

Regulation of pro-inflammatory and pro-fibrotic factors by CCN2/CTGF in H9c2 cardiomyocytes

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Abstract Connective tissue growth factor (CTGF), also known as CCN2, is implicated in fibrosis through both extracellular matrix (ECM) induction and inhibition of ECM degradation. The role of CTGF in inflammation in cardiomyocytes is unknown. In some mesenchymal cell systems, CTGF mediates effects through TGF- β or tyrosine kinase cell surface receptor, TrkA, signalling. In this study, cellular mechanisms by which CTGF regulates pathways involved in fibrosis and inflammation were explored. Murine H9c2 cardiomyocytes were treated with recombinant human (rh)CTGF and ECM formation gene expression: fibronectin, collagen type -I and -III and ECM degradation genes: TIMP-1, TIMP-2 and PAI-1 were found to be induced. CTGF treatment also increased pro-inflammatory cytokines TNF- α , IL-6, MCP-1 and IL-8. CTGF upregulated TGF- β 1 mRNA and rapidly induced phosphorylation of TrkA. The CTGF-induced pro-fibrotic and pro-inflammatory effects were blocked by anti-TGF- β neutralizing antibody and Alk 5 inhibitor (SB431542). A specific blocker of TrkA activation, k252a, also abrogated CTGF-induced effects on fibrosis and gene expression of MCP-1 and IL-8, but not TNF- α or IL-6. Collectively, this data implicates CTGF in effects on pro-fibrotic genes and

pro-inflammatory genes via TGF- β pathway signalling and partly through TrkA.

Keywords CTGF · TrkA · TGF- β · Fibrosis · Inflammation · Cardiomyocytes

Introduction

The predominant cell type in the heart, the cardiomyocyte, is implicated in the pathologies of both fibrosis and inflammation that can occur in the myocardium. For example, the heart disease of diabetic cardiomyopathy (DCM) (Fang et al. 2004) involves inflammatory (Westermann et al. 2007; Wang & Cai 2006) and fibrotic changes (Samuel et al. 2008) including effects mediated by cardiomyocytes (Wang and Cai 2006; Wang et al. 2009). Pro-fibrotic factors such as collagen-I, collagen-III and fibronectin (Wang et al. 2009) and pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumour necrosis factor- α (TNF- α), are increased in DCM (Westermann et al. 2006). However the cellular mechanisms mediating these changes are still not completely understood.

Also known as CCN2, the protein CTGF is considered to be a mediator involved in fibrotic processes (Leask and Abraham 2004) and it stimulates fibroblast growth and ECM protein up-regulation in vitro and in vivo. CTGF over-production has been proposed to play a major role in pathways that lead to fibrosis in many organs (Frazier et al. 1996; Igarashi et al. 1993). Increased CTGF expression has been described in diabetes including DCM (Wang et al. 2009; Way et al. 2002) and post-myocardial infarct (Dean et al. 2005). CTGF has been shown to induce the inhibitors of matrix metalloproteinases known as TIMPS, leading to ECM accumulation in renal cells (McLennan et al. 2004).

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Cellular mechanisms exploring how CTGF regulates ECM changes in cardiomyocytes remain minimally explored and whether CTGF has any effects on inflammatory processes in cardiomyocytes has not been reported.

In some mesenchymal cell systems, CTGF appears to function by modulating bioactivity of other cytokines, especially TGF- β (Abreu et al. 2002), indicating that TGF- β protein is required for CTGF to exert its effect. In contrast, in a renal based cell system, CTGF was reported to function through the cell surface receptor, TrkA (tyrosine kinase A), leading to activation of TGF- β inducible early gene (TIEG), and TGF- β pathway signalling (Wahab et al. 2005). Thus in some cells both TrkA and TGF- β based pathways have been implicated in CTGF effects.

With this background, the aims of this study in cardiomyocytes were to test whether (a) CTGF regulates pro-fibrotic and pro-inflammatory gene expression in cardiomyocytes; (b) whether CTGF effects on fibrosis and inflammation are TGF- β dependent; and/or through the cell surface receptor TrkA in cardiomyocytes.

Materials and methods

Reagents

Recombinant human CTGF (rhCTGF) protein was produced using an adenoviral expression system, purified with heparin-Sepharose affinity chromatography using HiTrap Heparin HP columns (Amersham Biosciences, Piscataway, NJ, USA) and quantified as previously described (Tan et al. 2008). A chemical inhibitor of TrkA activity, k252a, purchased from Merck-Calbiochem, Melbourne, Australia was prepared by dissolving in DMSO, with final DMSO concentration in cell culture at 0.001%. Pan anti-TGF- β neutralizing antibody was purchased from R&D systems Inc (Minneapolis, USA) and Alk 5 inhibitor (SB 431542) was purchased from Tocris Cookson Inc, Ellisville, Missouri USA. Rat nerve growth factor (NGF) was purchased from Sigma (St. Louis, Mo, USA). Antibodies for detection of phospho-TrkA (Tyr490) and total TrkA were purchased from Cell Signaling Technology, Danvers, MA, USA. The α -tubulin antibody was purchased from Abcam Inc (Cambridge, MA, USA).

