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Molecular basis and functional significance of Angiotensin II-induced increase in Discoidin Domain Receptor 2 gene expression in cardiac fibroblasts

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ABSTRACT

Delineation of mechanisms underlying the regulation of fibrosis-related genes in the heart is an important clinical goal as cardiac fibrosis is a major cause of myocardial dysfunction. This study probed the regulation of Discoidin Domain Receptor 2 (DDR2) gene expression and the regulatory links between Angiotensin II, DDR2 and collagen in Angiotensin II-stimulated cardiac fibroblasts. Real-time PCR and western blot analyses showed that Angiotensin II enhances DDR2 mRNA and protein expression in rat cardiac fibroblasts via NADPH oxidase-dependent reactive oxygen species induction. NF-κB activation, demonstrated by gel shift assay, abolition of DDR2 expression upon NF-κB inhibition, and luciferase and chromatin immunoprecipitation assays confirmed transcriptional control of DDR2 by NF-κB in Angiotensin II-treated cells. Inhibitors of Phospholipase C and Protein kinase C prevented Angiotensin II-dependent p38 MAPK phosphorylation that in turn blocked NF-κB activation. Angiotensin II also enhanced collagen gene expression. Importantly, the stimulatory effects of Angiotensin II on DDR2 and collagen were inter-dependent as siRNA-mediated silencing of one abolished the other. Angiotensin II promoted ERK1/2 phosphorylation whose inhibition attenuated Angiotensin II-stimulation of collagen but not DDR2. Furthermore, DDR2 knockdown prevented Angiotensin II-induced ERK1/2 phosphorylation, indicating that DDR2-dependent ERK1/2 activation enhances collagen expression in cells exposed to Angiotensin II. DDR2 knockdown was also associated with compromised wound healing response to Angiotensin II. To conclude, Angiotensin II promotes NF-κB activation that up-regulates DDR2 transcription. A reciprocal regulatory relationship between DDR2 and collagen, involving cross-talk between the GPCR and RTK pathways, is central to Angiotensin II-induced increase in collagen expression in cardiac fibroblasts.

1. Introduction

Delineation of molecular mechanisms relevant to tissue repair and fibrosis is a clinically important goal since parenchymal tissue destruction and fibrosis are a major cause of organ dysfunction. In the heart, fibroblast proliferation associated with augmented matrix production is a critical event in wound healing following parenchymal cell loss [1]. However, excessive hyperplasia of fibroblasts, in conjunction with unchecked matrix deposition, results in myocardial fibrosis and compromised ventricular function. Cardiac fibroblast response to injury is a complex process involving many factors and the intricate interplay between them that controls matrix homeostasis. In this regard, a major focus of research over the years in several laboratories has been the regulation of collagen turnover by Angiotensin II (Ang II) in cardiac fibroblasts, which are the only source of type I and type III collagens in the heart [2–3]. These investigations have convincingly demonstrated that Ang II, whose intracardiac generation is reported to be enhanced following myocardial injury [4], is a potent pro-fibrogenic factor in the myocardium with marked stimulatory effects on collagen expression in cardiac fibroblasts [4–5].

In the overall context of matrix biology, there has also been a surge of interest in collagen receptors that mediate fibroblast responses during tissue remodeling [6]. Discoidin domain receptor 2 (DDR2) is a collagen-specific receptor noted for its association with fibrotic diseases besides regulation of a wide array of fundamental cellular processes [7–9], including extracellular matrix remodeling and cell proliferation, differentiation, migration and adhesion [7,10]. It is a unique tyrosine kinase receptor (RTK) expressed predominantly in mesenchymal cells, with a marked regulatory role in epithelial-mesenchymal transition [11–13]. Over-expression of DDR2 is associated with pathological scarring in non-cardiac tissues and fibrotic diseases of the lung, kidney and liver [6,14–15] besides atherosclerosis, osteoarthritis and several tumors [16–19]. Sporadic reports suggest that the relationship between DDR2 and collagen may be of immense relevance in a setting of tissue injury [20–21]. In fact, collagen-DDR2 interaction as a key determinant

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of matrix remodeling *post injury* is an emerging paradigm. In the heart, DDR2 is expressed predominantly in fibroblasts that are of mesenchymal origin [22] and is likely to play a key role in tissue repair and fibrogenesis. Surprisingly, however, regulation of DDR2 gene expression and its possible involvement in collagen production have not been investigated yet in relation to the heart.

Against this backdrop, the objective of this study was to examine the regulation of DDR2 expression in cardiac fibroblasts by Ang II and probe its functional link with collagen. We present evidence, for the first time, that Ang II acts via protein kinase C to trigger p38 mitogen-activated protein kinase (p38 MAPK)-mediated activation of Nuclear Factor-κB (NF-κB) that in turn associates with the DDR2 promoter to enhance DDR2 transcription in cardiac fibroblasts. Importantly, using an siRNA-based gene silencing approach, we have demonstrated that DDR2 and collagen type 1 are locked in a cycle of mutual regulation in Ang II-stimulated cardiac fibroblasts, which can potentially impact tissue response to injury.

