at: Heart and Vascular Institute, Tulane University School of Medicine, 1430 Tulane Avenue, SL-48, New Orleans, LA 70112, United States. Tel.: +1 504 988 3034.
E-mail address: bchandra@tulane.edu (B. Chandrasekar).

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but not *TIMP* gene deletion, is embryonically lethal [9], implicating a critical role for RECK in development and growth.

RECK is a 110 kDa glycosylated protein that inhibits MMP9 activity and pro-MMP9 release [8]. It also inhibits both the maturation and activity of MMP2 by targeting a MMP2/TIMP2/MMP14 ternary complex [9]. To date, RECK has been shown to inhibit gelatinase MMPs 2 and 9, the transmembrane-type MMP MT1-MMP (MMP14) and the matrilysin MMP7 in cancer cells [8–10]. Of note, persistent activation of MMPs 2, 7, 9 and 14 contributes causally to cardiac fibrosis and adverse remodeling [11–14]. Recently we reported that chronic infusion of the vasoactive peptide angiotensin II (Ang II) activates MMPs, but markedly inhibits RECK in the hypertrophied heart [15]. We also demonstrated that Ang II-induced CF migration *in vitro* is characterized by AP-1- and NF-κB-dependent MMP induction and Sp1-mediated RECK suppression [15].

RECK expression is regulated at multiple levels; while Sp1 activation represses *RECK* transcription [16,17], post-transcriptional modifications such as acetylation, methylation and modulation by microRNAs have been shown to target RECK. Multiple microRNAs, including miR-21, target *RECK* 3′UTR, and repress its expression in various cancer cells. Of note, miR-21 is enriched in CF, and targeting miR-21 has been shown to attenuate pressure overload-induced cardiac fibrosis [18]. Since Ang II activates AP-1 [15], and AP-1 is known to positively regulate miR-21 induction [19], we examined whether Ang II-induced RECK suppression is mediated by miR-21 induction, and investigated the underlying mechanisms. Since the omega-3 polyunsaturated fatty acids ($\omega-3$ fatty acids) are known to attenuate miR-21 induction in a breast cancer cell line [20] and inhibit CF activation [21], we further investigated whether the $\omega-3$ fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) reverse Ang II-induced miR-21 induction, MMP expression, RECK suppression and CF migration.

2. Materials and methods

2.1. Materials

Human angiotensin II (Ang II; #A9525), lentiviral shRNA targeting RECK (#TRCN0000080129), c-Jun (JUN; #TRCN0000229528), Sp1 (#TRCN0000071603) and GFP (#SHC005V), anti-actin antibody (#A2066), protease inhibitor cocktail (#P8340), and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Eicosapentaenoic acid (EPA, #90110), docosahexaenoic acid (DHA, # 90310), and oleic acid (#90260) were purchased from Cayman Chemical Co. (Ann Arbor, MI). Antibodies against RECK (#3433), Akt (#9272), p-Akt (Thr308, # 9275), α-tubulin (# 2144) and lamin A/C (# 2032) were from Cell Signaling Technology, Inc. (Beverly, MA). Antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; #MAB374), total MMP9 (#AB19016), and MMP2 (#AB19167), and S31-201 (#573102) were purchased from Millipore (Billerica, MA). Anti-phospho-Sp1 (Thr453) antibodies were from Novus Biologicals (Littleton, CO). Anti-Sp1 (#14027), STAT3 (#sc-482), p-STAT3 (Ser727; #sc-8001-R), PI3Kp85α (#sc-423), PTEN (#sc-6817), and Spry1 (#sc-30048) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-MMP2 antibody (#NB200-114G) that detects both pro and active forms was from Novus Biologicals (Littleton, CO). Wortmannin (#681675), Ly294002 (#440202), SH-5 (#124008), PD98059 (#513000) and DMSO were purchased from EMD Biosciences (San Diego, CA). Nuclear Extraction Kit (#AY2002) was purchased from Panomics/Affymetrix (Freemont, CA). Enhanced chemiluminescence detection kit was from Amersham Pharmacia Biotech. pMIR-REPORT™ miRNA Expression Reporter Vector System was from Ambion®/Life Technologies.