Cell treatment

The well studied murine H9c2 cells derived from embryonic rat heart myocardium were purchased from ATCC (Rockville, MD, USA). Cells were cultured in DMEM containing normal 5.5 mM glucose, 4.4 mM L-glutamine and 1.7 g/L bicarbonate supplemented with 10% FBS, 50 U/ml of penicillin and 50 μ g/ml of streptomycin. Each experiment was performed at

least three times independently with in each experiment, triplicate wells of treatments.

Cells were grown in 6-well plates until ~80% confluent and then treated with reagents in serum-free fresh DMEM media containing 0.1% BSA (Sigma, St Louis, MO, USA). Cells were harvested at 24 h. Treatments included control media, rhCTGF, k252a (TrkA chemical blocker), Pan anti-TGF- β neutralizing antibody, SB 431542 (Alk 5 inhibitor) and combination treatments of rhCTGF with k252a or TGF- β antibody or SB 431542. In the case of k252a or SB 431542 the highest relevant concentration of DMSO was used as control. DMSO alone was shown in preliminary studies not to affect mRNA levels measured compared with no DMSO addition (not shown).

Total RNA isolation and analysis by quantitative real-time PCR

Total RNA of each cell treatment was isolated from cells using the RNeasy Mini kit (Qiagen). RNA amount was quantitated spectrophotometrically (Bio-Rad Laboratories Inc, CA, USA), and the purity was determined from the A_{260}/A_{280} ratio (at or above 1.80, to 2.00). RNA was then reverse transcribed to cDNA using Random Hexamer Primer (Invitrogen, CA, USA) and SuperScriptTM III Reverse Transcriptase (Invitrogen). The resulting cDNA was analyzed by quantitative real-time PCR using Rotor Gene 6000 (Corbett Research), using SYBR green as fluorescence dye, as described previously (Tan et al. 2008). Relative quantitation of mRNA expression in the gene of interest was calculated using the comparative threshold cycle number for each sample. In each case, the gene of interest expression level was normalized to 18s ribosomal RNA and related to the relevant control, as previously described (Twigg et al. 2002; Twigg et al. 2001). Four groups of genes were measured (a) CTGF and TGF- β 1; (b) ECM genes: fibronectin, collagen type - I and - III; (c) ECM protease inhibitors: tissue inhibitors of matrix metalloproteinases, TIMP-1, TIMP-2, TIMP-3, TIMP-4 and plasminogen activator inhibitor 1, PAI-1; and (d) pro-inflammatory cytokines: TNF- α , MCP-1, IL-6 and IL-8. The primer sequences used are listed in Table 1.

TNF- α detection in conditioned media

A commercial ELISA assay (R&D Systems, Minneapolis, MN, USA) was used to measure the protein level of rat TNF- α in conditioned media after CTGF treatment (0–500 ng/mL), as per the manufacturer's instructions.

Preparation of cell lysates for protein quantitation

Cells were washed with cold Phosphate Buffer Saline (PBS), then 150 μ l of cold RIPA lysis buffer with a

Table 1 Primer sequences used in qRT-PCR to assess mRNA levels of respective genes

Genes	Forward sequences	Reverse sequences
CTGF	5'-gaggaaacattaagaaggcaaa	5'-cggcacaggtcttgatga
FN	5'-gctgctgggactccacgt	5'-tctgttccgggagggtgca
Collagen I	5'-gcgaaggcaacagtcgattc	5'-ccaagtccgggtgctgac
Collagen III	5'-cagctggcctcctcagact	5'-gctgttttgcagtggtatgtaag
PAI-1	5'-ggcatggaacagaatg	5'-ctgagactagaatggctg
TIMP1	5'-aattgcacatcactgcc	5'-gtgatcgtctgtgtagc
TIMP2	5'-atttatctacaccggcccc	5'-caagaaccatcacttctctg
TNF- α	5'-agtctccagctggagaagg	5'-gccactactcagcatctcg
MCP-1	5'-tctcttccaccactatgca	5'-ggctgagacagcagctggat
IL-6	5'-gcccttcaggaacagctatga	5'-tgtcaacaacaacatcagtccaaga
IL-8	5'-aaaccgcctgtgttoga	5'-tcagtctggtctgggttcctg
TGF- β 1	5'-aggtcaccgcgtgctaata	5'-tcaaccatgccgcacaact
18s	5'-cggctaccacatccaaggaa	5'-gctggaattaccgcggct

complete protease inhibitor cocktail at concentration recommended by the manufacturer (Roche Diagnostics, NSW, Australia) was added to each well. Plates were left on ice for 20 min, and lysates were collected after centrifugation at 10,000 g for 10 min at 4°C. Total protein concentration was measured using the DC Protein Assay (Bio-Rad Laboratories, Richmond, CA) and was used for sample loading for SDS-PAGE.

