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

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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 (CF) 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 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.

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Keywords

Fibrosis; RECK; microRNA; migration; ω -3 lipids; PTEN; SPRY1

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 (CF), 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, 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 known MMPs [3–5]. Since therapeutic strategies that target MMPs using various broad-spectrum inhibitors resulted in musculoskeletal complications [6, 7] and therefore necessitated withdrawal, identifying and characterizing newer MMP inhibitors is important to attenuate progression of cardiac fibrosis. In 1998, Noda and colleagues identified a membrane protein in NIH 3T3 cells transduced with the protooncogene v-Ki-RAS, and named it **r**eversion-inducing-cysteine-rich protein with **K**azal motifs (RECK) based on its role in the flat transformation of cells and structure [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 MMPs 9 and 2 [8, 9]. This unique membrane anchoring distinguishes it from other endogenous MMP inhibitors. Of note, *RECK*, but not *TIMP* gene deletion is embryonically lethal [9], implicating a critical role in development.

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 the gelatinases MMPs 2 and 9, the transmembrane-type 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 expression in the hypertrophic heart [15]. We also demonstrated that Ang II-induced CF migration *in vitro* is characterized by AP-1- and NF- κ B-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 (ω -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 ω -3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) reverse Ang II-induced miR-21 induction, MMP expression, RECK suppression and CF migration.

Materials and methods

Materials

Human angiotensin-II (Ang-II; #A9525), lentival 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), PI3Kp85 α (#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.

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 CF were used in all experiments. At 50–70% confluency, the cells were made quiescent by incubating in medium containing 0.5% BSA (serum-free) for 48 h.

Adeno and lentiviral transductions

Adenovirus that express 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) were 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).

CF were infected with adenoviral vectors and lentiviral shRNA as previously reported [15, 22]. At the indicated moi and the duration, adeno or lentiviral infection displayed no off-target effects and failed to affect cell viability and adherence (data not shown).

Transfections and promoter-reporter analysis

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]. CF were transfected with the reporter constructs or the empty vector pGL4.12 using the Neon® transfection system (MPK-5000, Invitrogen). CF were microporated (pulse voltage: 1300 volts, 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.

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

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). CF were transfected with the mimic, inhibitor or controls (80 nM) using the Neon® transfection system (MPK-5000, Invitrogen). CF were microporated (pulse voltage: 1300 volts, pulse width: 20 ms, pulse number: 2, and the tip type: 10 µl) and then cultured for 24 h. CF 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).

mRNA expression

Total RNA was isolated using the TRIzol method. One microgram of DNA-free total RNA was used for the first strand cDNA synthesis using Quantitect cDNA Synthesis Kit [15]. MMP2 and RECK mRNA levels were analyzed by RT-qPCR using Gene Expression TaqMan® probes (Applied Biosystems) and Eppendorf Realplex₄ system [15]. 18S served as an invariant control. Data are shown as fold change ($2^{-\Delta\Delta C_t}$). For miRNA expression, small RNA-enriched total RNA isolated using the *mirVana*™ miRNA Isolation Kit (Ambion®) was used. miR-21 expression was analyzed by RT-qPCR using miRNA TaqMan® probes (Applied Biosystems). Its expression was analyzed by Northern blotting using radiolabeled StarFire oligonucleotide probes. Radiolabeling of miRNA antisense oligonucleotides was performed with a StarFire labeling kit (IDT, Coralville, IA), according to the manufacturer's instructions. Twenty micrograms of small RNA-enriched total RNA were resolved through 12.5% urea-polyacrylamide gel under denaturing conditions, transferred onto GeneScreen Plus membranes (PerkinElmer, Waltham, MA), UV-cross-linked, pre-hybridized, hybridized, and washed as described. U6 served as a loading control.

Immunoblotting

Preparation of whole cell homogenates, immunoblotting, detection of the immunoreactive bands by enhanced chemiluminescence (ECL Plus; GE Healthcare), and their quantification by densitometry were described previously [15, 22]. MMP2 activation was analyzed by immunoblotting using antibodies that detect both pro and active forms [15]. Nuclear extracts were prepared using the Panomics Nuclear Extraction kit according to the manufacturer's instructions (#AY2002, Panomics/Affymetrix, Freemont, CA), and nuclear phospho-Sp1 (p-Sp1) levels were analyzed by immunoblotting. Lamin A/C (nuclear) and GAPDH (cytoplasmic) served as loading and purity controls.