2.2. Animals and isolation of adult mouse cardiac fibroblasts

All animal studies were approved by the Institutional Care and Use Committees at the University of Texas Health Science Center in San Antonio and Tulane University in New Orleans, and conformed to the *Guide for the Care and Use of Laboratory Animals* published by the NIH. Fibroblasts were isolated from the hearts of 8–10-wk old male wild type C57Bl/6, as previously described [15]. Freshly isolated and up to second passage CFs were used in all experiments. At 50–70% confluency, the cells were made quiescent by incubating in a medium containing 0.5% BSA (serum-free) for 48 h.

2.3. Adenoviral and lentiviral transductions

Adenovirus that expresses full-length mouse RECK ORF (GenBank accession #NM_016678.2) under control of the CMV promoter (Ad.RECK), PTEN (Ad.PTEN), dominant negative mutant of Akt (Ad.dnAkt), and siRNA against MMP2 (Ad.siMMP2) was previously described [15,22]. Ad.GFP and Ad.siGFP served as controls. Adenovirus expressing wild type mouse SPRY1 (Ad.SPRY1) was provided by Robert Friesel (Center for Molecular Medicine, Maine Medical Center Research Institute, Scarborough, ME). Lentiviral shRNA against c-Jun and Sp1 were previously described [15]. STAT3-Dominant Negative Lentivirus (dn.STAT3; #EH0007) was purchased from KeraFAST (Boston, MA). The CFs were infected with adenoviral vectors and lentiviral shRNA as previously reported [15,22]. At the indicated moi and duration, adenoviral or lentiviral infection displayed no off-target effects and failed to affect cell viability and adherence (data not shown).

2.4. Transfections and promoter-reporter analysis

2.4.1. MIR21 promoter analysis

The consensus *MIR21* promoter construct (miPPR-21-410) containing four AP-1 binding sites and its step-wise deletion constructs lacking each of the four AP-1 sites have been previously reported [19]. The CFs were transfected with the reporter constructs or the empty vector pGL4.12 using the Neon® transfection system (MPK-5000, Invitrogen). The CFs were microporated (pulse voltage: 1300 V, pulse width: 20 ms, pulse number: 2, tip type: 10 μl) with 3 μg of plasmid DNA for 24 h, co-transfected with the *Renilla* luciferase vector (pRL-TK, 100 ng) prior to Ang II addition.

2.4.2. RECK 3′UTR analysis

A 1172 nucleotide mouse *RECK* 3′UTR was amplified from total cDNA from mouse cardiac fibroblasts using the sense (ccacccacaactggaaggaactgca) and antisense (ggtataataagcatattgctatat) primers, and cloned into pMIR-REPORT vector (The Ambion® pMIR-REPORT™ miRNA Expression Reporter Vector System). Mutations in miR-21 binding site in *RECK* 3′UTR were introduced by site-directed mutagenesis using the QuikChange kit (Stratagene), and confirmed by nucleotide sequencing. The CFs were transfected with the wild type (*RECK*-3′ UTR) or mutant (m*RECK*-3′UTR) *RECK* pMIR-REPORTER vectors using the Neon® transfection system described above. Twenty-four hours after transfection the CFs were treated with Ang II (10^{-7} M for 24 h) and analyzed for reporter activity.

2.5. miRNA mimic and inhibitor, and transfections

Mouse miR-21 mimic (#C-310515-05), miR-21 inhibitor (#IH310515-07), inhibitor control (#IN-001005-01) and mimic control (#CN-001000-01) were purchased from Thermo Scientific-Dharmacon (Lafayette, CO). The CFs were transfected with the mimic, inhibitor or controls (80 nM) using the Neon® transfection system (MPK-5000, Invitrogen). The CFs were microporated (pulse voltage: 1300 V, pulse width: 20 ms, pulse number: 2, and the tip type: 10 μl) and then cultured for 24 h. The CFs showed transfection efficiency of 49% with only 7% cell death as determined using the pEGFP-N1 vector. Transfections at the indicated concentration and for the duration of treatment failed to significantly modulate CF adherence, shape or viability (trypan blue-dye exclusion; data not shown).