Western immunoblot analysis

Cell lysate samples were loaded at 30 μ g of total protein per lane and separated on a 4% to 12% gradient SDS-PAGE gel (Invitrogen, Melbourne, Australia). Proteins were electrotransferred onto nitrocellulose, and membranes were blocked with 5% nonfat dry milk/TBS with 0.1% (Vol/Vol) Tween 20 for 1 h, followed by incubation in either anti-phospho-TrkA (1:150), or anti-total TrkA (1:300) as previously described (Wang et al. 2009). Briefly, after incubation of membranes with the appropriate horseradish peroxidase (HRP)-labeled secondary antibody (1:10,000, Vector Laboratories) for 1 h at 21°C. Immunoreactive protein bands were then detected using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA). In each case, after stripping of nitrocellulose membranes with stripping buffer, anti- α -tubulin (1:5000) at 4°C overnight followed by the relevant secondary antibody then enhanced chemiluminescence as described above, was used to detect α -tubulin as the loading control.

Statistical analysis

Results are expressed as the mean value \pm SD. All numerical data were derived from three or more independent experiments. Differences between groups were assessed using one way ANOVA, or Student's two-tailed

paired *t* test where only two groups were to be compared, as indicated in the text. $P < 0.05$ was considered statistically significant.

Results

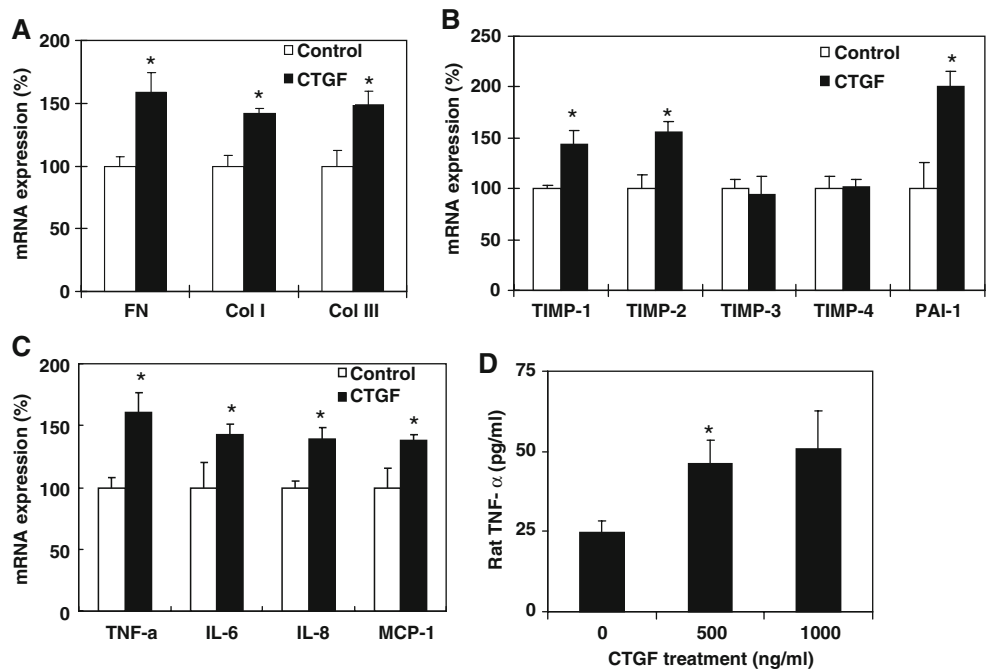
CTGF increased ECM accumulation and degradation genes as well as inflammatory genes markers in cardiomyocytes

To assess whether CTGF can induce fibrosis and inflammation gene expression in cardiomyocytes, H9c2 cells were treated with 500 ng/ml of CTGF for 24 h in serum free conditions. Gene expression of ECM formation and degradation markers and pro-inflammatory cytokines were measured by RT-qPCR. CTGF treatment increased the ECM formation genes of fibronectin (FN), Collagen type-I (Col-I) and Collagen type -III (Col-III) to 160%, 143% and 149% compared with no CTGF addition 100% control for each gene respectively (Fig. 1a). Then, mRNA levels of matrix protease inhibitors TIMP-1 to TIMP-4 and PAI-1 were also measured. TIMP-1 and TIMP-2 and PAI-1 were up-regulated by rhCTGF while TIMP-3 and TIMP-4 were not changed (Fig. 1b). Furthermore, elevated mRNA levels of TNF- α , IL-6, IL-8 and MCP-1 occurred by the same treatment (Fig. 1c). The protein concentration of TNF- α in condition media measured using rat TNF- α ELISA kit was induced ~2-fold induction by 24 h of CTGF treatment (Fig. 1d).