Cell migration

CF migration was quantified as described previously [15] using BioCoat™ Matrigel™ invasion chambers and 8.0-μm pore polyethylene terephthalatemembranes with a thin layer of Matrigel™ basement membrane matrix. Cultured CF were trypsinized and suspended in RPMI + 0.5% bovine serum albumin, and 1 ml containing 2.0×10^5 cells/ml was layered on the coated insert filters. Cells were stimulated with Ang II (10^{-7} M). The lower chamber contained 10% serum. Plates were incubated at 37 °C for 12 h. Membranes were washed with PBS, and noninvading cells on the upper surface were removed using cotton swabs. CF invading into and through the Matrigel™ matrix were quantified by MTT assay. Numbers of CF migrating in response to Ang II were normalized to those of untreated cells and expressed as fold change from untreated.

Cell death analysis

To determine whether transduction of viral vectors, pharmacological inhibitors or over-expression of mutant proteins affected cell viability, cell death was analyzed using the Cell Death Detection ELISA^{PLUS}, trypan blue dye exclusion, and microscopic visualization of cell shape and for cells floating in the media.

Statistical analysis

Results are expressed as means $\pm$ S.E. For statistical analysis we used analysis of variance followed by an appropriate post hoc multiple comparison test (Tukey method). Data were considered statistically significant at $P < 0.05$.

Results

DHA induces RECK expression and reverses Ang II-mediated RECK suppression

Previously, we demonstrated that Ang II differentially regulates MMPs, and their regulator RECK, in cardiac fibroblasts [15]. While inducing MMPs, Ang II inhibits RECK expression, and forced expression of RECK attenuates Ang II-induced MMPs 2, 9 and 14 expression, and cardiac fibroblast migration [15]. Since ω -3 polyunsaturated fatty acids exert a number of protective effects, including the inhibition of inflammation and fibrosis [21, 23–27], we investigated whether EPA and DHA regulate basal and Ang II-induced RECK expression in CF. Treatment of quiescent CF with 100 μM EPA or DHA upregulated basal RECK expression (Fig. 1A). The response to DHA was more pronounced than with EPA. In line with our previous studies, the addition of Ang II (10^{-7} M) caused a marked reduction in RECK expression in the CF, but pretreatment with either EPA or DHA reversed Ang II-induced RECK suppression (Fig. 1B). Since DHA was again more effective than EPA, we focused our subsequent experiments on DHA. Importantly, DHA pre-treatment attenuated Ang II-induced CF migration, as did the forced expression of RECK (Fig. 1C). However, the ω -9 fatty acid oleic acid (OA) failed to affect either basal RECK expression (Supplementary Fig. S1-A), Ang II-induced RECK suppression (Supplementary Fig. S1-B),

or miR-21 induction (Supplementary Fig. S1-C). Together, these results indicate that the ω -3 fatty acid DHA reverses Ang II-induced RECK suppression and inhibits Ang II-induced CF migration (Fig. 1).

Ang II induces CF migration via miR-21-dependent RECK suppression

The non-coding microRNA miR-21 has been shown to target RECK expression in various cancer cells [28, 29]. miR-21 also plays a role in cardiac fibrosis [18, 30]. Interestingly, *MIR21* is an AP-1 responsive gene [19], and we have previously reported that Ang II activates AP-1 in CF via ERK [15]. Therefore we investigated whether Ang II suppresses RECK expression in CF via ERK/AP-1-dependent miR-21 induction. Transfection of CF with a miR-21 inhibitor, but not its scrambled control, reversed both Ang II-induced RECK suppression (Fig. 2A) and CF migration (Fig. 2B). In contrast, a miR-21 mimic attenuated basal RECK expression (Fig. 2C), and moderately stimulated CF migration (Fig. 2D). Further, Ang II suppressed wild type *RECK* 3'UTR-driven reporter activity, and this effect was reversed when the miR-21 binding sequence was mutated (Fig. 2E). Together these results indicate that miR-21 modulates Ang II-mediated RECK suppression (Fig. 2).

