Published in final edited form as:

J Cardiovasc Pharmacol. 2016 February ; 67(2): 110–120. doi:10.1097/FJC.0000000000000322.

mTOR Complexes Repress Hypertrophic Agonist-Stimulated Expression of Connective Tissue Growth Factor in Adult Cardiac Muscle Cells

Kamala Sundararaj[‡], Dorea L. Pleasant[‡], Phillip C. Moschella[‡], Kavin Panneerselvam[‡], Sundaravadivel Balasubramanian[‡], Dhandapani Kuppuswamy^{‡,*}

[‡]Cardiology Division of the Department of Medicine, Gazes Cardiac Research Institute, Medical University of South Carolina, Charleston, SC 29425-2221

Abstract

Connective tissue growth factor (CTGF) is a fibrogenic cytokine that promotes fibrosis in various organs. In the heart, both cardiomyocytes (CM) and cardiac fibroblasts (CFb) have been reported as a source of CTGF expression, aiding cardiac fibrosis. Since the mammalian target of rapamycin (mTOR) forms two distinct complexes, mTORC1 and mTORC2, and plays a central role in integrating biochemical signals for protein synthesis and cellular homeostasis, we explored its role in CTGF expression in adult feline CM. CM were stimulated with 10 μ M phenylephrine (PE), 200 nM angiotensin (Ang) or 100 nM insulin for 24 h. PE and Ang, but not insulin, caused an increase in CTGF mRNA expression with the highest expression observed with PE. Inhibition of mTOR with torin1 but not rapamycin significantly enhanced PE-stimulated CTGF expression. Furthermore, silencing of Raptor and Rictor using shRNA adenoviral vectors to suppress mTORC1 and mTORC2, respectively, or blocking phosphatidylinositol 3-kinase (PI3K) signaling with LY294002 (LY) or Akt signaling by dominant negative Akt (DN-AKT) expression caused a substantial increase in PE-stimulated CTGF expression as measured by both mRNA and secreted protein levels. However, studies with dominant negative PKC δ (DN-PKC δ) demonstrate that PKC δ is required for both agonist-induced CTGF expression and mTORC2/Akt-mediated CTGF suppression. Finally, PE-stimulated CTGF expression was accompanied with a corresponding increase in Smad3 phosphorylation and pretreatment of cells with SIS3, a Smad3 specific inhibitor, partially blocked the PE-stimulated CTGF expression. Therefore, a PI3K/mTOR/Akt axis plays a suppressive role on agonist-stimulated CTGF expression where the loss of this mechanism could be a contributing factor for the onset of cardiac fibrosis in the hypertrophying myocardium.

Keywords

Cardiomyocytes; CTGF; phenylephrine; mTOR; PKC δ ; Smad3

*Corresponding author at: Gazes Cardiac Research Institute, Medical University of South Carolina, 114 Doughty Street, Charleston, South Carolina 29425-2221 Telephone: 843-876 5067; Fax: 843-876 5068; kuppud@musc.edu.

Disclosures
None.

INTRODUCTION

A major hallmark of pathological cardiac hypertrophy is myocardial fibrosis which contributes to interstitial stiffness, impaired electrical conductance and regional ischemia, leading to the progression of diastolic dysfunction and compromised systolic mechanics¹⁻⁴. Cardiac fibrosis develops as a response to multiple stimuli, such as mechanical stress, neurohumoral activation, growth factors and cytokines⁵⁻⁷. In this context, connective tissue growth factor (CTGF, also known as CCN2), a matricellular protein and potent fibrogenic cytokine belonging to the CCN protein family, has been shown to regulate a wide range of biological functions including ECM deposition, wound repair, angiogenesis, migration, differentiation, survival and proliferation^{8,9}. Research has shown that elevated CTGF is highly correlated with fibrotic diseases¹⁰⁻¹², and thus has emerged as a new therapeutic target¹³. Cardiac biopsies of patients with ventricular hypertrophy show at least a five fold increase in CTGF expression, as compared to nonhypertrophic control ventricles¹³. While CTGF overexpression in fibroblasts has been shown to be primarily responsible for fibrosis in various organs, both cardiomyocytes (CM) and cardiac fibroblasts (CFb) serve as major sources of CTGF in the heart¹⁴⁻¹⁸. Several studies have shown that an increase in CTGF expression in CM by hypertrophic stimuli could act on fibroblasts in a paracrine manner leading to cardiac fibrosis¹⁴⁻¹⁶. Furthermore, a recent study shows that CTGF transgenic mice did not show elevated fibrosis at baseline but substantially developed cardiac fibrosis upon pressure overload (PO) accompanied by deteriorating cardiac function¹⁹. Finally, a monoclonal antibody against CTGF was shown to protect from adverse left ventricular (LV) remodelling and deteriorating function in pressure overloaded myocardium via transverse aortic constriction (TAC)²⁰. Therefore, understanding the molecular mechanism concerning the regulation of CTGF expression specifically in CM could lead to identifying potential targets for treating cardiac fibrosis.

The mammalian target of rapamycin (mTOR), a serine/threonine kinase, has been described as a cellular sensor of nutrition and energy status and a mediator of hormone-induced cellular processes, including cell growth/size, protein synthesis/degradation, cell survival/programmed cell death, cytoskeletal changes, etc (for reviews^{21,22}). Since mTOR is reported to regulate hypertrophic agonist-induced signaling²³⁻²⁶, we hypothesized that mTOR might play a critical role for CTGF expression in CM during hypertrophic agonist stimulation.

mTOR forms two independent complexes by associating with distinct proteins Raptor and Rictor in mTOR complex-1 (mTORC1) and complex-2 (mTORC2), respectively^{21,22,27}. mTORC1 controls cell growth and size via regulating protein translation through phosphorylation of p70 S6 kinase (S6K1), 4E-binding protein (4EBP) and protein phosphatase-2A (PP2A)²⁸. Additionally, recent studies have shown a new role of mTORC1 in gene expression²⁹. On the other hand, mTORC2 regulates both cell survival via phosphorylation of Akt^{22,30} and the actin cytoskeleton through the activation of the alpha isoform of protein kinase C (PKC α)^{31,32}. Acute treatment with a pharmacological antagonist rapamycin specifically blocks the activity of mTORC1 whereas torin1 inhibits both mTORC1 and mTORC2. Our recent study shows that blocking mTORC1 with acute rapamycin treatment results in an increased mTORC2 activity in both PO myocardium *in*

vivo and in agonist-stimulated adult CM *in vitro*³³ and that distinct PKC isoforms are involved in the signaling mechanisms by both these complexes^{26,34}. Several studies implicate a link between the delta isoform of protein kinase C (PKC δ) and mTOR^{35,36} and our earlier work has shown that it plays an upstream role in mTOR phosphorylation in hypertrophic agonist stimulated adult CM³⁴. Since mTOR complexes have been shown to regulate both gene expression and protein translation, we explored the role of both of these complexes on the expression of CTGF during hypertrophic stimulation of isolated adult CM.