Effects of TGF- β and TrkA signalling on CTGF gene expression

CTGF is able to induce its own gene expression in mesenchymal derived cells as previously described (McLennan et al. 2004). To study if CTGF mRNA is

Fig. 1 CTGF increases fibrotic and inflammatory gene expression in H9c2 cardiomyocytes. Cells were treated with CTGF 500 ng/ml in serum free media with 0.1% BSA at 80% confluence, and mRNA expression was measured by real-time RT-PCR after 24 h. A, mRNA expression of ECM formation genes of FN, Col-I and Col-III. B, mRNA expression of ECM degradation genes TIMP-1 to TIMP-4 and PAI-1. C, Gene expression of inflammatory markers of TNF- α , IL-6, IL-8 and MCP-1. D, TNF- α concentration was quantitated by ELISA in conditional media collected 24 h after CTGF treatment(500 ng/ml and 1000 ng/ml). Data is mean \pm SD. * P <0.05 vs respective control normalised to 100%



regulated via TGF- β pathways or through TrkA signalling, to potentially augment fibrotic and inflammatory markers in cardiomyocytes, blockers of TGF- β (anti-TGF- β antibody), TGF- β receptor-I (Alk5) and TrkA activity (K252a) were studied, each pre-treated on H9c2 cells for 1 h before rhCTGF (0–500 ng/mL) was added. Induced CTGF mRNA by rhCTGF treatment was fully inhibited to baseline by pan-specific anti-TGF- β antibody (Fig. 2a) and by the Alk5 blocker SB431542 (Fig. 2b). An inhibitory effect was also seen by blocking TrkA activity, using k252a at 100 nM and 200 nM (Fig. 2c). In each case, the basal steady state CTGF mRNA level in the absence of added rhCTGF was also

significantly inhibited by each reagent, consistently implicating TGF- β and TrkA signalling in CTGF mRNA regulation.

TGF- β dependent effects of rhCTGF on fibrotic and inflammatory gene expression

To further test if CTGF regulation of fibrotic and inflammatory factors is through TGF- β pathways, anti-TGF- β Ab and the Alk 5 inhibitor (SB431542) each alone or combined with rhCTGF were applied to the H9c2 cells.

Table 2 lists the gene expression of ECM formation, degradation and pro-inflammatory genes examined under

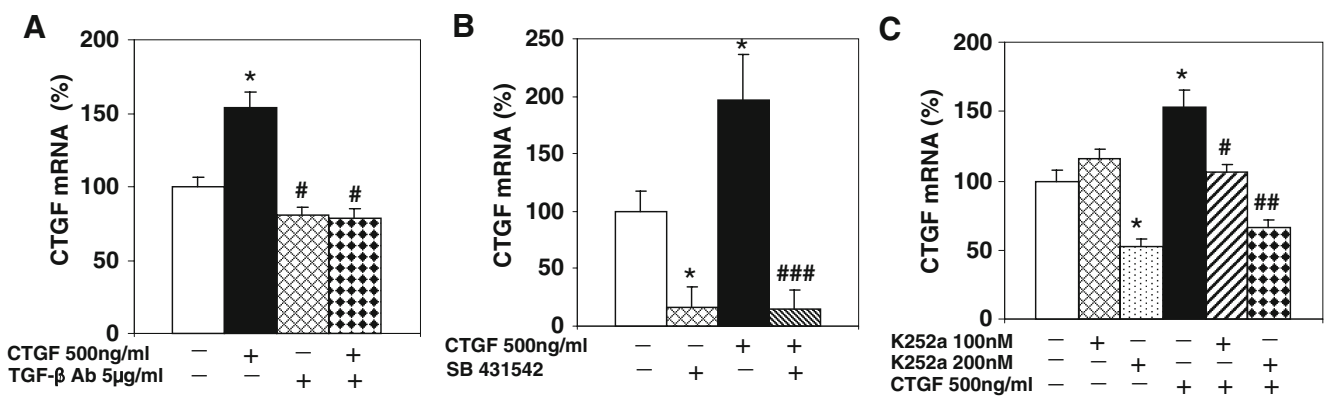


Fig. 2 Effects of TGF- β and TrkA signalling on CTGF gene expression in H9c2 cells. Cells were pre-treated with anti-TGF- β neutralizing antibody at 5 μ g/ml (TGF- β Ab), Alk-5 inhibitor (SB431542) at 5 μ M, or TrkA blocker (k252a) at 100 nM and 200 nM alone in some wells for 1 h followed by addition of unconditioned media alone or CTGF 500 ng/ml in serum free media with 0.1% BSA for 24 h. CTGF mRNA levels were measured by qRT-

PCR and are shown compared with control at 100%. A, CTGF mRNA measurement by treatment with CTGF, anti-TGF- β Ab or combinations of CTGF with anti-TGF- β Ab. B, Alk 5 inhibitor. C, k252a effects on CTGF gene expression. Data shown as mean \pm SD. * P <0.05 vs respective control. # P <0.05, ### P <0.01, #### P <0.001 vs CTGF treatment