DHA inhibits Ang II-induced ERK/AP-1-dependent miR-21 expression

Since miR-21 regulated RECK expression in the CF (Fig. 2), and Ang II has been shown to induce miR-21 expression in cardiac cells [18], we investigated the effect of DHA on the Ang II-miR-21- RECK pathway. Consistent with earlier reports, Ang II induced miR-21 expression in CF (Fig. 3A), an effect that was markedly attenuated by the ERK inhibitor PD98059 (Fig. 3A) and c-Jun knockdown (Fig. 3B). Further, Ang II-stimulated *MIR21* promoter-reporter activity was gradually reduced by the stepwise deletion of each of the 4 AP-1 binding sites in a *MIR21* promoter construct (Fig. 3C). Deletion of all 4 AP-1 binding sites abrogated Ang II-induced *MIR21* promoter activity. Importantly, DHA inhibited Ang II-induced *MIR21* promoter activity (Fig. 3D) and expression (Fig. 3E). These results indicate that DHA not only upregulates basal RECK expression, but it also reverses Ang II-induced RECK suppression by inhibiting miR-21 expression (Fig. 3).

DHA inhibits PTEN/Akt/Sp1-mediated MMP2 expression

miR-21 has multiple well-defined targets, including PTEN, the negative regulator of PI3K/Akt signaling. Interestingly, inhibition of PTEN has been shown to result in the increased expression of the metalloproteinase MMP2 [31], an important regulator of ECM homeostasis. MMP2 also plays a role in cell migration, and knockdown of MMP2 markedly reduced CF migration in response to Ang II (Fig. 4A). Therefore we investigated whether DHA inhibits Ang II-induced MMP2 expression. Ang II treatment increased both MMP2 mRNA and activity in CF, and this was inhibited by the forced expression of PTEN or a dominant negative form of Akt (Fig. 4B). However, at the indicated moi and duration of treatment, PTEN overexpression failed to affect cell viability (Supplementary Fig. S2). Further, CF express PTEN and Akt under basal conditions, and Ang II, while inhibiting PTEN expression, induced Akt activation (Fig. 4C). Importantly, pretreatment with DHA partially reversed both of these responses (Fig. 4C).

The induction of MMP2 in response to Ang II is mediated by a number of transcription factors including the ubiquitously expressed Sp1. In fact, knockdown of Sp1 reduced Ang II-induced MMP2 expression and activity in the CF (Fig 4D). Further, forced expression of PTEN (Fig. 4E) and the PI3K inhibitor wortmannin and the Akt inhibitor SH-5 (Fig. 4F) each attenuated Ang II-induced Sp1 activation. Importantly, DHA inhibited Ang II-induced Sp1 activation (Fig. 4G). Together, these results demonstrate that Ang II induces MMP2 expression in CF in part via PTEN/PI3K/Akt-mediated Sp1 activation, and that DHA inhibits this pathway (Fig. 4).

SPRY1 regulates RECK expression

We have demonstrated that Ang II suppresses RECK expression via ERK activation (Fig. 3A). SPRY1 is an ERK inhibitor [32], and a target of miR-21 [18]. Of note, CF, but not cardiomyocytes, express SPRY1. Therefore, we investigated whether SPRY1 regulates RECK expression in CF. Similar to its effects on PTEN (Fig. 4C), Ang II suppressed SPRY1 protein expression in CF, and this effect was reversed by the miR-21-inhibitor (Fig. 5A). Further, the miR-21 mimic inhibited basal SPRY1 expression (Fig. 5B), and forced expression of wild type mouse SPRY1 reversed Ang II-induced ERK activation (Fig. 5C) and RECK suppression (Fig. 5D), suggesting that SPRY1 regulates RECK expression by inhibiting Ang II-induced ERK activation (Fig. 5).

DHA inhibits Ang II-induced STAT3-mediated miR-21 expression and CF migration

The transcription factor STAT3 plays a role in MMP induction [33]. Because STAT3 positively regulates miR-21 induction [34], we next investigated whether STAT3 regulates Ang II-induced MMP2 expression and CF migration, and the effect of DHA. Treatment with Ang II induced rapid phosphorylation of STAT3, with a marked increase seen as early as 15 min (Supplementary Fig. S3), and this effect was reversed by the ERK inhibitor PD98059 (Fig. 6A). Similarly, forced expression of a dominant negative mutant of STAT3 (Fig. 6B), and the pharmacological STAT3 inhibitor S31-201 (Fig. 6C) each attenuated Ang II-induced STAT3 phosphorylation and miR-21 expression (Fig. 6D). Moreover, S31-201 attenuated Ang II-induced MMP2 expression (Fig. 6E) and CF migration (Fig. 6F). Importantly, DHA attenuated Ang II-induced STAT3 phosphorylation (Fig. 6G), indicating that Ang II-mediated miR-21 induction, MMP2 expression, and CF migration are mediated in part via STAT3, and DHA inhibits STAT3 activation (Fig. 6).

