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Neuregulin-1β induces proliferation, survival and paracrine signaling in normal human cardiac ventricular fibroblasts

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ABSTRACT

Neuregulin-1β (NRG-1β) is critical for cardiac development and repair, and recombinant forms are currently being assessed as possible therapeutics for systolic heart failure. We previously demonstrated that recombinant NRG-1β reduces cardiac fibrosis in an animal model of cardiac remodeling and heart failure, suggesting that there may be direct effects on cardiac fibroblasts. Here we show that NRG-1β receptors (ErbB2, ErbB3, and ErbB4) are expressed in normal human cardiac ventricular (NHCV) fibroblast cell lines. Treatment of NHCV fibroblasts with recombinant NRG-1β induced activation of the AKT pathway, which was phosphoinositide 3-kinase (PI3K)-dependent. Moreover, the NRG-1β-induced PI3K/AKT signaling in these cells required phosphorylation of both ErbB2 and ErbB3 receptors at tyrosine (Tyr)1248 and Tyr1289 respectively. RNASeq analysis of NRG-1β-treated cardiac fibroblasts obtained from three different individuals revealed a global gene expression signature consistent with cell growth and survival. We confirmed enhanced cellular proliferation and viability in NHCV fibroblasts in response to NRG-1β, which was abrogated by PI3K, ErbB2, and ErbB3 inhibitors. NRG-1β also induced production and secretion of cytokines (interleukin-1α and interferon-γ) and pro-reparative factors (angiopoietin-2, brain-derived neurotrophic factor, and crypto-1), suggesting a role in cardiac repair through the activation of paracrine signaling.

1. Introduction

Cardiac muscle is highly susceptible to injury caused by ischemia, inflammation, pressure overload and/or volume overload. Cardiac damage incurred during injurious processes, such as myocardial infarction (MI), results in the initiation of the wound healing processes in response to cardiomyocyte death leading to replacement of normal cardiac tissue with a stiff, collagenous scar. While this fibrotic scar is necessary to the integrity of the heart, its extreme rigidity and lack of functioning cardiomyocytes perturb the heart's natural mechanical properties, accelerating pathogenic remodeling and promoting development of heart failure. The conversion of fibroblasts to collagen-producing myofibroblasts (myoFbs) is a hallmark of cardiac tissue remodeling subsequent to injury, but their persistence after this initial healing phase contributes to adverse extracellular matrix (ECM) remodeling and collagen deposition that interferes with normal heart functions [1].

NRG-1β (neuregulin-1β) is an epidermal growth factor family member, which is absolutely required for embryonic heart development [2–5]. More recently, it has become evident that NRG-1β also plays a role in cardiac repair in the adult heart [4,6] and may contribute to the beneficial effects of exercise on cardiac and skeletal muscles [7,8]. The main source of NRG-1β in the heart is the cardiac microvascular endothelium [6,9]. Endothelial cell-derived NRG-1β is activated by proteolysis, after which it induces both autocrine and paracrine signals within the myocardium [10–14] and possibly distant signaling, as circulating levels are associated with ischemic and coronary artery disease [15,16].

NRG-1β transduces signaling in cardiac myocytes and endothelial cells via erythroblastic leukemia viral oncogene homolog tyrosine kinase receptors (ErbB2, ErbB3, and/or ErbB4). NRG-1β-bound ErbB4 forms a homo-dimer or hetero-dimerizes with one of the other ErbBs, leading to activation of the intracellular tyrosine kinase domain and signal transduction. ErbB2 contains a functional tyrosine kinase domain but lacks a NRG binding site. ErbB3, on the other hand is kinase dead,

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but when bound by NRG-1β can dimerize with another ErbB receptor (typically ErbB2) and thereby relay the signal. Upon ligand binding, ErbB receptors are activated via autophosphorylation, which results in one of several downstream signaling cascades, dependent upon cell type and condition [17–19]. Known NRG-inducible cardiac signaling pathways include phosphoinositide 3-kinase (PI3K)/AKT, Ras/ERK, and Src/FAK [17–19]. In cardiomyocytes, for example, NRG-1β influences sarcomeric organization, induces proliferation, and increases survival [10,20–23]. In embryonic stem cells, NRG-1β promotes differentiation into cardiomyocytes [24–26]. NRG-1β can also influence blood pressure via the rostral ventrolateral medulla and modulate circulating immune cell functions [6].

Our laboratory previously reported improved cardiac function in both rat and swine models of myocardial infarction (MI) in animals treated with the glial growth factor 2 (GGF2) [27] isoform of NRG-1β, which contains a unique kringle domain in addition to the Ig-like and EGF-like domains that are common to other isoforms [19]. These studies led to the currently ongoing clinical trial examining the safety and tolerability of GGF2 in human subjects with systolic heart failure. We also demonstrated previously ErbB receptor expression and signaling in primary rodent cardiac stromal cells [27], as well as reduced fibrosis and MyoFb percentages in remote-from-infarct cardiac tissues of post-MI swine [27]. These preliminary experiments suggested NRG-1β may regulate cardiac fibroblast signaling and activity.

In this study, we investigated the effects of NRG-1β treatment on primary human cardiac ventricular fibroblasts and herein describe the presence of functional ErbB signaling in human fibroblasts and involvement of NRG/ErbB in fibroblast-mediated signaling, a significant finding given their importance in cardiac remodeling.