Table 2 Anti-TGF- β neutralising antibody effects on CTGF regulated fibrotic and inflammatory gene expression in H9c2 cardiomyocytes

	mRNA species detected	Cell Treatments		
		CTGF (500ng/ml)	TGF- β Ab (5 μ g/ml)	CTGF +TGF- β Ab
ECM formation genes	FN	159 \pm 15.2*	105 \pm 18.9	97 \pm 14.7#
	Col I	152 \pm 4.28*	85 \pm 14.9	94 \pm 7.3#
	Col III	149 \pm 10.2	94 \pm 13.2	94 \pm 8.8#
ECM remodeling genes	PAI-1	189 \pm 16.7*	87 \pm 11.2	102 \pm 6.8#
	TIMP-1	145 \pm 8.3*	68 \pm 7.4**	60 \pm 9.7#
	TIMP-2	149 \pm 11.4*	93 \pm 15.6	108 \pm 7.6#
Pro-inflammatory genes	TNF- α	151 \pm 20.9*	107 \pm 28.6	125 \pm 23.5
	IL-6	147 \pm 8.5	71 \pm 11.5*	110 \pm 16.5#
	IL-8	156 \pm 9.2*	90 \pm 16.1	71 \pm 14.5#
	MCP-1	169 \pm 10.2*	80 \pm 10.8	73 \pm 19.5#

Data is expressed as % of control (at 100%). * $P < 0.05$, ** $P < 0.01$ vs control; # $P < 0.05$ vs CTGF treatment

treatments of rhCTGF (500 ng/ml), anti-TGF- β Ab (5 μ g/ml) and a mixture of CTGF and anti-TGF- β Ab. FN, Col-I and Col-III mRNA species induced by CTGF were fully blocked by anti-TGF- β Ab compared with the no treatment control respectively. Similar results were found for PAI-1, TIMP-1, TIMP-2 mRNA levels. For inflammatory genes, CTGF induction compared with control was inhibited using the anti-TGF- β Ab as a blocker of endogenous TGF- β .

Table 3 Effects of TGF- β type I receptor (Alk-5) blocker on CTGF regulated fibrotic and inflammatory gene expression in H9c2 cardiomyocytes

	mRNA species detected	Cell Treatments		
		CTGF (500ng/ml)	Alk 5 inhibitor (SB431542) (5 μ M)	CTGF+Alk 5 inhibitor SB431542 (5 μ M)
ECM formation genes	FN	177 \pm 29.6*	110 \pm 62.5	51 \pm 19.7#
	Col I	151 \pm 22.3*	103 \pm 35.8	128 \pm 26.9#
	Col III	147 \pm 12.9	131 \pm 45.8	87 \pm 23.6#
ECM remodelling genes	PAI-1	203 \pm 34.1*	4 \pm 1.2***	8 \pm 1.8# ##
	TIMP-1	203 \pm 21.5*	45.8 \pm 25.3*	49 \pm 41.0#
	TIMP-2	197 \pm 29.0*	121 \pm 18.8	153 \pm 24.3#
Pro-inflammatory genes	TNF- α	198 \pm 22.9*	74.9 \pm 8.3*	78 \pm 43
	IL-6	167 \pm 30.5	41.2 \pm 8.0**	87 \pm 31.6
	IL-8	155 \pm 19.8*	175 \pm 46.8	137 \pm 58.9
	MCP-1	175 \pm 33.8	256 \pm 32.1*	99 \pm 13.9#

Data is expressed as % of control (at 100%). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs control; # $P < 0.05$, ### $P < 0.001$ vs CTGF treatment

The effect of Alk 5 inhibitor (SB431542) was then studied. The induction of most gene expression in the three groups by rhCTGF was prevented by the Alk 5 inhibitor (Table 3). PAI-1 mRNA showed a very strong inhibitory effect with both Alk 5 inhibitor alone in the basal state and in combination treatment with rhCTGF, suggesting that PAI-1 mRNA is strongly dependent on the TGF- β signalling pathway (Table 3).

In combination the data in Tables 2 and 3 indicate that CTGF regulation of fibrotic and inflammatory genes is TGF- β and TGF- β pathway dependent.

CTGF signalling through TrkA to induce TGF- β 1

To confirm whether CTGF signals through TrkA, the H9c2 cells were treated with rhCTGF for up to 120 min and cell lysate was isolated and probed by Western immunoblot. Phosphorylation of TrkA was induced by added CTGF from 15 min (Fig. 3a). In contrast total TrkA was unchanged. These findings are consistent with those that we recently reported, in a less detailed time course (McLennan et al. 2004). As TGF- β 1 gene expression is induced by TGF- β pathway activation, subsequent studies focussed on TGF- β 1 mRNA as a read out at 24 h. These experiments showed that rhCTGF induced TGF- β 1 mRNA levels, which was blocked by the TrkA activity inhibitor, k252a and also by anti-TGF- β antibody (Fig. 3b). Collec-