Earlier studies show both excessive ECM deposition and increased cardiomyocyte loss in chronically PO myocardium¹⁻³. Importantly, Akt deficient mice experiencing cardiac PO via transverse aortic constriction (TAC) develop increased pathological hypertrophy and fibrosis compared to WT TAC controls^{37,38}. In this study, we sought to explore whether the loss of mTOR signaling is also linked to increased cardiac fibrosis. We demonstrate that the loss of the PI3K/mTOR/Akt axis in CM leads to increased expression of hypertrophic agonist-stimulated CTGF, a major profibrotic factor involved in cardiac fibrosis.

MATERIALS AND METHODS

Chemicals

The following chemicals were purchased: phenylephrine (PE) and alfuzosin (Alfuz) were from Sigma (St. Louis, MO), wortmannin and LY294002 were from Calbiochem (San Diego, CA), rapamycin was from LC laboratory (Woburn, MA) and SIS3 (Smad3 inhibitor) was from Cayman chemical (Ann Harbor, MI). torin1 was obtained from Dr. N. S. Grey (currently available as torin1 at TOCRIS Bioscience, Bristol, UK). All other chemicals were purchased from Sigma, St. Louis, MO.

Antibodies

Antibodies used for Western blot analyses were purchased from the following companies: Akt, pS473-Akt, Smad3, pSmad3, YAP1 and p(S127)YAP1 (Cell Signaling, Beverly, MA); CTGF (Abcam, Cambridge, MA); horseradish peroxidase-labeled secondary antibodies (Promega, Madison, WI). Primary antibodies were used at a 1:1000 dilution for immunoblotting.

Adenoviruses

Wild-type PKC δ (WT-PKC δ) and dominant negative PKC δ (DN-PKC δ) adenoviruses were originally generated in the laboratory of Dr. Jeff Molkentin³⁹. Adenovirus harboring nonphosphorylatable dominant negative Akt (DN-Akt) was obtained from Dr. Mark Sussman⁴⁰. Adenoviruses for the expression of Rictor-shRNA (shRict) and Raptor-shRNA (shRapt) were purchased from Vector Biolabs (Philadelphia, PA). β -galactosidase (β -gal) adenovirus was used as a control adenovirus, at matching multiplicity of infection (MOI). A MOI of 200 was used for all adenoviral infections.

Adult feline cardiomyocyte culture model

Adult ventricular feline CM were isolated using enzymatic digestion as described previously⁴¹. All operative procedures were carried out under full surgical anesthesia, consisting of

ketamine HC1 (20 mg/kg im), acepromazine maleate (0.33 mg/kg im) and meperidine (2.2 mg/kg IM); after intubation and respiratory support, the cats were given 3% isoflurane supplemented with 100% O₂. The heart was harvested via a left thoracotomy while the animal was under full surgical anesthesia. Ventricular CM were isolated and maintained initially for 1 h at 37°C in 2.5 mM calcium and mitogen-free M199 medium at pH 7.4 in order to remove the fibroblasts. Isolated CM were then suspended in 1.8 mM calcium containing mitogen-free M-199 medium (M199, GIBCO-BRL, Inc., Grand Island, NY) at pH 7.4. For experimental conditions, cells were plated at a density of 1.0×10^5 cells/35 mm dish and incubated at 37°C in humidified air with 5% CO₂. After 4 h of attachment, the medium was changed with M199 medium containing 200 units/ml penicillin and 200 µg/ml streptomycin (GIBCO-BRL). All animal procedures were conducted in accordance with the accepted standards of humane care, as outlined in the ethical guidelines (National Research Council, National Academy Press, Washington, DC, 1996) and were approved by the MUSC Institutional Animal Care and Use Committee (Approval ID: ACORP443).

For treatment with inhibitors Alfuz, LY294002, rapamycin, torin1 and SIS3, freshly isolated adult feline CM were cultured overnight and stimulated with 10 µM PE for 24 h in the presence or absence of various pharmacologic inhibitors. Vehicle-treated cells served as controls. For treatment, cells were pre-incubated for 30 min with inhibitor and then stimulated with PE for 24 h. For adenoviral-mediated gene delivery, freshly isolated CM were plated on laminin-coated trays and cultured for 4 h prior to infection. Cells were then incubated overnight in M-199 media containing the adenovirus at 200 MOI as described in the figure legends. Cells infected with an equal MOI of β-gal adenovirus served as control. The medium was replaced after 24 h and allowed to incubate for an additional 24 h prior to agonist stimulation. For SIS3 treatment in adenovirus infected cells, cells were treated with 5 µM SIS3 or vehicle (DMSO, final concentration <0.1%) for 30 min prior to adenoviral infection and the inhibitor was maintained during the infection period.

Real-time PCR

For quantitative assessment of relative mRNA level, total RNA was isolated from CM using RNeasy mini kit (Qiagen, Santa Clarita, CA). cDNA was synthesized using 0.5 µg total RNA using iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) containing iScript reaction mixture and iScript reverse transcriptase. The entire reaction was cycled for 5 minutes at 25°C, 30 minutes at 42°C and 5 minutes at 85°C. Real time PCR analysis was performed using SYBR® Green Master Mix Kit (Bio-Rad Laboratories, Hercules, CA) and analyzed using iCycler iQ real-time PCR detection system (Bio-Rad Laboratories, Hercules CA). Forward and reverse primer sequences for CTGF and GAPDH were obtained from PrimerBank and primers were purchased from Integrated DNA Technologies Inc. (Coralville, IA). The thermal cycling parameters were as follows: 32 cycles of denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec, extension at 72°C for 1 min with a first denaturation at 95°C for 10 min, and final extension at 72°C for 7 min. GAPDH was used as an internal control. The starting quantities (SQ) of target gene expression were normalized to the corresponding GAPDH expression in the same sample.

Western blotting

CM were lysed in 2% Triton X-100 lysis buffer containing protease and phosphatase inhibitors (Sigma protease inhibitor cocktail and phosphatase inhibitor cocktails I and II) and centrifuged at 14,000 ×g for 15 min. The protein concentration of the supernatant was determined by the Bradford method (Bio-Rad, Hercules, CA). Total protein (10 µg) was mixed with an equal volume of 2× SDS-sample buffer, boiled for 5 min and electrophoresed in a 4-20% Bis-Tris polyacrylamide gel (Bio-Rad, Hercules, CA).

To determine the levels of secreted CTGF, cell conditioned media was ultra-filtered using 10 kDa cut-off ultrafiltration devices (Vivaspin4, Sartorius, Germany) according to the manufacturer's protocol. After concentrating approximately 20 times the original volume, equal amount of 2X SDS sample buffer was added and boiled for 5 min. Samples were then loaded onto a 4-20% Bis-Tris polyacrylamide Gel (Bio-Rad, Hercules, CA) along with a molecular weight marker (Fisher Scientific, Pittsburgh, PA). Gels were processed for Coomassie blue staining or Western blot analysis.