Discussion

RECK, a unique membrane-anchored MMP regulator, is expressed in various organs, including heart [8]. Here we show that Ang II suppresses RECK expression in CF via ERK-dependent AP-1- and STAT3-mediated miR-21 induction. Moreover, Ang II suppresses the pro-apoptotic PTEN and the ERK negative regulator SPRY1, but induces MMP2, all in a miR-21-dependent manner. Further, forced expression of PTEN inhibits Akt phosphorylation, Sp1 activation, and MMP2 induction. Importantly, for the first time we show that the ω -3 polyunsaturated fatty acid DHA not only enhances basal RECK expression, but also reverses Ang II-induced miR-21 expression, RECK suppression, MMP2 induction, and CF migration. In contrast to DHA, EPA and oleic acid had minimal or no effect on these Ang II-induced changes. These results indicate that Ang II induces CF migration by suppressing the MMP inhibitor RECK. Notably, DHA reverses Ang II-induced RECK suppression and cardiac fibroblast migration (Fig. 7). DHA may thus exert potential beneficial effects in cardiac fibrosis by upregulating the MMP inhibitor RECK.

Although RECK is expressed in various organs under physiological conditions, its expression and regulation has been investigated primarily in tumor cell biology. RECK expression is greatly reduced in various tumors, perhaps a contributing factor to their aggressiveness and metastatic potential. In contrast, information about its expression, role and regulation in heart and other organs is limited. Recently, we reported that infusion of Ang II while activating four major MMPs, *e.g.*, MMPs 2, 7, 9 and 14, markedly attenuated RECK expression in the heart [15]. Consistent with these results obtained *in vivo*, we found that Ang II also suppresses RECK transcription in cultured CF via ERK-dependent Sp1 activation. In the current study, we show that the suppression of RECK in Ang II-treated CF involves ERK-dependent miR-21 induction. Mutation of the miR-21 recognition sequence in *RECK* 3'UTR reverses its suppression by Ang II. Consistent with these observations, a

miR-21 mimic attenuated basal RECK expression, and a miR-21 inhibitor reversed Ang II-induced RECK suppression, suggesting that miR-21 plays an important role in the post-transcriptional regulation of RECK in CF. Of note, miR-21 expression was reported to be enhanced in failing human hearts, specifically in cardiac fibroblasts, and targeting miR-21 by a modified antagomir attenuated cardiac fibrosis in pressure overloaded hearts [18]. In this report, it was also demonstrated that overexpression of a miR-21 mimic activates ERK and promotes CF survival in vitro, indicating that ERK may act downstream of miR-21. Here we show that Ang II-induced miR-21 induction is ERK-dependent, suggesting a positive amplification in ERK signaling following Ang II treatment.

We also show that Ang II activates AP-1 in cardiac fibroblasts in an ERK-dependent manner, and forced expression of a dominant negative mutant form of c-Jun attenuates Ang II-induced miR-21 induction. Moreover, Ang II induces STAT3 phosphorylation/activation in an ERK-dependent manner, and induces miR-21 in a STAT3-dependent manner, indicating that both AP-1 and STAT3 contribute to Ang II-mediated miR-21 induction. Supporting our results, a role for phospho-STAT3 was recently demonstrated in β -catenin-mediated miR-21 induction and RECK suppression in a model of glioma cell invasion [35]. Of note, NF- κ B activation also positively regulates miR-21 induction [36], and we showed previously that Ang II activates NF- κ B in CF in an ERK-dependent manner [15]. This suggests that ERK, through its activation of multiple transcriptional regulators, including AP-1, NF- κ B and STAT3 plays a central role in Ang II-induced miR-21 induction. We also previously reported that the non-steroidal anti-inflammatory drug aspirin induces RECK and inhibits CF migration [37]. Interestingly, aspirin is known to suppress phosphorylation and nuclear translocation of STAT3 in CF by attenuating IFN- γ -induced ROS generation and ERK activation [38].