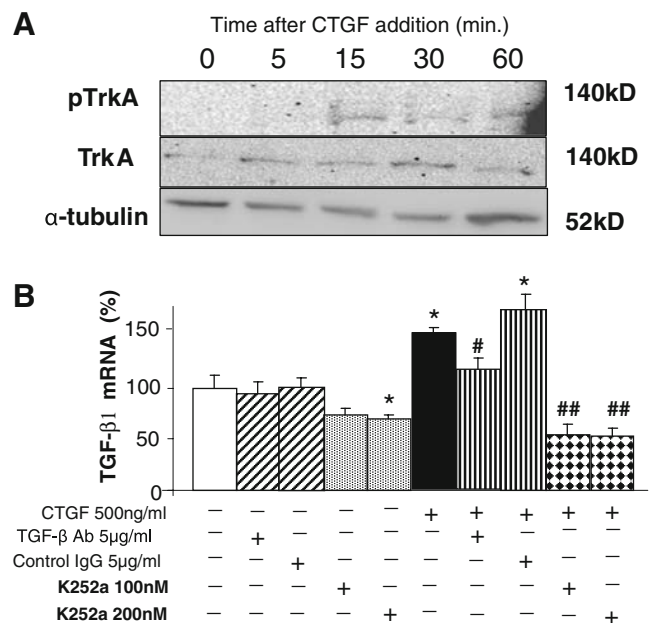


Fig. 3 CTGF phosphorylation of TrkA and upregulation of TGF- β 1 mRNA. A, Addition of rhCTGF to H9c2 cells induced rapid phosphorylation of TrkA but did not increase total TrkA. B, rhCTGF application to cells induced TGF- β 1 mRNA, which was inhibited by anti-TGF- β 1 antibody or k252a. Data is mean \pm SD. *, $P < 0.05$, vs control. # $P < 0.05$, ## $P < 0.01$ vs CTGF treatment

Table 4 Effects of blocking TrkA signaling on CTGF regulated fibrotic gene expression in H9c2 cardiomyocytes

mRNA species detected		Cell Treatments				
		CTGF	TrkA blocker (k252a)		CTGF+TrkA blocker (k252a)	
		500ng/ml	100 nM	200 nM	100 nM	200 nM
ECM formation genes	FN	161±15.5*	107±11.5	80±7.1*	144±39.7	87±9.6#
	Col I	141±3.5*	80±6.3*	71±12.9*	71±15.8#	88±8.8#
	Col III	139±10.7	71±5.5**	54±3.0**	65±2.1#	70±6.0##
ECM remodeling genes	PAI-1	177±16.1*	30±4.1***	12±2.7***	28±16.7###	16±1.8####
	TIMP-1	143±8.3*	80±3.2**	74±24.3*	76±6.8#	70±1.4##
	TIMP-2	148±11.4*	122±13.0	86±10.9	106±10.3	116±2.4#

Data is expressed as % of control (at 100%). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs control; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs CTGF treatment

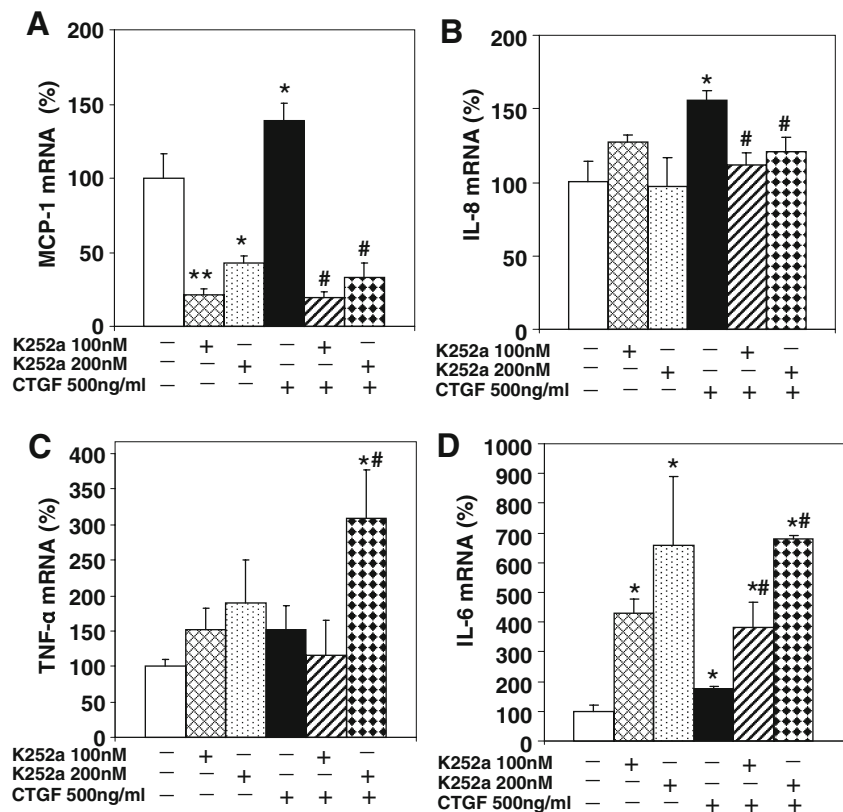
tively, this data suggests that CTGF rapidly signals through TrkA activation and then through TGF- β protein dependent processes in H9c2 cells.