Proteins from the gels were transferred to Immobilon-P membrane (Millipore, Bedford, MA). Membranes were blocked in 2% BSA in TBST buffer (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl and 0.1% Tween-20) for 1 h and then incubated with primary antibodies (1:1,000) overnight at 4°C followed by incubation with peroxidase-conjugated secondary antibodies (1:10,000; Promega) in TBST buffer for 1 h at room temperature. After 3-5 washes in TBST for 5 min each, proteins were detected using the enhanced chemiluminescence detection method according to the manufacturer's instructions (PerkinElmer, Wellesley, MA) with subsequent exposure of membrane to X-OMAT imaging film (Kodak). The density of bands on the images was quantified using NIH ImageJ. Where necessary, summary data for the experiments are expressed in graphical form as means ± SEM.

For Coomassie Blue staining, gels following electrophoresis were incubated with Coomassie Blue staining solution overnight. Gels were destained until the bands were visible. Protein bands were scanned in grey scale using the HP Scanjet software.

Statistics

Values were presented as mean ± SEM. Differences were analyzed between groups using one-way analysis of variance (ANOVA) followed by a *post hoc* Tukey's multiple comparison test to determine the statistical significance of the respective experiments. A value of $p < 0.05$ was considered significant.

RESULTS

Role of mTOR in PE stimulated CTGF expression in adult CM:

To study the role of mTOR in CTGF expression, we first determined if hypertrophic agonists could induce CTGF expression in adult CM. Previous studies have shown that α -adrenergic agonists, such as PE and angiotensin, stimulate CTGF expression in CM¹⁴. In the experiments shown in Figure-1A, stimulation of cells for 24 h with 10 µM PE or 200 nM angiotensin, but not 100 nM insulin, increased CTGF mRNA expression. Our results show

that PE stimulated robust CTGF mRNA expression when compared to angiotensin (Fig. 1A). On the other hand, insulin stimulation was found to significantly blunt the basal expression of CTGF mRNA. To confirm that PE stimulated CTGF mRNA expression occurs specifically via the alpha-1 adrenergic receptor, we used alfuzosin, a non-selective alpha-1 adrenergic receptor antagonist. For this, CM were pretreated with alfuzosin (2.5 μ M) for 30 min and then stimulated with 10 μ M PE for 24 h (Fig. 1B). Results showed that alfuzosin blocked, almost completely, PE-stimulated CTGF mRNA expression, indicating that the expression occurred via the alpha-1 adrenergic receptor.

To study the role of mTOR in the PE-stimulated CTGF mRNA expression, we used mTOR pathway inhibitors, torin1 (a specific inhibitor of both mTORC1 and mTORC2) and rapamycin (a specific inhibitor of mTORC1). For this, CM were pretreated with 100 nM torin1 or 5 nM rapamycin for 30 min and then stimulated with 10 μ M PE for 24 h. Torin1 pretreatment significantly augmented PE-stimulated CTGF mRNA expression (Fig. 1C). On the other hand, the CTGF mRNA expression was not significantly affected in rapamycin treated cells (Fig. 1D). Other profibrogenic factors, including collagen, fibronectin and TGF β , were analyzed and their mRNAs were not significantly altered under PE stimulation of CM (data not shown).

To demonstrate that PE stimulated CTGF expression and its further augmentation by torin1 is reflected at the protein level, culture media from respective conditions were concentrated and used for Western blot analysis with anti-CTGF antibody (Fig. 1E). As expected, treatment of adult CM with PE resulted in an increase in the secretion of CTGF, which was blunted by the treatment of alfuzosin. Furthermore, similar to changes observed at the message level, pretreatment of cells with torin1 but not rapamycin significantly enhanced PE stimulated extracellular secretion of CTGF (Fig. 1E). These data demonstrate that changes in CTGF gene expression correlates directly with the secreted level of CTGF. Importantly, these initial findings indicate that the loss of mTOR function can cause enhanced CTGF mRNA expression and increase the level of CTGF in the extracellular environment.

Repressive role of mTOR complexes on PE stimulated CTGF expression in adult CM:

Our studies (Fig. 1) reveal enhancement of PE stimulated CTGF expression by torin1, which suggests a possible negative role of mTOR on CTGF expression. Therefore, we performed additional studies to explore the independent roles of the two mTOR complexes in PE stimulated adult CM. We first explored if the loss of mTORC2 activity was responsible for the augmented effect on PE stimulated CTGF expression. For this, we suppressed Rictor, a specific component of mTORC2, in CM by expressing Rictor-specific shRNA using an adenoviral construct (Fig. 2A). Our earlier studies show that Rictor-specific shRNA adenovirus (shRict) infection for 36 h reduces the level of Rictor significantly in CM²⁶. In the present study, knockdown of Rictor by this approach significantly enhanced PE-stimulated CTGF mRNA (> 13 fold) and protein expression (Fig. 2A) and the data is similar to those obtained with torin1 treatment (Fig. 1D). Therefore, loss of mTORC2 function by either pharmacological inhibition with torin1 or genetic means via shRictor causes a significant augmentation of PE-stimulated CTGF expression, indicating a negative role of mTORC2 on agonist-induced CTGF expression. Next, we performed a similar experiment

using a Raptor shRNA (shRapt) expressing adenoviral construct. Our studies once again clearly show that PE stimulated CTGF mRNA expression and CTGF protein level in Raptor shRNA expressing CM were substantially (> 8 fold) increased when compared to β -gal expressing control virus infected CM (Fig. 2B). The loss of Raptor and Rictor in the respective shRNA expressing cells was confirmed at mRNA levels (data not shown). These studies demonstrate that both mTORC1 and mTORC2 suppress PE stimulated CTGF expression via negative regulatory mechanisms.

Loss of upstream activators of mTOR promotes PE stimulated CTGF expression:

To further confirm the negative role of mTOR complexes during α -agonist stimulation of CTGF expression, we blocked the activity of PI3K and Akt that are known to function upstream of mTOR, although Akt has been shown also to function downstream of mTORC2. Pretreatment of cells with 10 μ M or 50 μ M of LY, a specific inhibitor of PI3K, significantly increased the basal level of CTGF expression in a dose dependent manner (Fig. 3A). Furthermore, PE-stimulated CTGF was also substantially increased in LY treated cells in a dose dependent manner. This pattern of an augmented effect by LY on PE stimulated CTGF expression was also observed at the extracellularly secreted CTGF protein level (Fig. 3A, bottom panel). Similar results were obtained with 200 nM wortmannin treatment (data not shown). These data strongly indicate that PI3K, which mediates activation of both mTOR complexes, negatively controls CTGF expression.

Next, to explore whether the mTOR-mediated negative effect on PE stimulated CTGF expression requires Akt, we used an adenoviral vector that expresses a dominant negative, mutant form of Akt (DN-Akt)⁴⁰. For this, CM were infected with DN-Akt adenovirus or β -gal control virus. Following a 36 h incubation period, cells were stimulated with PE for an additional period of 24 h and processed for RNA isolation (Fig. 3B). Conditioned media was also saved during PE stimulation in order to measure secreted CTGF expression. The data shown in Fig. 3B demonstrate that the expression of DN-Akt to suppress endogenous Akt activity, significantly augments PE stimulated CTGF mRNA expression. This augmented expression was also reflected in the level of CTGF secreted in the media (Fig. 3B, lower panel). Therefore, the PI3K/Akt/mTOR axis controls CTGF expression where its loss could augment agonist-stimulated CTGF expression.