In addition to RECK, Ang II also suppresses SPRY1 in CF. SPRY1 is a miR-21 target [18] and a negative regulator of ERK [32]. In fact, while a miR-21 mimic suppressed SPRY1 basal expression, a miR-21 inhibitor reversed Ang II-induced SPRY1 suppression. Further, SPRY1 overexpression attenuated Ang II-induced ERK activation, and importantly reversed Ang II-induced RECK suppression. Of note, knockdown of SPRY1 has been shown to enhance phospho-ERK levels in CF [18], and increased levels of SPRY1 mRNA and protein were reported in the human heart in response to mechanical unloading with a left ventricular assist device [39]. In those hearts, the phospho-ERK levels were markedly attenuated. Here we demonstrate that SPRY1 overexpression reverses RECK suppression, most likely by inhibiting ERK activation.

The pro-apoptotic factor PTEN is also a miR-21 target, and Ang II suppresses its expression in CF. PTEN is a negative regulator of PI3K/Akt signaling. Here we show that forced expression of wild type PTEN inhibited Ang II-induced PI3K activation, and basal as well as inducible Akt phosphorylation. Further, PTEN overexpression attenuated MMP2 expression via Akt-dependent Sp1 activation. PTEN overexpression also attenuated CF migration, without affecting cell viability. Taken together, these results indicate that Ang II suppresses RECK, SPRY1 and PTEN in CF, but induces MMP2, all in a miR-21-dependent manner (Fig. 7). Importantly, the ω -3 fatty acid DHA reversed these effects.

Pretreatment with DHA reversed Ang II-induced RECK suppression by attenuating AP-1 and STAT3 activation, and miR-21 induction. EPA, another biologically active ω -3 fatty acid, was found to be less effective. At a similar dose, EPA moderately upregulated basal RECK expression and reversed Ang II-induced RECK suppression. However, oleic acid, a ω -9 fatty acid, failed to modulate either basal RECK expression or Ang II-induced RECK suppression. These data suggest that DHA may exert potential beneficial effects in hypertensive heart diseases. In fact, a diet rich in ω -3 fatty acids has been shown to

markedly attenuate hypertension and renal ERK activation in the Dahl rat model of salt-sensitive hypertension [27]. Further, co-treatment with DHA+EPA has been shown to markedly attenuate Ang II- and arachidonic acid-induced profibrotic gene expression in mesangial cells [23], and IL-6-induced STAT3 activation and C-reactive protein expression in a liver cell line [24]. Moreover, DHA has been shown to inhibit migration and invasion of a breast cancer cell line by attenuating the pro-fibrotic MMP9 mRNA and protein expression, and enzyme activity [25]. Together, our present results and the published reports demonstrate potential anti-inflammatory and antifibrotic effects of the ω -3 fatty acids.

Recently, ω -3 fatty acids have also been shown to prevent pressure overload-induced cardiac fibrosis through activation of cGMP/PKG signaling in cardiac fibroblasts [21]. In that study, both DHA and EPA, but not oleic acid, inhibited TGF- β 1-induced cardiac fibroblast transformation, proliferation, and collagen synthesis. Importantly, in the GISSI-HF trial (The Gruppo Italiano per lo Studio della Streptochinasi nell'Infarto Miocardico Italian [GISSI] heart failure [HF]), a simple and safe treatment with ω -3 fatty acids was shown to moderately reduce mortality and admissions to hospital for cardiovascular reasons in patients with heart failure [40], suggesting beneficial effects of ω -3 fatty acids in cardiac diseases. Here we show for the first time that ω -3 fatty acids upregulate basal RECK expression, and reverse Ang II-induced RECK suppression. Thus ω -3 fatty acids may have therapeutic potential in the progression of cardiac fibrosis and adverse remodeling by upregulating RECK, an endogenous MMP inhibitor, and by targeting multiple proinflammatory and profibrotic factors in cardiac fibroblasts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Ang II	angiotensin II
AP-1	activator protein 1
CF	cardiac fibroblasts
CMV	cytomegalovirus
DHA	docosahexaenoic acid
ECM	extracellular matrix
EPA	eicosapentaenoic acid
GFP	green fluorescent protein
GPI	glycophosphatidylinositol
HA	hemagglutinin
JNK	c-Jun amino terminal kinase
miRNA	microRNA