Effect of TrkA on CTGF regulated fibrosis and inflammation

The ability of k252a to regulate other effects induced by rhCTGF was examined. As shown in Table 4, FN, Col-I and Col-III induction by CTGF treatment was found to be blocked by k252a. Similar profiles of results were obtained

for TIMP-1 and TIMP-2, while PAI-1 showed very strong inhibitory effects with k252a treatment with or without rhCTGF. For the pro-inflammatory cytokine gene expression studied, as shown in Fig. 4, two differential profiles were observed: MCP-1 and IL-8 showed the same trends as for the fibrotic markers (Fig. 4a–b); in contrast, for TNF- α and IL-6 the k252a reagent added alone showed an increased trend compared with control, and ~ additive effects with CTGF and k252a combined compared with CTGF addition alone (Fig. 4c–d). This data for TNF- α and IL-6 suggests that TrkA activity has a tonic inhibitory effect on steady state

Fig. 4 Differential effects of TrkA on CTGF regulation of inflammatory gene expression. H9c2 cells were pretreated for 1 hr with K252a at 100 nM and 200 nM and then conditioned media with no treatment or CTGF (500 ng/ml) was added. The RNA was isolated at 24 h and mRNA levels of respective species was determine by RT-qPCR A, MCP-1 B, IL-8 C, TNF- α and D IL-6 mRNA. Data is mean±SD. *, $P < 0.05$, vs control respectively. # $P < 0.05$, ## $P < 0.01$, vs. CTGF treatment



TNF- α and IL-6 and that upregulatory effects of rhCTGF on TNF- α and IL-6 (Fig. 1c) are not TrkA mediated and they persist when TrkA activity is blocked.

Nerve growth factor up-regulates MCP-1 and IL-8 but not TNF- α or IL-6

As TrkA is a receptor for nerve growth factor (NGF) (Wahab et al. 2005), to further examine gene expression regulation through TrkA, 10 ng/ml and 100 ng/ml of NGF were applied to cells and change in CTGF and inflammatory cytokines gene expression was measured. NGF at 100 ng/ml up-regulated CTGF mRNA (Fig. 5a); while MCP-1 and IL-8 (Fig. 5b and c) were increased at 10 ng/ml by NGF treatment. No change was observed for TNF- α and IL-6 (Fig. 4d and e). This data is consistent with the TrkA activity blocker k252a treatment showing that observed CTGF up-regulatory effects on TNF- α and IL-6 (Fig. 1c) are TrkA independent (Fig. 1d).

Discussion

This study demonstrates that CTGF induces pro-fibrosis and pro-inflammation gene expression in cultured cardiac

myocytes. CTGF gene expression is TrkA and TGF- β dependent. CTGF effects on fibrosis and inflammatory markers was found to consistently be TGF- β dependent. However for the TrkA pathway in our study, while CTGF effects on fibrotic gene expression was TrkA dependent a differential pattern was found for the pro-inflammatory genes. MCP-1 and IL-8 mRNA were up-regulated by rhCTGF through TrkA signalling, but TNF- α and IL-6 mRNA species induced by rhCTGF were not through TrkA signalling. The ability of the k252a blocker alone to potently induce TNF- α and IL-6 mRNA implicates a basal inhibitory effect of TrkA signalling on TNF- α and IL-6 mRNA. As TrkA signalling appears to inhibit TNF- α and IL-6 mRNA it is then not surprising that the observed rhCTGF induction of these two genes was not found to be TrkA signalling dependent.

CTGF as a down stream mediator of TGF- β has been extensively studied, including in diabetes in renal pathology. CTGF up-regulation was reported including in diabetic animal heart (Way et al. 2002) and by stimuli such as aldosterone (Lee et al. 2004), and endothelin-1 (Kemp et al. 2004). in cardiomyocytes. Some studies also showed CTGF is involved in fibrosis induction in cardiomyocytes (Dean et al. 2005; Yun et al. 2007; Matsui and Sadoshima 2004), but how CTGF regulates fibrotic processes in cardiomyocytes

