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Brief Communication

Phenylephrine and endothelin-1 upregulate connective tissue growth factor in neonatal rat cardiac myocytes

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Abstract

Cardiac hypertrophy is associated with hypertrophic growth of cardiac myocytes and increased fibrosis. Much is known of the stimuli which promote myocyte hypertrophy and the changes associated with the response, but the links between the two are largely unknown. Using subtractive hybridization, we identified three genes which are acutely (<1 h) upregulated in neonatal rat ventricular myocytes exposed to the α-adrenergic agonist, phenylephrine. One represented connective tissue growth factor (CTGF) which is implicated in fibrosis and promotes hypertrophy in other cells. We further examined the expression of CTGF mRNA and protein in cardiac myocytes using quantitative PCR and immunoblotting, confirming that phenylephrine increased CTGF mRNA (maximal within 1 h) and protein (increased over 4 - 24 h). Endothelin-1 promoted a greater, though transient, increase in CTGF mRNA, but the increase in CTGF protein was sustained over 8 h. Neither agonist increased CTGF mRNA in cardiac non-myocytes. By increasing the expression of CTGF in cardiac myocytes, hypertrophic agonists such as phenylephrine and endothelin-1 may promote fibrosis. CTGF may also propagate the hypertrophic response initiated by these agonists.

Keywords: Fibrosis; Hypertrophy; Gene expression; Cardiac myocytes; Subtractive hybridization; CTGF

1. Introduction

In response to an increase in work-load, terminally-differentiated cardiac myocytes undergo hypertrophic growth to maintain/increase cardiac output. In the short term, this produces beneficial "compensated" cardiac hypertrophy, but this may degenerate into "decompensation" and heart failure, a response which is accompanied by increased fibrosis [1]. Cardiac myocyte hypertrophy is associated with morphological changes and changes in gene/protein expression. A number of stimuli promote hypertrophy including α-adrenergic agonists (e.g. phenylephrine, PE) and endothelin-1 (ET-1) which activate specific receptors and acutely stimulate intracellular signaling pathways [2]. However, the links between acute signaling events and the established hypertrophic phenotype are largely unknown. Many signaling pathways lead to the activation of transcription factors and hypertrophic signaling probably results in changes in gene and, subsequently, protein expression which are either directly hypertrophic or which propagate the response by promoting further changes. Little is known about the changes in gene expression which occur immediately downstream of the signaling pathways. Using subtractive hybridization, we identified 3 genes which are induced acutely (< 1 h) by PE in cardiac myocytes.

2. Materials and methods

2.1. Cell cultures

Primary cultures of neonatal ventricular myocytes were prepared from Sprague-Dawley rats [3,4], plated at confluence (4×10^6 cells/60 mm dish) and used within 3 d. To prepare cardiac non-myocytes, cells from the myocyte preparation were plated at 0.5×10^6 cells/60 mm dish to allow proliferation of contaminating non-myocytes. The terminally-differentiated myocytes do not divide. When cells

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were confluent, they were trypsinised and re-plated at 20% confluence. This destroyed the myocytes and non-myocytes were grown to confluence. Serum was withdrawn prior to experimentation (24 h).

2.2. Suppression subtractive hybridization, cDNA cloning, secondary screening and sequence analysis

Poly(A)$^+$ RNA was extracted (mRNA microisolation kit, Invitrogen) and 2.25 μg from each of 3 myocyte preparations was pooled. First and second strand cDNA synthesis was performed and double-stranded cDNA was subtracted using PCR Select Kit (Clontech) [5]. Tester and driver populations consisted of cDNA derived from myocytes stimulated with PE (100 μM, 1 h) and unstimulated myocytes, respectively. Differentially expressed cDNAs were selectively amplified, and subtracted cDNAs were cloned [5]. Clones containing inserts were selected, transferred to nylon membranes and incubated (4 h) on 1.5 % LB-agar containing 50 mg/ml ampicillin.