2. Materials and methods

2.1. Materials

Angiotensin II, Tri reagent, Bay 11–7085, PD 98059, Chelerythrine, U73122, VAS2870, Diphenyleneiodonium chloride, NAC and M199 were obtained from Sigma-Aldrich, USA. Dual Luciferase assay kit, Random primers, Reverse transcriptase, RNAase inhibitor, dNTPs and SB203580 were obtained from Promega. Low cell# ChIP kit protein A × 48 was from Diagenode. NE-PER Nuclear and Cytoplasmic Extraction Reagents and Chemiluminescent nucleic acid detection module were from Thermoscientific. DDR2 siRNA and Col1a1 siRNA were from Ambion. Lipofectamine was from Invitrogen. Opti-MEM and fetal bovine serum were from GIBCO. All cell culture ware was purchased from BD Falcon, USA. Primary antibodies against DDR2, p38 MAPK and ERK1/2 MAPK were obtained from Cell Signaling Technology, USA. Primary antibody for Collagen type I was from Santa Cruz Biotechnology, USA, and that for p65 NF-κB was from Abcam. All antibodies were used after dilution (1:1000), except anti-p65 NF-κB (1:30). This study was approved by the Institutional Animal Ethics Committee (SCT/IAEC122/AUGUST/2014/85).

2.2. Isolation of fibroblasts

Cardiac fibroblasts were isolated from young adult male Sprague–Dawley rats (2–3 months) as described earlier [23]. Sub-confluent cultures of cardiac fibroblasts from passage 2 or 3 were used for the experiments.

2.3. Real-time polymerase chain reaction analysis

Sub-confluent cultures of cardiac fibroblasts were subjected to the indicated treatments and total RNA was isolated using TRI Reagent, according to the manufacturer's instructions. Following DNase I treatment, 2 μg of total RNA was reverse transcribed to cDNA with random primers and M-MLV reverse transcriptase. TaqMan quantitative Real-time polymerase chain reaction (RT-PCR) analysis was carried out using the ABI prism 7500 Sequence Detection System (Applied Biosystems, CA) with specific FAM-labeled probes. PCR reactions were performed over 40 cycles, as per the manufacturer's instructions. DDR2 expression was normalized to β-actin.

2.4. Western blot analysis

Sub-confluent cultures of cardiac fibroblasts in serum-free M199 were treated with Ang II and relative DDR2 protein abundance was determined by western blot analysis following standard protocols, using β-actin as loading control. Enhanced chemiluminescence reagent was used to detect the proteins, and protein expression was quantified by densitometric scanning (Bio-Rad Laboratories).

2.5. Electrophoretic mobility shift assay

DNA-binding activity of NF-κB was assessed by electrophoretic mobility shift assay (EMSA) using the LightShift Chemiluminescent EMSA Kit. Sub-confluent cardiac fibroblast cultures were serum-starved for 24 h followed by incubation in M199 with or without Ang II, and nuclear extracts were prepared using the NE-PER nuclear extraction kit. Protein concentration of the nuclear extracts was determined using the BCA protein assay method. Primers for NF-κB were biotinylated using the Thermo Scientific 3′-end biotin labeling kit. The nuclear extracts were incubated with the biotinylated probes and components of the Light Shift Chemiluminescent kit at 37 °C for 60 min and electrophoresed on 6% non-denaturing gel. After transfer to nylon membrane, DNA was UV cross-linked at a wavelength of 254 nm for 10 min. After blocking, streptavidin-conjugated HRP was applied and bands were visualized by enhanced chemiluminescence.

2.6. Luciferase assay

Rat genomic DNA was subjected to PCR to amplify the DDR2 gene promoter region with forward primer, 5'GGTGGTAAGCTTTCTAGTCG AGGACCCAAACAG 3′, and reverse primer, 5'GGTGGTACGCGTTGCAGG CCACCAATAATGC 3′, and subsequently cloned into TA cloning vector, pCRII (Invitrogen). DDR2 promoter luciferase (pDDR2-Luc) plasmid was constructed by directionally cloning HindIII and MluI-digested 1.5 Kb promoter fragment from pCRII-DDR2 into pGL3 basic vector (Promega). The orientation and fidelity of the sequence was confirmed using restriction enzyme digestion and gene sequencing. Cardiac fibroblasts were plated on a 24-well plate and co-transfected with the constructed luciferase plasmid, pGL3-DDR2, in the presence of Renilla control vector (Promega, Madison, WI, USA). Total cell lysate was prepared 48 h after transfection and Luciferase activity was determined using the Luciferase reporter assay system (Promega) and normalized to Renilla.

2.7. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) was performed using the low cell ChIP kit (Diagenode), according to the manufacturer's protocol. After treatment of cardiac fibroblasts with Ang II, immunoprecipitation was carried out overnight at 4 °C using 10 μl of anti-p65 NF-κB antibody (Abcam). Immune complexes were pulled down with Protein A-coated magnetic beads. DNA cross-links of the immune complexes were reverted by proteinase K digestion followed by heating. DNA isolated from an aliquot of the total sheared chromatin was used as loading control for PCR (input control). The retrieved DNA was analyzed by PCR amplification using the following DDR2 promoter-specific primers containing binding site for NF-κB: 5′-AGCGAATCAACATGGCAGATAC-3′ (Forward), 5′-ACTCCTAACTCCTCACATATAAGC 3′ (Reverse). The PCR products were subjected to electrophoresis on 1% agarose gel. Chromatin immunoprecipitation using a non-specific antibody (normal human IgG) served as negative control.

2.8. RNA interference

RNA interference protocol was as reported by us earlier [24]. Briefly, cells were seeded on 12-well plates at 8×10^4 cells/well. After 24 h, the cells were incubated in Opti-MEM with Ambion pre-designed Silencer-Select siRNA [5 pmol DDR2, 5 pmol collagen α1 type1 or scrambled siRNA, control] and Lipofectamine (2 μl) for 19 h. Following an additional incubation in M199 with 10% FBS for 12 h, the cells were treated with Ang II for the indicated duration. Cell lysate was prepared in SDS lysis buffer, denatured and used for western blot analysis.