DN-PKC δ suppresses PE-induced CTGF expression:

Previous studies have shown that PKC δ contributes to TGF β -mediated CTGF expression⁴². Therefore, we explored whether PKC δ is required for PE-stimulated CTGF expression and whether it has any effect over mTOR-mediated negative regulation. For this, we expressed PKC δ -WT (wild type) and DN-PKC δ (dominant negative) in adult CM using adenoviral vectors (Fig. 4A). Our results show that while WT-PKC δ augments PE-stimulated CTGF expression, DN-PKC δ viral infection significantly reduced PE-stimulated CTGF mRNA expression (Fig. 4A). Western blot analysis using concentrated conditioned media (lower panel) further showed a similar trend where WT-PKC δ augments and DN-PKC δ reduces PE-stimulated CTGF expression. Next, we explored whether Akt activation (S473 phosphorylation) is altered during PKC δ expression. Our previous studies show that PKC δ plays an upstream role on mTOR³⁴. Results show that PE stimulated Akt S473

phosphorylation was significantly augmented in PKC δ -WT expressing cells while it is significantly blunted in CM infected with DN-PKC δ adenovirus when compared with β -gal expressing control cells (Fig. 4B). These results show that PKC δ plays an upstream role for the activation of Akt. Together, these data suggest that PKC δ is critical for both CTGF expression and Akt activation during agonist stimulation, although under these conditions, mTOR/Akt suppresses CTGF expression.

Role of Smad3 phosphorylation in PE-stimulated CTGF expression:

We next explored whether PE-stimulated CTGF expression and its augmentation due to PI3K/mTOR inhibition are accompanied by corresponding increase in Smad3 phosphorylation and whether blocking Smad3 phosphorylation results in the loss of PE-stimulated CTGF expression. Our studies show that PE-stimulated CTGF expression was accompanied with increased Smad3 S423/425 phosphorylation (Fig. 5). Furthermore, blocking either PI3K function (Fig. 5A) or mTOR signaling (Fig. 5B) significantly enhanced Smad3 phosphorylation. We also analyzed total and phosphorylated Smad3 levels in WT and DN-PKC δ expressing CM, following their stimulation with PE. These studies (Fig. 5C) showed that the expression of WT PKC δ moderately increased the PE-stimulated Smad3 phosphorylation. On the other hand, expression of DN-PKC δ suppressed the PE-stimulated Smad3 phosphorylation, indicating that PKC δ plays an upstream role for Smad3 phosphorylation similar to its effect on CTGF expression. To explore the role of Smad3 phosphorylation on CTGF expression, we used a specific inhibitor of Smad3 (SIS3). A recent study shows that SIS3 treatment at 3-10 μ M concentration in dermal fibroblasts attenuates the TGF β -induced Smad3 phosphorylation, Smad3/Smad4 interaction, myofibroblast differentiation and procollagen transcription⁴³. For the experiment shown in Fig. 5D, CM were treated with ± 5 μ M SIS3 30 min prior to β -gal or shRict adenoviral infections as detailed in Methods. These data showed that SIS3 treatment suppressed the PE stimulated Smad3 phosphorylation and partially blocked the basal and PE-stimulated CTGF expression. We also explored the effect of SIS3 on the augmentation of PE-stimulated CTGF expression when mTOR complexes are suppressed. The augmented CTGF expression due to the loss of mTORC2 (via Rictor knockdown) was also partially affected by SIS3. A similar observation was obtained with torin1 that blocked both mTORC1 and mTORC2 (data not shown).

Finally, we also tested another potential mechanism known to contribute to CTGF expression. That is, the association of a transcription coactivator Yes-associated protein (YAP) with TEAD transcriptional factor in the nucleus has been shown to contribute to CTGF expression⁴⁴. Phosphorylated YAP at specific sites by the Hippo pathway is held in the cytoplasm by 14-3-3 proteins and the loss of such specific phosphorylation is critical for nuclear localization of YAP. Therefore, to explore whether YAP is involved in the PE-stimulated CTGF expression, we analyzed the phosphorylation state of YAP1 at serine-127. Our studies (data not shown) showed that YAP1 is basally phosphorylated at S127 and PE stimulation of CM in the presence or absence of torin1 did not alter YAP1 phosphorylation or the protein levels.

DISCUSSION

In patients with aortic stenosis or hypertensive heart disease, cardiac fibrosis contributes significantly to interstitial stiffness, impaired conductance, and regional ischemia, which are often accompanied by cardiac dysfunction, especially in elderly patients^{3,4,6,45}. The release of autocrine/paracrine and humoral factors during cardiac stress is known to play a major role in profibrotic signaling^{6,14,46–49}. In this context, the expression of CTGF, which is released as an autocrine/paracrine factor, has been found to be deregulated, resulting in tissue fibrosis^{50–52} as observed in heart failure^{53,54}. The link between increased CTGF expression and fibrosis, which has been reported in several tissues (such as lung, kidney, skin, etc.), indicate an important role for this matricellular protein in the excessive deposition of ECM proteins¹⁰. For example, a recent study demonstrates that CTGF collaborates with TGF β and β 3-integrin to mediate a signaling cascade leading to excessive matrix protein deposition⁵². In support of these data, our recent work⁵⁵ demonstrates the loss of PO-induced cardiac fibrosis in β 3-integrin null mice. Furthermore, suppression of CTGF with antagonists has been shown to prevent or reverse fibrosis in several *in vivo* animal models⁵⁶. In the heart, a recent study demonstrates that CTGF transgenic mice substantially develops cardiac fibrosis upon PO accompanied by deteriorating cardiac function¹⁹. Furthermore, neutralizing CTGF in mice using humanized monoclonal antibody has been shown to suppress PO-induced expression of ECM markers and collagen and protects from adverse LV remodeling and dysfunction²⁰. However, a few related studies also show that CTGF offers a cardioprotective effect in certain cardiac hypertrophic transgenic animal models⁵⁷, and this could be due to the high level expression of CTGF in these transgenic mice. In isolated CM, our present work shows that suppression of baseline and PE-stimulated CTGF levels using shRNA adenoviral vector did not alter cellular morphology or viability (data not shown). Therefore, CTGF may serve as a potential drug target to treat fibrosis, and suppression of CTGF expression through mTOR activation would serve as a novel mechanism to reduce cardiac fibrosis and the associated pathological ventricular remodeling.