miR-21	microRNA-21
miPPR-21	the <i>MIR21</i> promoter
miR-21-I	miR-21 inhibitor
MMP	matrix metalloproteinase
moi	multiplicity of infection
MT1-MMP	Membrane type 1 metalloproteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NF-κB	nuclear factor κB
PI3K	phosphoinositide 3-kinase
PTEN	Phosphatase and tensin homolog
RECK	reversion-inducing-cysteine-rich protein with Kazal motifs
ROS	reactive oxygen species
shRNA	short hairpin RNA
siRNA	small interfering RNA
snRNA	Small nuclear ribonucleic acid
Sp1	Specificity protein 1
SPRY1	Sprouty homologue 1
STAT3	signal transducer and activator of transcription 3
TIMP	tissue inhibitor of metalloproteinase
UTR	untranslated region

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Highlights

1. RECK is a unique membrane-anchored MMP inhibitor.
2. Angiotensin II suppresses RECK, PTEN and SPRT1 via miR-21 induction.
3. Forced expression of SPRY1 upregulates RECK.
4. RECK overexpression inhibits angiotensin II-induced fibroblast migration.
5. Docosahexaenoic acid reverses angiotensin II-induced RECK suppression.

Fig. 1. ω -3 polyunsaturated fatty acids reverse Ang II-induced miR-21 expression and RECK suppression

A, EPA and DHA upregulate basal RECK expression. At 50-70% confluency, adult mouse cardiac fibroblasts (CF) were made quiescent by incubating in medium containing 0.5% BSA (serum-free) for 48 h. The quiescent CF were then treated with EPA or DHA (100 μ M) for 8 h. RECK expression levels were analyzed by immunoblotting. Densitometric analysis of immunoreactive bands from three independent experiments is summarized in the adjacent panel. * P < at least 0.05 vs. untreated (n=3). B, EPA and DHA reverse Ang II-induced RECK suppression. The quiescent CF were treated with EPA or DHA (100 μ M for 8 h) prior to Ang II addition (10^{-7} M for 6 h). RECK expression was analyzed as in A, and the

densitometric analysis is shown on the right. $*P < 0.05$ vs. untreated, $\dagger P < 0.05$ vs. Ang II (n=3). C, Both DHA and forced expression of RECK attenuate Ang II-induced CF migration. CF infected with Ad.RECK (moi 40) for 24 h, or pretreated with DHA as B, were layered on Matrigel™ basement membrane matrix-coated filters, and then treated with Ang II (10^{-7} M for 12 h). The lower chamber contained medium with 10% serum. Cells migrating to the other side of the membrane were quantified using MTT assay. C, $*P < 0.01$ vs. untreated, $\dagger P < 0.05$ vs. Ang II, $\S P < 0.01$ vs. Ang II+DHA (n=6).

Fig. 2. Ang II induces CF migration via miR-21-dependent RECK suppression

A, miR-21 inhibitor reverses Ang II-induced RECK suppression. CF transfected with miR-21 inhibitor (miR-21-I; 80 nM for 24 h) were treated with Ang II (10^{-7} M) for 6 h, and then analyzed for RECK expression by immunoblotting. Inhibition in miR-21 expression was confirmed by Northern blotting as shown on the right (n=3). B, miR-21 inhibitor reverses Ang II-induced CF migration. CF transfected with the miR-21 inhibitor (80 nM for 24 h) were layered on Matrigel™ basement membrane matrix-coated filters, incubated with Ang II (10^{-7} M) for 12 h, and then analyzed for migration by MTT assay. * $P < 0.01$ vs. untreated; † $P < 0.05$ vs. Ang II, § $P < 0.05$ vs. Ang II + miR-21-I (n=6). C, miR-21 mimic inhibits basal RECK expression. CF transfected with miR-21 mimic or its scrambled control (80 nM for 24 h) were analyzed for RECK expression by immunoblotting (n=3). D, miR-21 mimic stimulates CF migration. CF transfected with miR-21 mimic (80 nM) for 24 h were analyzed for CF migration as in B. * $P < 0.05$ vs. untreated (n=6). E, Targeting miR-21 reverses Ang II-mediated RECK suppression. CF transfected with wild type (pGL3-RECK-3'UTR) or miR-21 mutated (pGL3-mRECK-3'UTR) RECK 3'UTR reporter vector (2 µg) along with pRL-Tk vector (100 ng) for 24 h were treated with Ang II (10^{-7} M for 12 h), and then harvested for dual luciferase activity. * $P < 0.05$ vs. Saline-untreated, †† $P < 0.01$ vs. mutant RECK (n=6).