2. Materials and methods

2.1. Cell culture and reagents

Primary human cardiac ventricular fibroblasts (NHCF-V, cc-2904) were purchased from Lonza Walkersville Inc. (Walkersville, MD), expanded per manufacturer's protocol, and stored in 1 mL aliquots in Recovery Cell Culture Freezing Medium (Gibco/Thermo Fisher Scientific, Waltham, MA) in liquid nitrogen. All media, additives, and sub-culturing reagents were purchased from Lonza. Cells were routinely cultured in FGM-3 BulletKit (cc-cc4526), which includes Fibroblast Basal Medium (cc-3301) supplemented with FGM-3 SingleQuots (cc-4525). FGM-3 SingleQuots include recombinant human insulin (cc-4021), gentamycin sulfate amphotericin-B (cc-4081), and recombinant human fibroblasts growth factor-B (cc-4065). Expansion/splitting was accomplished by rinsing cells in HEPES Buffered Saline Solution (cc-5024) followed by detachment with Trypsin/EDTA Solution (cc-5012), inactivation of trypsin with Trypsin Neutralization Solution (TNS, cc-5002) and centrifugation at 1000*g* for 5 min. Unless otherwise stated, cells were serum-starved for 24 h followed by treatments in Fibroblast Basal Medium supplemented with gentamycin only (50 μg/mL, 17-528Z, Lonza). A total of five different NHCF-V cell lines (cc-2904, Lonza) were used in this study. Donor characteristics are provided in Table 1.

2.2. RNA sequencing (RNASeq)

Total RNA was isolated using the RNEasy Midi kit (Qiagen, Valencia, CA) per manufacturer's instruction. Quality control, mRNA enrichment and cDNA library preparation were performed by the Vanderbilt Technologies and Genomics (VANTAGE) Core at Vanderbilt. RNA integrity was assessed using the Bioanalyzer 2100 (Agilent). The Illumina Truseq RNA sample prep kit was employed for targeted analysis of polyadenylated transcripts. Paired-end sequencing was conducted on the Illumina HiSeq 2500. The resulting FASTQ data files for each sample were transferred to the Basespace Sequence Hub and aligned to the hg19 human reference genome assembly using the STAR aligner (Dobin et al., Bioinformatics 2012) with the following parameters: (1) pseudoautosomal regions masking, (2) mapping quality score of at least 37 (3) and paired end reads mapped to same chromosome with expected orientations.

Resulting BAM files were imported into Partek Genomics Suite 6.6 software, followed by quantification, normalization, and differential gene expression. Reads were quantified based on the RefSeq transcriptome, and normalized reads were represented as RPKM (Reads Per Kilobase of transcript per Million mapped reads).

2.3. Quantitative polymerase chain reaction (qPCR)

Total RNA was converted to cDNA with iScript (Bio-Rad). Pre-validated primers for human ErbB2, ErbB3, and ErbB4 receptors were purchased from Qiagen. Relative gene expression for each receptor was assessed using iTaq Universal SYBR Green Supermix (Bio-Rad) in a Bio-Rad CFX instrument, according to manufacturer's protocol. Briefly, ~200 ng of cDNA was mixed with 2× supermix mix, RNase-free water and 1 μM of primers, for a total reaction volume of 10 μL. A typical protocol included for polymerase activation at 95 °C for 30 s, and 40 cycles as follows: denaturation (5 s at 95 °C), annealing/extension (30 s at 60 °C), followed by melt-curve analysis. The comparative threshold method used to calculate fold-differences in Excel. Pre-validated human GAPDH primers served as internal controls to normalize target gene expression across different samples. Levels of each ErbB receptor was analyzed at P0, P1, and P2 (where P0 = pre-passaged cells from Lonza before subsequent passage in our laboratory). The experiment was performed for three cell lines acquired from different individuals. At least three technical replicates were also included to ensure reproducibility.

2.4. Western blot analysis

Cells were lysed with RIPA buffer (Millipore), supplemented with protease and phosphatase inhibitors (Roche) and mechanical disrupted using 29 ½ gauge needles (Becton Dickinson). Lysates were then centrifuged at 14,000 RPM for 15 min followed by measurement of proteins in the resulting supernatants by Bradford assay (Bio-Rad). Proteins (30 μg/lane) were mixed with 4× loading buffer (Bio-Rad), loaded into 4–20% TGX polyacrylamide gels (Bio-Rad) and subjected to gel electrophoresis. Proteins were then transferred to PVDF membranes (0.45 μm pore size, Millipore) at 100 V for 1 h at 4 °C. Membranes were rinsed in tris-buffered saline (TBS) and then blocked in 5% blocking buffer in TBS with

Table 1
General characteristics of the human cardiac ventricular fibroblast donors.

Tissue acq. no.	Age	Race	Sex	Experiments
20731	42 Y	Caucasian	Male	Preliminary optimization experiments
25981	45 Y	Caucasian	Male	Optimization, Western blotting, qPCR, preliminary cytokine profiling
27277	32 Y	Caucasian	Female	Western blotting, qPCR, RNASeq, flow, cytokine arrays
27741	52 Y	Caucasian	Male	Western blotting, qPCR, RNASeq, flow, proliferation and viability, cytokine arrays
27321	31 Y	Caucasian	Female	Western blotting, qPCR, RNASeq, flow, proliferation and viability, cytokine arrays