Although CTGF expression has been reported in both CM and CFb^{17,58}, α -adrenergic agonists such as PE and angiotensin are shown to promote CTGF expression primarily in CM¹⁸. Therefore, we used CM to explore the intermediary components involved in agonist-induced CTGF expression. Furthermore, previous studies mostly utilized neonatal CM. For the present work we utilized adult feline CM, as cardiac fibrosis is mostly observed with aging and heart failure. Our studies show that PE and angiotensin but not insulin stimulate CTGF expression. We used PE as the choice of agonist, since the CTGF expression was found to be substantially increased when compared to angiotensin stimulated CM. Pretreatment of cells with 2.5 μ M alfuzosin completely abolished PE stimulated CTGF expression indicating that the activation strictly occurs via a PE-specific α -adrenergic receptor.

mTOR serves as a key intermediate, regulating protein synthesis, gene expression, cell growth and survival^{21,22}. Of the two independent signaling complexes of mTOR, Raptor constitutes mTORC1 that promotes protein translation through phosphorylation of (S6K1), 4E-binding protein (4EBP) and protein phosphatase-2A (PP2A)²⁸, whereas Rictor

constitutes mTORC2 and promotes Akt activation as part of cell survival ^{22,30}. For the initial studies, we investigated the differential effects of torin1 and rapamycin on these complexes. Pretreatment of cells with torin1 blocks both of the mTOR complexes and was found to augment PE-stimulated CTGF expression. However, pretreatment of cells with rapamycin which was shown to affect mTORC1 but promoted mTORC2 ³³, did not affect PE-stimulated CTGF-gene expression. In this context, chronic *in vivo* treatment of rats for 3 wk with everolimus, a derivative of rapamycin, was found to augment CTGF expression ⁵⁹. Although rapamycin and its derivatives are known to block specifically mTORC1, their prolonged treatment is known to affect both mTORC1 and mTORC2 ⁶⁰. Since both mTOR complexes were found to negatively regulate CTGF expression, it is possible that the CTGF expression may remain unchanged during acute rapamycin treatment due to the loss of mTORC1 and augmentation of mTORC2.

To further confirm that both the mTOR complexes suppress CTGF expression, we undertook the shRNA approach to study the role of independent mTOR complexes. We expressed shRNA to knockdown Rictor and Raptor that block mTORC2 and mTORC1 activities, respectively, prior to stimulating cells with PE. We confirmed the loss of Rictor and Raptor under these conditions by measuring their mRNA levels. Furthermore, our previous work shows that this approach effectively knocks down Rictor and affects mTORC2-mediated Akt activation when we used Rictor shRNA adenovirus ²⁶. Our studies clearly show that the loss of mTORC2 or mTORC1 substantially augments PE-stimulated CTGF expression and secretion into conditioned media (Fig. 2). In the canonical mTOR signaling pathway, tyrosine phosphorylation of IRS-1 (insulin receptor substrate-1) and the subsequent activation of PI3K and Akt is critical for agonist-stimulated mTOR activation. Therefore, we explored whether the loss of PI3K and Akt also augments PE-stimulated CTGF expression. Our studies clearly show that blocking PI3K with LY294002 or wortmannin or blocking Akt with dominant negative Akt expression significantly augments PE-stimulated CTGF expression. These data clearly indicate that PI3K/Akt/mTOR signaling axis plays a negative regulatory role and restricts PE-stimulated CTGF expression.

Earlier studies using specific inhibitors or siRNA show that PKC δ mediates TGF β -mediated CTGF expression in HepG2 cells ⁴². Therefore, we used adenoviral constructs that express WT-PKC δ or DN-PKC δ to explore whether PKC δ regulates CTGF expression in agonist stimulated adult CM. Our studies show that WT-PKC δ overexpression augments and DN-PKC δ blunts PE-stimulated CTGF expression (Fig. 4A), indicating the critical need of PKC δ for PE-stimulated CTGF expression. However, our earlier work shows that the activation of PKC δ is also required for mTOR/S6K1 activation in PE, ET1 and insulin stimulated adult CM ³⁴. Furthermore, our present work using WT-PKC δ and DN-PKC δ adenoviral constructs shows the critical need of PKC δ for the PE-stimulated Akt activation (Fig. 4B). In support of this observation, a recent study using rottlerin (a PKC δ specific inhibitor) demonstrates that PKC δ is required for mTORC2-mediated Akt activation ³⁶. Together, these data indicate that PKC δ independently promotes PE-stimulated CTGF expression while at the same time facilitating the mTOR complexes-mediated negative regulation on CTGF expression. The net effect by these two opposing mechanisms by PKC δ leads to a partial expression and release of CTGF during PE stimulation.

Our present work also shows that PE stimulated CTGF expression and its augmentation during the loss of mTOR activation are accompanied by a corresponding increase in the phosphorylation of Smad3 (Fig. 5) but not Smad2 (data now shown). Furthermore, similar to CTGF expression, Smad3 phosphorylation was found to require PKC δ (Fig. 5C). Previous studies show that CTGF expression in kidney and tubular epithelial cells require Smad3 phosphorylation⁶¹. Furthermore, a recent study in muscle cells demonstrates the presence of Smad binding element in the 5'UTR of CTGF gene and that the Smad3 signaling is required for CTGF expression⁶². Therefore, we explored whether the increase in Smad3 phosphorylation was critical for the PE-stimulated CTGF expression in our studies. For this, we used a Smad3-specific inhibitor, SIS3⁴³. These studies (Fig. 5D) show that SIS3 treatment partially affects both basal and PE-stimulated CTGF expression. Importantly, we explored the effect of SIS3 treatment on the augmented effect on PE-stimulated CTGF expression by mTOR inhibition. For this, we suppressed endogenous Rictor expression for the loss of mTORC2. Our studies clearly show that the augmented expression of PE-stimulated CTGF following the loss of mTORC2 is also partially blocked by the SIS3 treatment. A partial effect by SIS3 was also observed when we used torin1 to block both mTORC2 and mTORC1 (data not shown). Therefore, these studies show that the negative regulation by mTOR complexes on the PE-stimulated CTGF expression proceeds at least partly via Smad3 activation (phosphorylation). Several earlier studies indicate both Smad-dependent and -independent mechanisms contribute to CTGF expression^{61–64}. In this context, recent studies show that a TEAD-dependent transcriptional mechanism for CTGF expression that requires the action of YAP1 as a coactivator⁴⁴. Specific phosphorylation of YAP1 at the serine 127 site blocks its nuclear localization⁶⁵ and thus prevention of CTGF expression. Therefore, we analyzed for the changes in the phosphorylation state of YAP1 at the S127 site using a specific antibody. Our studies (data not shown) showed that YAP1 in CM are basally phosphorylated and both YAP1 at protein and phosphorylation levels did not appreciably change in PE stimulated cells, treated with or without torin1, indicating YAP1 independent mechanisms that contribute to CTGF expression. Therefore, in addition to Smad3-mediated transcriptional mechanism, other noncanonical mechanism(s) independent of YAP1 is also involved in the PE-stimulated CTGF expression.

During hypoxia and other forms of cell stress, Akt activation is known to counteract proapoptotic molecules in order to protect cells from programmed cell death. However, if the stress is severe and sustains longer, Akt signaling is gradually lost, allowing cell death to occur⁴². Under such conditions of Akt loss, stress signals are expected to cause excessive CTGF expression due to the loss of Akt/mTOR mediated suppressive effect. Therefore, during chronic heart disease, the accompanying loss of prosurvival mechanism, especially mTORC2/Akt signaling, in CM could be a major contributing factor for the excessive release of profibrogenic cytokines such as CTGF, leading to the development of cardiac fibrosis. Therefore, sustaining basal Akt/mTOR signaling and the associated reduction in the expression of profibrogenic factor such as CTGF is expected to reduce adverse ventricular remodeling during chronic PO.