Fig. 3. DHA reverses Ang II-induced miR-21 expression

A, Ang II induces miR-21 via ERK. The quiescent CF were treated with PD98059 (10 μ M) for 1 h prior to Ang II addition (10⁻⁷M for 1 h). miR-21 expression was analyzed by RT-qPCR and Northern blotting (inset). U6 snRNA served as a loading control (n=3). B, Ang II-induced miR-21 expression is AP-1 dependent. CF transduced with lentiviral c-Jun shRNA (moi 0.5 for 48 h) were incubated with Ang II (10⁻⁷M for 1 h). miR-21 expression was analyzed as in A (n=3). C, Ang II-induced *MIR21* promoter-reporter activity is AP-1-dependent. CF transfected with miPPR-21-410 promoter reporter or its deletion constructs (2 μ g) along with the *Renilla* luciferase vector (pRL-TK, 100 ng) for 24 h were treated with Ang II (10⁻⁷ M for 12 h), and harvested for the dual-luciferase assay. D, DHA inhibits Ang

II-induced miR-21 promoter reporter activity. CF were transfected with miPPR-21-410 promoter reporter (2 μ g) along with pRL-Tk (100 ng) for 24 h, incubated with DHA (100 μ M for 8 h) and then treated with Ang II (10^{-7} M) for 12 h prior to harvesting for the dual-luciferase assay. E, DHA suppresses Ang II-induced miR-21 induction. The quiescent CF were treated with DHA (100 μ M for 8 h) prior to Ang II addition. miR-21 expression was analyzed as in A. A, B, D, E, $*P < \text{at least } 0.05 \text{ vs. untreated}$, $\dagger P < 0.05 \text{ vs. Ang II}$ (n=3-6).

Fig. 4. DHA inhibits Ang II-induced PTEN/Akt-dependent MMP2 expression

A, Ang II induces CF migration in part via MMP2. CF transduced with adenoviral MMP2 siRNA (moi 100 for 24 h) were layered on matrix-coated filters, incubated with Ang II (10^{-7} M for 12 h), and then analyzed for migration as in Fig. 2B. Knockdown of MMP2 was confirmed by immunoblotting as shown on the right (n=3). MMP9 served as an off-target. * $P < 0.01$ vs. untreated; † $P < 0.01$ vs. Ang II (n=6). B, Forced expression of PTEN or dnAkt attenuates Ang II-induced MMP2 expression. CF transduced with Ad.PTEN (moi 20) or Ad.dnAkt (moi 100) for 24 h were incubated with Ang II (10^{-7} M) for 1 h (mRNA expression) or 24 h (immunoblotting). MMP2 mRNA expression was analyzed by RT-qPCR and MMP2 levels in culture supernatants by immunoblotting using antibodies that detect both pro and active forms (inset; n=3). * $P < 0.01$ vs. untreated; † $P < 0.05$ vs. Ang II ± Ad.GFP (n=6). C, DHA reverses Ang II-induced PTEN suppression and Akt activation. The quiescent CF were treated with DHA (100 mM for 8 h) prior to Ang II addition (10^{-7} M for 2 h). PTEN and p-Akt expressions were analyzed by immunoblotting (n=3). D, Ang II-induced MMP2 expression and activation is Sp1-dependent. CF transduced with lentiviral Sp1 shRNA (moi 0.5) for 48 h prior to Ang II addition (10^{-7} M) for either 1 (mRNA expression) or 24 h (immunoblotting) were analyzed for MMP2 mRNA expression by RT-qPCR and activity by immunoblotting using equal amounts of culture supernatants and antibodies that detect both pro and active forms (inset; n=3). Knockdown of Sp1 was