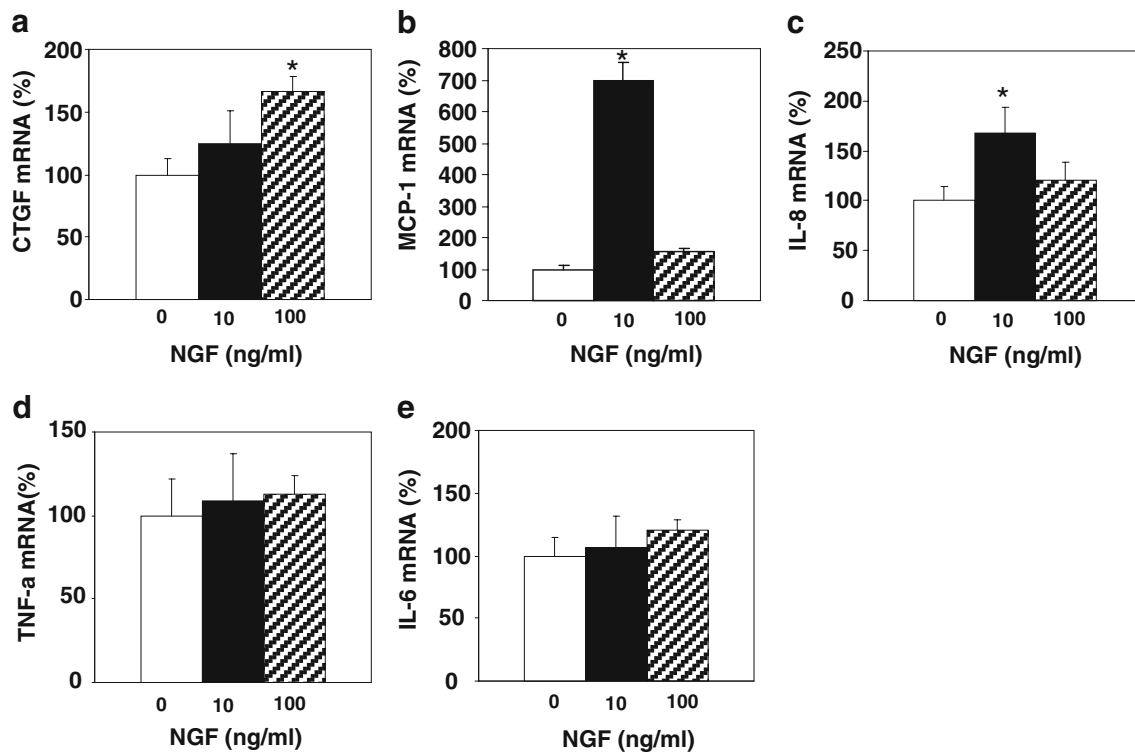


Fig. 5 Gene expression of CTGF and inflammatory cytokines by nerve growth factor treatment. H9c2 Cells were treated with nerve growth factor (NGF) 10 ng/ml and 100 ng/ml for 24 h and mRNA levels of CTGF (a), MCP-1 (b), IL-8 (c), TNF- α (d) and IL-6 (e) were

measured by RT-qPCR and compared to control at 100%. Data is mean \pm SD. * $P < 0.05$ shows the difference of each treatment vs control for the respective mRNA species

is unclear. In this study, we examined two potential pathways describing CTGF regulation of pro-fibrotic and pro-inflammatory gene expression. The current data is the first work in cardiomyocytes to implicate both TGF- β and TrkA pathways in these CTGF regulated processes and the first to show that CTGF induces a profile of pro-inflammatory genes in cardiomyocytes. CTGF has been shown to induce MCP-1 and IL-6 in some cells (Sánchez-López et al. 2009) but not in others (Qi et al. 2006), reflecting the cell type dependence of the findings.

Like TGF- β 1 mRNA and TGF- β protein, CTGF mRNA is known to be induced in a 'feed forward' process by soluble CTGF protein treatment (McLennan et al. 2004). The current work shows that this auto-induction effect in H9c2 cells is TGF- β and TrkA dependent. The induction of CTGF mRNA and then endogenous CTGF protein would be predicted to amplify the cellular responses seen for CTGF protein addition although this was not explored in the current work. It is notable in our studies that CTGF protein rapidly induced phosphorylation of TrkA, and that CTGF mRNA increase was TrkA dependent. In contrast, TNF- α and IL-6 mRNA increases by rhCTGF protein occurred in the absence of TrkA signalling and in the absence of CTGF mRNA induction by rhCTGF. Thus rhCTGF protein inducing effects on TNF- α and IL-6 mRNA is both TrkA and CTGFmRNA independent in H9c2 cardiomyocytes. The NGF treatment data also suggest that TrkA activation does not upregulate TNF- α and IL-6 mRNA, although interpretation of the NGF effect is complicated by the potential presence of a low affinity binding NGF receptor, p75NTR, in heart cells (Zhou et al. 2004), in addition to TrkA (Wang et al. 2009), which may also be contributing to the bimodal NGF effect seen in Fig. 5 on MCP-1 and IL-8 induction.

The current data as shown in Fig. 3 suggests that rhCTGF may signal sequentially through TrkA and then TGF- β pathways. To confirm this finding detailed time course studies of TGF- β pathway regulation would be required, which is beyond the scope of the current work. Thus while we found that rhCTGF rapidly induces TrkA phosphorylation in H9c2 cells, others have shown in other mesenchymal cell types that CTGF rapidly activates TGF- β pathway signalling (Abreu et al. 2002).

That TrkA has differential effects in regulating CTGF induction of pro-inflammatory cytokines could have relevance to cell and tissue pathology. Thus TrkA may be necessary for effects of CTGF such as macrophage chemoattraction (MCP-1) and cellular hypertrophy (IL-6), but not for neutrophil chemotaxis (IL-8 dependent) or caspase 8 dependent apoptosis (TNF- α mediated).

In summary, the main novel finding in this work is that in H9c2 cardiomyocytes, CTGF mediates pro-fibrosis gene expression via both TrkA and TGF- β signalling, while

induction of pro-inflammatory cytokines by CTGF is through TGF- β in each case. For TrkA, CTGF effects on proinflammatory cytokines are differentially regulated. The significance of these findings including the differential regulation of CTGF effects can now be explored in cardiac pathologies and in more depth at the level of differentially regulated genes.

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