In summary (Fig. 6), our studies present a novel finding that the PI3K/Akt/mTOR axis negatively regulates hypertrophic agonist-induced CTGF expression. Our studies also show that PKC δ on one hand plays a mandatory role for CTGF expression, and on the other hand

promotes an mTOR-mediated suppressive mechanism on CTGF expression. Therefore, during cardiac stress such as PO, the accompanying activation of Akt might initially suppress CTGF expression, but the loss of such mechanism under chronic conditions could result in the excessive release of CTGF, causing cardiac fibrosis and the associated maladaptive remodeling.

Acknowledgement

This work was supported by the National Institutes of Health postdoctoral fellowship (T32HL07260 to K.S.) and predoctoral fellowship (T32HL07260 to D.L.P. and P.C.M.).

REFERENCES

1. Weber KT, Anversa P, Armstrong PW, Brilla CG, Burnett JC Jr., Cruickshank JM, Devereux RB, Giles TD, Korsgaard N, Leier CV, et al. Remodeling and reparation of the cardiovascular system. *J Am Coll Cardiol* 1992;20:3–16. [PubMed: 1318886]
2. Conrad CH, Brooks WW, Hayes JA, Sen S, Robinson KG, Bing OH. Myocardial fibrosis and stiffness with hypertrophy and heart failure in the spontaneously hypertensive rat. *Circulation* 1995;91:161–170. [PubMed: 7805198]
3. Gaasch WH, Zile MR. Left ventricular diastolic dysfunction and diastolic heart failure. *Annu Rev Med* 2004;55:373–394. [PubMed: 14746527]
4. Zile MR, Baicu CF, Ikonomidis J, Stroud RE, Nietert PJ, Bradshaw AD, Slater R, Palmer BM, Van Buren P, Meyer M, Redfield M, Bull D, Granzier H, LeWinter MM. Myocardial Stiffness in Patients with Heart Failure and a Preserved Ejection Fraction: Contributions of Collagen and Titin. *Circulation* 2015.
5. Essick EE, Sam F. Cardiac hypertrophy and fibrosis in the metabolic syndrome: a role for aldosterone and the mineralocorticoid receptor. *Int J Hypertens* 2011;2011:346985. [PubMed: 21747976]
6. Gonzalez A, Lopez B, Diez J. Fibrosis in hypertensive heart disease: role of the renin-angiotensin-aldosterone system. *The Medical clinics of North America* 2004;88:83–97. [PubMed: 14871052]
7. Schnee JM, Hsueh WA. Angiotensin II, adhesion, and cardiac fibrosis. *Cardiovasc Res* 2000;46:264–268. [PubMed: 10773230]
8. Leask A, Abraham DJ. All in the CCN family: essential matricellular signaling modulators emerge from the bunker. *Journal of cell science* 2006;119:4803–4810. [PubMed: 17130294]
9. Perbal B. CCN proteins: multifunctional signalling regulators. *Lancet* 2004;363:62–64. [PubMed: 14723997]
10. Sonnylal S, Shi-Wen X, Leoni P, Naff K, Van Pelt CS, Nakamura H, Leask A, Abraham D, Bou-Gharios G, de Crombrughe B. Selective expression of connective tissue growth factor in fibroblasts in vivo promotes systemic tissue fibrosis. *Arthritis and rheumatism* 2010;62:1523–1532. [PubMed: 20213804]
11. Lipson KE, Wong C, Teng Y, Spong S. CTGF is a central mediator of tissue remodeling and fibrosis and its inhibition can reverse the process of fibrosis. *Fibrogenesis & tissue repair* 2012;5 Suppl 1:S24. [PubMed: 23259531]
12. Mason RM. Fell-Muir lecture: Connective tissue growth factor (CCN2) -- a pernicious and pleiotropic player in the development of kidney fibrosis. *International journal of experimental pathology* 2013;94:1–16. [PubMed: 23110747]
13. Duisters RF, Tijssen AJ, Schroen B, Leenders JJ, Lentink V, van der Made I, Herias V, van Leeuwen RE, Schellings MW, Barenbrug P, Maessen JG, Heymans S, Pinto YM, Creemers EE. miR-133 and miR-30 regulate connective tissue growth factor: implications for a role of microRNAs in myocardial matrix remodeling. *Circulation research* 2009;104:170–178, 176p following 178. [PubMed: 19096030]