confirmed by immunoblotting and is shown on the right. $*P < 0.01$ vs. untreated; $\dagger P < 0.05$ vs. Ang II $\pm$ GFP (n=6). E, Forced expression of PTEN attenuates Ang II-induced Sp1 activation via PI3K and Akt. CF transduced with Ad.PTEN (moi 20 for 24 h) prior to Ang II addition were analyzed for phospho-Sp1 levels (Thr453) by immunoblotting using nuclear protein extracts and antibodies that detect activation-specific antibodies (n=3). Lamin A/C and GAPDH served as purity and loading controls. F, Ang II induces Sp1 activation via PI3K and Akt. The quiescent CF were treated with wortmannin or SH-5 as in A and B prior to Ang II addition. Phospho-Sp1 levels were analyzed by immunoblotting as in E (n=3). G, DHA inhibits Ang II-induced Sp1 activation. The quiescent CF were incubated with DHA (100 μ M for 8 h) prior to Ang II addition (10^{-7} M). Phospho-Sp1 levels were analyzed as in E (n=3).

Fig. 5. Sprouty homologue 1 (SPRY1) regulates RECK expression

A, miR-21 inhibitor reverses Ang II-induced SPRY1 suppression. CF transfected with miR-21 inhibitor (miR-21-I; 80 nM) for 24 h were incubated with Ang II (10^{-7} M) for 1 h, and then analyzed for SPRY1 expression by immunoblotting using cleared whole cell lysates (n=3). miR-21 scrambled inhibitor served as a control. B, miR-21 mimic inhibits basal SPRY1 expression. CF transfected with miR-21 mimic (80 nM) for 24 h were analyzed for SPRY1 expression as in A (n=3). C, Forced expression of SPRY1 reverses Ang II-induced ERK activation. CF transduced with Ad.SPRY1 (moi100) for 24 h were incubated with Ang II (10^{-7} M for 1 h). Phospho-ERK levels were analyzed by immunoblotting (n=3). SPRY1 overexpression was confirmed by immunoblotting as shown on the right. D, Forced expression of SPRY1 attenuates Ang II-induced RECK suppression. CF treated as in C, but for 6 h with Ang II (10^{-7} M) were analyzed for RECK expression by immunoblotting. Densitometric analysis of immunoreactive bands from three independent experiments is summarized on the right. * $P < 0.05$ vs. untreated; † $P < 0.05$ vs. Ang II (n=3).

Fig. 6. DHA inhibits Ang II-induced STAT3-mediated miR-21 expression and CF migration

A, Ang II induces STAT3 phosphorylation via ERK. The quiescent CF were incubated with PD98059 (10 μ M in DMSO for 1 h) prior to Ang II addition (10⁻⁷ M for 15 min). Total and phospho-STAT3 levels were analyzed by immunoblotting (n=3). B, Forced expression of mutant STAT3 attenuates Ang II-induced STAT3 phosphorylation. CF transduced with lentiviral dominant negative STAT3 (moi 0.5 for 48 h) were treated with Ang II (10⁻⁷ M) for 15 min, and then analyzed for phospho-STAT3 levels as in A (n=3). C, S31-201 inhibits Ang II-induced STAT3 phosphorylation. The quiescent CF treated with Ang II (10⁻⁷ M) and S31-201 (10 μ M) simultaneously were analyzed for total and phospho-STAT3 levels as in A (n=3). D, Targeting STAT3 attenuates Ang II-induced miR-21 expression. CF treated as in B and C, but for 1 h with Ang II (10⁻⁷ M) were analyzed for miR-21 expression by RT-qPCR. E, STAT3 inhibition attenuates Ang II-induced MMP2 expression. The quiescent CF treated with S31-201 and Ang II simultaneously for 2 h were analyzed for MMP2 mRNA expression by RT-qPCR. F, STAT3 inhibition attenuates Ang II-induced CF migration. The quiescent CF layered on matrix-coated filters were incubated with Ang II (10⁻⁷ M) and S31-201 (10 μ M) together for 12 h were analyzed for migration as in Fig. 2B. G, DHA

inhibits Ang II-induced STAT3 phosphorylation. The quiescent CF incubated with DHA (100 μ M for 8 h) prior to Ang II addition (10^{-7} M for 15 min) were analyzed for total and phospho-STAT3 levels as in A (n=3). D, E, F, * $P < 0.01$ vs. untreated; † $P < 0.05$ vs. Ang II (n=6).

Fig. 7. Schema showing possible signal transduction pathways involved in Angiotensin II-induced miR-21 induction, RECK, SPRY1 and PTEN inhibition, MMP2 expression, and cardiac fibroblast migration.