Clones were replica plated and incubated overnight (25°C). DNA was transferred to membranes by placing sequentially onto Whatman 3MM paper saturated with 10 % (w/v) SDS (10 s), 2 M NaOH/1.5 M NaCl (5 min) and 1.5 M NaCl/0.5 M Tris pH 8.0 (5 min). Cellular debris was removed. Membranes were air-dried and baked (80°C, 2 h). Secondary screening of DNA on replica membranes was performed using labelled cDNA from unstimulated myocytes or myocytes exposed to PE (100 μM, 1 h). Total RNA was extracted [4] and cDNA incorporating [α-^{32}P]-dCTP was generated by incubating total RNA (1 μg) with 10 pM poly-dT primer, 1 mM each of dATP, dGTP, dTTP, 25 μCi [α-^{32}P]-dCTP, 1 × 1st strand buffer (Gibco BRL), 200 U MMLV reverse transcriptase, 40 U RNAse inhibitor (Gibco BRL) in 19 μl final volume. Samples were incubated (37°C, 10 min), supplemented with dCTP (1 mM final concentration) and incubated further (37°C, 50 min). Unincorporated [α-^{32}P]-dCTP was removed by centrifugation through fine Sephadex G50 (Sigma Biosciences) in 1 mM Tris pH 8.0/0.1 mM EDTA. Sonicated herring sperm DNA was added to the probes (250 μg/ml). Membranes were prehybridised (Rapid Hyb solution, Amersham Biosciences; 30 min, 65°C). The probes were boiled, added to the prehybridization solution and hybridized to filters with constant agitation (65°C, 2 h). Filters were washed [2 × SSC (30 mM sodium citrate, 300 mM NaCl, 21°C) then 2 × SSC containing 1 % (w/v) SDS (65°C, 15 min)]. Membranes were autoradiographed (-80°C, 4 h) and spot intensities between replica filters (unstimulated vs PE-treated) were compared to identify differentially-expressed clones. Cloned gene fragments were sequenced [5]. Sequences were aligned, compared and edited using Sequence Editor Version 1.0.3 (Perkin-Elmer Applied Biosystems, Inc.). GenBank and EMBL databases were searched for sequence identity at nucleotide and amino acid levels using BLAST Version 2.0 basic alignment search tool (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/BLAST).

2.3. Ratiometric RT-PCR analysis and quantitative PCR

Total RNA isolation and ratiometric RT-PCR was carried out [4,5] using primers designed to C208 (sense, 5′-ACATGGAGTAATTCAACTGAATA-3′; antisense, 5′-AGCACTTGCTAAGTTAGCAAC-3′; 200 bp amplicon), connective tissue growth factor (CTGF:sense, 5′- CTGAAAGAATAGCTGGCTTCA-3′; antisense, 5′-CTGGTACTAGCTGAGGTCAT-3′; 158 bp amplicon), C64 (sense, 5′-GCCTTAGCACAAGACATACC -3′; antisense, 5′-CTTGTTCACGAGGGATTCAC-3′; 282 bp amplicon) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, sense, 5′- ACCACAGTCCATGCCATCAC-3′; antisense, 5′-TCCACCACCCTGTTGCTGTA-3′; 451 bp amplicon).

Rat CTGF mRNA expression was analyzed using quantitative PCR with fluorescent-labeled TaqMan probes [6] using CTGF primers [forward primer, 5′-CAAGGACCGCACAGTGGTT-3’ (Tm, 58.8°C); reverse primer, 5′-GCAGTTGGCTCGCATCATAG-3′ (Tm, 59.4°C)]; probe [5′-CCCTAGCTGCCTACCGACTGGAAGACAC-3′ (Tm, 65.6°C); 91 bp amplicon] designed from the published rat CTGF sequence (AF120275). Internal control 18S rRNA primers and the TaqMan probe were from a pre-optimized kit (Cat no. 4310893E). PCR amplifications were performed in duplicate in 25 μl containing 2.5 μl cDNA template in 2 × PCR Master Mix (PE Biosystems). Reaction conditions as follows: 50°C, 2 min; 95°C, 10 min; 40 cycles of 95°C for 15 s followed by 60°C for 1 min. Reactions were performed and products detected using an ABI-Prism 7700 sequence detector. Omission of reverse transcriptase demonstrated no

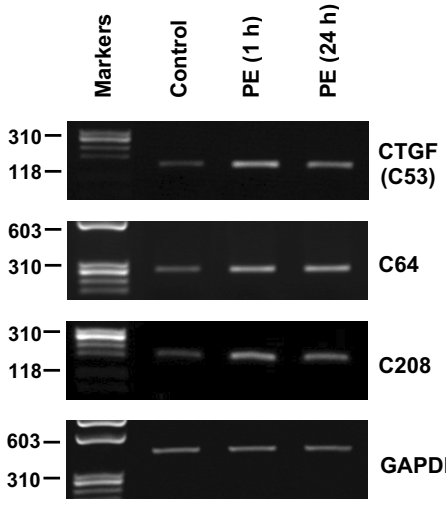

Fig. 1. CTGF (C53), and clones C64 and C208 are upregulated in cardiac myocytes by PE. Myocytes were unstimulated or exposed to 100 μM PE. Expression of CTGF, C64, C208 and GAPDH mRNA was analyzed by ratiometric RT-PCR. The positions of bp markers are indicated on the left of each panel. Results are representative of 3 independent preparations of myocytes.

detectable interference by genomic DNA. The level of expression of CTGF mRNA was normalized to 18S rRNA. Amplification of 18S rRNA and CTGF mRNA was co-linear.