14. Matsui Y, Sadoshima J. Rapid upregulation of CTGF in cardiac myocytes by hypertrophic stimuli: implication for cardiac fibrosis and hypertrophy. *Journal of molecular and cellular cardiology* 2004;37:477–481. [PubMed: 15276017]
15. Messaoudi S, Gravez B, Tarjus A, Pelloux V, Ouvrard-Pascaud A, Delcayre C, Samuel J, Launay JM, Sierra-Ramos C, Alvarez de la Rosa D, Clement K, Farman N, Jaisser F. Aldosterone-specific activation of cardiomyocyte mineralocorticoid receptor in vivo. *Hypertension* 2013;61:361–367. [PubMed: 23297371]
16. Ko WC, Hong CY, Hou SM, Lin CH, Ong ET, Lee CF, Tsai CT, Lai LP. Elevated expression of connective tissue growth factor in human atrial fibrillation and angiotensin II-treated cardiomyocytes. *Circulation journal: official journal of the Japanese Circulation Society* 2011;75:1592–1600. [PubMed: 21576830]
17. Chen MM, Lam A, Abraham JA, Schreiner GF, Joly AH. CTGF expression is induced by TGF-beta in cardiac fibroblasts and cardiac myocytes: a potential role in heart fibrosis. *Journal of molecular and cellular cardiology* 2000;32:1805–1819. [PubMed: 11013125]
18. Kemp TJ, Aggeli IK, Sugden PH, Clerk A. Phenylephrine and endothelin-1 upregulate connective tissue growth factor in neonatal rat cardiac myocytes. *Journal of molecular and cellular cardiology* 2004;37:603–606. [PubMed: 15276029]
19. Yoon PO, Lee MA, Cha H, Jeong MH, Kim J, Jang SP, Choi BY, Jeong D, Yang DK, Hajjar RJ, Park WJ. The opposing effects of CCN2 and CCN5 on the development of cardiac hypertrophy and fibrosis. *Journal of molecular and cellular cardiology* 2010;49:294–303. [PubMed: 20430035]
20. Szabo Z, Magga J, Alakoski T, Ulvila J, Piuholo J, Vainio L, Kivirikko KI, Vuolteenaho O, Ruskoaho H, Lipson KE, Signore P, Kerkela R. Connective tissue growth factor inhibition attenuates left ventricular remodeling and dysfunction in pressure overload-induced heart failure. *Hypertension* 2014;63:1235–1240. [PubMed: 24688123]
21. Wullschlegel S, Loewith R, Hall MN. TOR signaling in growth and metabolism. *Cell* 2006;124:471–484. [PubMed: 16469695]
22. Sarbassov DD, Ali SM, Sabatini DM. Growing roles for the mTOR pathway. *Curr Opin Cell Biol* 2005;17:596–603. [PubMed: 16226444]
23. Iijima Y, Laser M, Shiraishi H, Willey CD, Sundaravadeivel B, Xu L, McDermott PJ, Kuppuswamy D. c-Raf/MEK/ERK pathway controls protein kinase C-mediated p70S6K activation in adult cardiac muscle cells. *J Biol Chem* 2002;277:23065–23075. [PubMed: 11940578]
24. Balasubramanian S, Kuppuswamy D. RGD-containing peptides activate S6K1 through beta3 integrin in adult cardiac muscle cells. *J Biol Chem* 2003;278:42214–42224. [PubMed: 12909616]
25. Naga Prasad SV, Perrino C, Rockman HA. Role of phosphoinositide 3-kinase in cardiac function and heart failure. *Trends Cardiovasc Med* 2003;13:206–212. [PubMed: 12837584]
26. Moschella PC, McKillop J, Pleasant DL, Harston RK, Balasubramanian S, Kuppuswamy D. mTOR complex 2 mediates Akt phosphorylation that requires PKC δ in adult cardiac muscle cells. *Cellular signalling* 2013;25:1904–1912. [PubMed: 23673367]
27. Bhaskar PT, Hay N. The two TORCs and Akt. *Dev Cell* 2007;12:487–502. [PubMed: 17419990]
28. Park IH, Erbay E, Nuzzi P, Chen J. Skeletal myocyte hypertrophy requires mTOR kinase activity and S6K1. *Exp Cell Res* 2005;309:211–219. [PubMed: 15963500]
29. Kamiya K, Ryer E, Sakakibara K, Zohlman A, Kent KC, Liu B. Protein kinase C delta activated adhesion regulates vascular smooth muscle cell migration. *The Journal of surgical research* 2007;141:91–96. [PubMed: 17574042]
30. Guertin DA, Stevens DM, Thoreen CC, Burds AA, Kalaany NY, Moffat J, Brown M, Fitzgerald KJ, Sabatini DM. Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKC α , but not S6K1. *Dev Cell* 2006;11:859–871. [PubMed: 17141160]
31. Jacinto E, Loewith R, Schmidt A, Lin S, Ruegg MA, Hall A, Hall MN. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat Cell Biol* 2004;6:1122–1128. [PubMed: 15467718]
32. Sarbassov DD, Ali SM, Kim DH, Guertin DA, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and

raptor-independent pathway that regulates the cytoskeleton. *Curr Biol* 2004;14:1296–1302. [PubMed: 15268862]

33. Harston RK, McKillop JC, Moschella PC, Van Laer A, Quinones LS, Baicu CF, Balasubramanian S, Zile MR, Kuppuswamy D. Rapamycin treatment augments both protein ubiquitination and Akt activation in pressure-overloaded rat myocardium. *American journal of physiology Heart and circulatory physiology* 2011;300:H1696–1706. [PubMed: 21357504]
34. Moschella PC, Rao VU, McDermott PJ, Kuppuswamy D. Regulation of mTOR and S6K1 activation by the nPKC isoforms, PKC ϵ and PKC δ , in adult cardiac muscle cells. *J Mol Cell Cardiol* 2007;43:754–766. [PubMed: 17976640]
35. Greene MW, Morrice N, Garofalo RS, Roth RA. Modulation of human insulin receptor substrate-1 tyrosine phosphorylation by protein kinase C δ . *The Biochemical journal* 2004;378:105–116. [PubMed: 14583092]
36. Choi YH, Jin GY, Li LC, Yan GH. Inhibition of protein kinase C δ attenuates allergic airway inflammation through suppression of PI3K/Akt/mTOR/HIF-1 α /VEGF pathway. *PLoS One* 2013;8:e81773. [PubMed: 24312355]
37. DeBosch B, Treskov I, Lupu TS, Weinheimer C, Kovacs A, Courtois M, Muslin AJ. Akt1 is required for physiological cardiac growth. *Circulation* 2006;113:2097–2104. [PubMed: 16636172]
38. Walsh K Akt signaling and growth of the heart. *Circulation* 2006;113:2032–2034. [PubMed: 16651482]
39. Braz JC, Bueno OF, De Windt LJ, Molkentin JD. PKC α regulates the hypertrophic growth of cardiomyocytes through extracellular signal-regulated kinase1/2 (ERK1/2). *The Journal of cell biology* 2002;156:905–919. [PubMed: 11864993]
40. Volkens M, Konstandin MH, Doroudgar S, Toko H, Quijada P, Din S, Joyo A, Ornelas L, Samse K, Thuermer DJ, Gude N, Glembotski CC, Sussman MA. Mechanistic target of rapamycin complex 2 protects the heart from ischemic damage. *Circulation* 2013;128:2132–2144. [PubMed: 24008870]
41. Kent RL, Mann DL, Urabe Y, Hisano R, Hewett KW, Loughnane M, Cooper GT. Contractile function of isolated feline cardiocytes in response to viscous loading. *Am J Physiol* 1989;257:H1717–1727. [PubMed: 2589523]
42. Lee SJ, Kang JH, Choi SY, Kwon OS. PKC δ as a regulator for TGF- β -stimulated connective tissue growth factor production in human hepatocarcinoma (HepG2) cells. *The Biochemical journal* 2013;456:109–118. [PubMed: 23988089]
43. Jinnin M, Ihn H, Tamaki K. Characterization of SIS3, a novel specific inhibitor of Smad3, and its effect on transforming growth factor- β 1-induced extracellular matrix expression. *Molecular pharmacology* 2006;69:597–607. [PubMed: 16288083]
44. Zhao B, Ye X, Yu J, Li L, Li W, Li S, Lin JD, Wang CY, Chinnaiyan AM, Lai ZC, Guan KL. TEAD mediates YAP-dependent gene induction and growth control. *Genes Dev* 2008;22:1962–1971. [PubMed: 18579750]
45. Copaja Soto M, Valenzuela R, Saldana A, Paz Ocaranza M, Jalil JE, Vio C, Lijnen P, Ordenes GE, Vivar Sanchez R, Lavandero S, Diaz-Araya G. Early expression of monocyte chemoattractant protein-1 correlates with the onset of isoproterenol-induced cardiac fibrosis in rats with distinct angiotensin-converting enzyme polymorphism. *J Renin Angiotensin Aldosterone Syst* 2008;9:154–162. [PubMed: 18957386]
46. Brown RD, Ambler SK, Mitchell MD, Long CS. The cardiac fibroblast: therapeutic target in myocardial remodeling and failure. *Annu Rev Pharmacol Toxicol* 2005;45:657–687. [PubMed: 15822192]
47. Leask A Potential therapeutic targets for cardiac fibrosis: TGF β , angiotensin, endothelin, CCN2, and PDGF, partners in fibroblast activation. *Circ Res* 2006;106:1675–1680.
48. Ponten A, Folestad EB, Pietras K, Eriksson U. Platelet-derived growth factor D induces cardiac fibrosis and proliferation of vascular smooth muscle cells in heart-specific transgenic mice. *Circ Res* 2005;97:1036–1045. [PubMed: 16224065]
49. Ares-Carrasco S, Picatoste B, Benito-Martin A, Zubiri I, Sanz AB, Sanchez-Nino MD, Ortiz A, Egido J, Tunon J, Lorenzo O. Myocardial fibrosis and apoptosis, but not inflammation, are present in long-term experimental diabetes. *American journal of physiology Heart and circulatory physiology* 2009;297:H2109–2119. [PubMed: 19820199]