2.4. Immunoblotting

Myocyte extracts were prepared and immunoblotting performed as described [4] with minor modifications. Proteins (40 μg) were separated on 10 % (w/v) resolving gels. Blots were incubated with antibodies to CTGF [Santa Cruz Biotechnology Inc. (sc-14939), 1/1500, 4°C, overnight], washed, then incubated with HRP-conjugated rabbit anti-goat antibodies (Santa Cruz Biotechnology Inc., 1/12000, 30 min, room temperature). Bands were detected by enhanced chemiluminescence and analyzed by scanning densitometry.

2.5. Statistical analysis

Combined data are presented as means ± S.E. for n independent preparations of myocytes. Results were analyzed using a one-sample t-test.

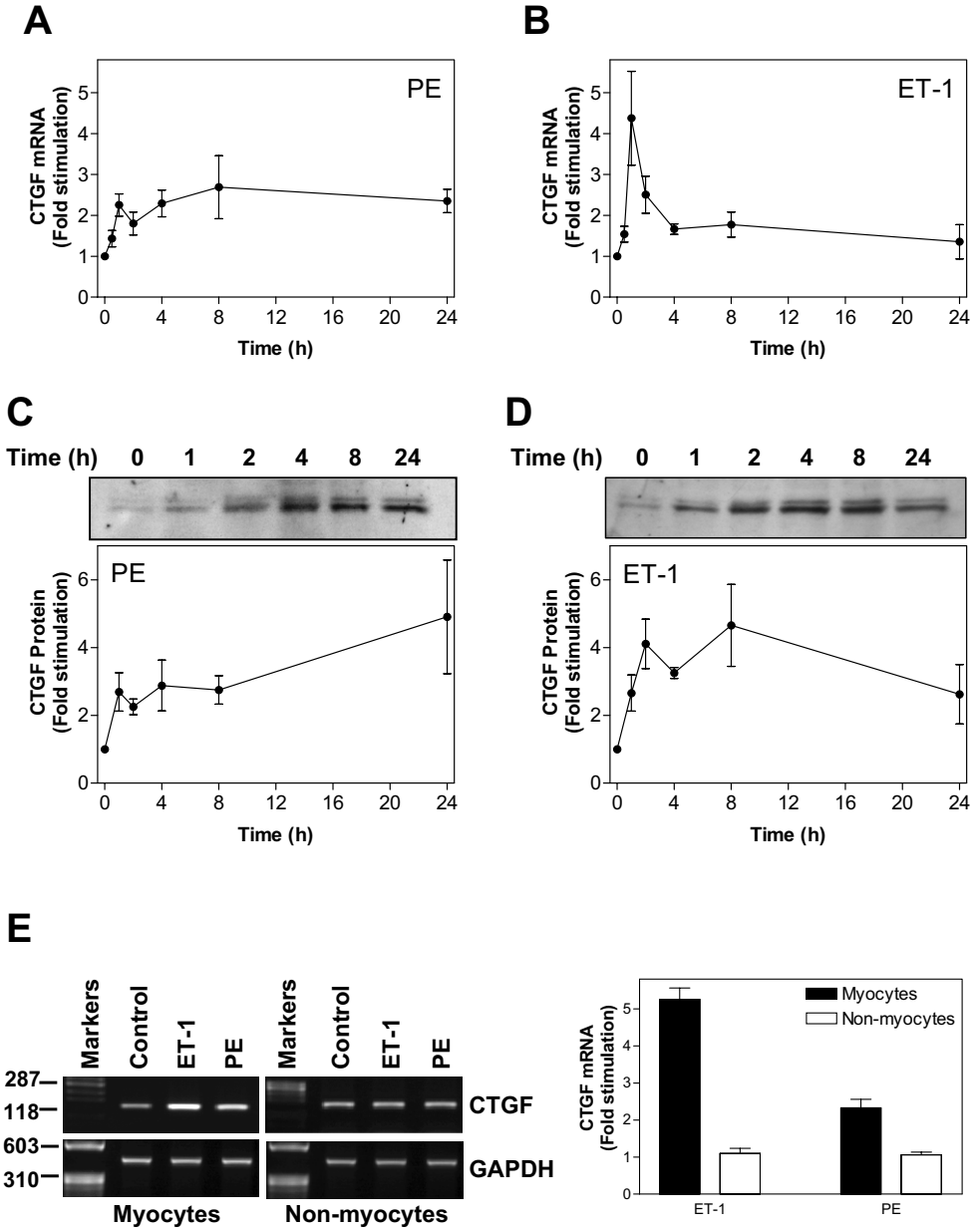

Fig. 2. Expression of CTGF mRNA and protein is increased in myocytes exposed to PE or ET-1. A - D, Myocytes were unstimulated or exposed to 100 μM PE (A and C) or 100 nM ET-1 (B and D). Expression of CTGF mRNA or protein was analyzed by quantitative PCR (A and B) or immunoblotting (C and D), respectively. Representative immunoblots are shown in the upper panels and densitometric analysis is shown in the lower panels. Results are means ± SEM for at least 3 independent preparations of cells. (E) Myocytes or non-myocytes were unstimulated or exposed to 100 μM PE or 100 nM ET-1 (1 h). CTGF mRNA expression was analyzed by ratiometric RT-PCR. Representative ethidium bromide gels are shown in the left panels and densitometric analysis is shown in the right panel. Results are means ± SEM for 3 independent preparations of cells.

3. Results and discussion

Although subtractive hybridization is a powerful approach for the identification of differentially-expressed mRNAs in the absence of prior sequence information, it is subject to a high rate of false positives [7] and further screening is required. From the shotgun ligation of subtracted PCR products, 300 clones were subjected to secondary screening and PCR-amplified. Thirty-six clones were selected as potentially upregulated in myocytes exposed to PE (100 μM, 1 h) compared with unstimulated cells. Twenty-three with a predicted insert size of >50 bp were sequenced. Twelve clones were suitable for PCR analysis. Ratiometric RT-PCR confirmed that the expression of three clones (C208, C53 and C64) was increased in myocytes exposed to PE (100 μM, 1 or 24 h) (Fig. 1). Database searches identified C53 as rat CTGF (accession no. AF120275). C64 had no identity to any characterized sequence in the database and was submitted to the EMBL Nucleotide Sequence Database as perc64 (Phenylephrine responsive c64, accession no. AJ601385). C64 was homologous to a rat genomic clone (chromosome 6; accession no. NW-047759.1). C208 had sequence identity to the 3' UTR of mouse Rac1 (accession no. XM_132485.3) but no corresponding sequence for rat Rac1 was available for comparison. This sequence is homologous to a rat genomic clone (chromosome 12; accession no. Rn12_1889) and has been submitted (accession no. AJ605749).

CTGF is widely expressed and is particularly implicated in fibrosis, promoting the expression of extracellular matrix proteins [8,9]. CTGF also induces hypertrophy in mesangial cells and chondrocytes [8,9]. As in other cells, CTGF expression is induced by TGFβ in cardiac fibroblasts and myocytes [10]. It is also increased in rodent hearts in experimental models of hypertension [11], diabetes [12] and myocardial infarction [10,13], and in human ischaemic heart samples [10]. It is therefore probable that expression of CTGF in the heart contributes to fibrosis in pathological situations. We further examined the regulation of CTGF mRNA and protein in myocytes exposed to PE or ET-1 using quantitive PCR and immunoblotting. Consistent with the previous data, PE (100 μM) stimulated an increase in CTGF mRNA [maximal within 1 h (2.25 ± 0.27-fold, n=8; $p<0.01$); sustained over 24 h] (Fig. 2A). ET-1 (100 nM) promoted a greater increase in expression of CTGF mRNA than PE (Fig. 2B) [maximal at 1 h (4.46 ± 0.89, n=5, $p<0.05$)], but this was transient and levels declined within 4 h. CTGF protein (a doublet of ~38 kDa) was increased in response to PE (4 - 24 h) (Fig. 2C) or ET-1 (2 - 8 h) (Fig. 2D). Neither agonist increased CTGF mRNA expression in cardiac non-myocytes (Fig. 2E). Since CTGF is implicated in fibrosis and hypertrophic growth of other cells, the increase in CTGF expression induced in cardiac myocytes by PE or ET-1 may not only propagate the hypertrophic response of the cardiac myocyte subsequent to the initial stimulus, but may also contribute to the increase in fibrosis which occurs in cardiac hypertrophy.

Acknowledgements

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