50. Bleau AM, Planque N, Perbal B. CCN proteins and cancer: two to tango. *Frontiers in bioscience : a journal and virtual library* 2005;10:998–1009. [PubMed: 15769600]
51. Shi-Wen X, Leask A, Abraham D. Regulation and function of connective tissue growth factor/CCN2 in tissue repair, scarring and fibrosis. *Cytokine & growth factor reviews* 2008;19:133–144. [PubMed: 18358427]
52. Nakerakanti SS, Bujor AM, Trojanowska M. CCN2 is required for the TGF-beta induced activation of Smad1-Erk1/2 signaling network. *PLoS One* 2011;6:e21911. [PubMed: 21760921]
53. Ahmed MS, Oie E, Vinge LE, Yndestad A, Oystein Andersen G, Andersson Y, Attramadal T, Attramadal H. Connective tissue growth factor--a novel mediator of angiotensin II-stimulated cardiac fibroblast activation in heart failure in rats. *Journal of molecular and cellular cardiology* 2004;36:393–404. [PubMed: 15010278]
54. Ahmed SH, Clark LL, Pennington WR, Webb CS, Bonnema DD, Leonardi AH, McClure CD, Spinale FG, Zile MR. Matrix metalloproteinases/tissue inhibitors of metalloproteinases: relationship between changes in proteolytic determinants of matrix composition and structural, functional, and clinical manifestations of hypertensive heart disease. *Circulation* 2006;113:2089–2096. [PubMed: 16636176]
55. Balasubramanian S, Quinones L, Kasiganesan H, Zhang Y, Pleasant DL, Sundararaj KP, Zile MR, Bradshaw AD, Kuppuswamy D. beta3 integrin in cardiac fibroblast is critical for extracellular matrix accumulation during pressure overload hypertrophy in mouse. *PLoS One* 2012;7:e45076. [PubMed: 22984613]
56. Brigstock DR. Connective tissue growth factor (CCN2, CTGF) and organ fibrosis: lessons from transgenic animals. *Journal of cell communication and signaling* 2010;4:1–4. [PubMed: 19798591]
57. Panek AN, Posch MG, Alenina N, Ghadge SK, Erdmann B, Popova E, Perrot A, Geier C, Dietz R, Morano I, Bader M, Ozcelik C. Connective tissue growth factor overexpression in cardiomyocytes promotes cardiac hypertrophy and protection against pressure overload. *PLoS One* 2009;4:e6743. [PubMed: 19707545]
58. Ohnishi H, Oka T, Kusachi S, Nakanishi T, Takeda K, Nakahama M, Doi M, Murakami T, Ninomiya Y, Takigawa M, Tsuji T. Increased expression of connective tissue growth factor in the infarct zone of experimentally induced myocardial infarction in rats. *Journal of molecular and cellular cardiology* 1998;30:2411–2422. [PubMed: 9925376]
59. Finckenberg P, Inkien K, Ahonen J, Merasto S, Louhelainen M, Vapaatalo H, Muller D, Ganten D, Luft F, Mervaala E. Angiotensin II induces connective tissue growth factor gene expression via calcineurin-dependent pathways. *The American journal of pathology* 2003;163:355–366. [PubMed: 12819040]
60. Sarbassov DD, Ali SM, Sengupta S, Sheen JH, Hsu PP, Bagley AF, Markhard AL, Sabatini DM. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol Cell* 2006;22:159–168. [PubMed: 16603397]
61. Chung AC, Zhang H, Kong YZ, Tan JJ, Huang XR, Kopp JB, Lan HY. Advanced glycation end-products induce tubular CTGF via TGF-beta-independent Smad3 signaling. *Journal of the American Society of Nephrology : JASN* 2010;21:249–260. [PubMed: 19959709]
62. Cordova G, Rochard A, Riquelme-Guzman C, Cofre C, Scherman D, Bigey P, Brandan E. SMAD3 and SP1/SP3 Transcription Factors Collaborate to Regulate Connective Tissue Growth Factor Gene Expression in Myoblasts in Response to Transforming Growth Factor beta. *J Cell Biochem* 2015.
63. Holmes A, Abraham DJ, Sa S, Shiwen X, Black CM, Leask A. CTGF and SMADs, maintenance of scleroderma phenotype is independent of SMAD signaling. *J Biol Chem* 2001;276:10594–10601. [PubMed: 11152469]
64. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 2003;425:577–584. [PubMed: 14534577]
65. Zhao B, Wei X, Li W, Udan RS, Yang Q, Kim J, Xie J, Ikenoue T, Yu J, Li L, Zheng P, Ye K, Chinnaiyan A, Halder G, Lai ZC, Guan KL. Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes Dev* 2007;21:2747–2761. [PubMed: 17974916]

Fig. 1.

Agonist stimulated CTGF expression is linked to mTOR signaling: Adult feline CM cultured on laminin-coated dishes under serum and growth factor free conditions were stimulated with PE (10 μ M), angiotensin (200 nM) or insulin (100 nM) for 24 h. (A) Cells were used for RNA extraction and the samples were processed for quantitative assessment of relative mRNA level by performing real time PCR using CTGF and GAPDH-specific primers. CTGF mRNA was normalized to GAPDH and the ratios from three independent experiments were summarized in the figure and represented as mean $\pm$ SEM. * p < 0.01 vs. Cont; ^ p < 0.01 vs. Ang; @ p < 0.01 vs. Cont. (B) CM were pretreated with alfuzosin (2.5 μ M) for 30 min prior to stimulation with 10 μ M PE for 24 h. Untreated cells with alfuzosin

or PE served as controls. CTGF mRNA expression was quantified as detailed in (A). $*p < 0.01$ vs. Cont; $\#p < 0.01$ vs. PE. (C) CM were pretreated with torin1 (100 nM) for 30 min prior to stimulation with 10 μ M PE for 24 h. Untreated cells served as controls. CTGF mRNA expression was quantified as detailed in (A). $*p < 0.01$ vs. Cont; $@p < 0.01$ vs. PE. (D) CM were pretreated with rapamycin (5 nM) for 30 min prior to stimulation with 10 μ M PE for 24 h. Vehicle treated cells served as controls. CTGF mRNA expression was quantified as detailed in (A). $*p < 0.01$ vs. Cont. (E) Conditioned media from the above experiments were collected and concentrated for Western blot analysis with anti-CTGF antibody as detailed in the Materials and Methods. In the bottom panel, equal loading of proteins was confirmed by Coomassie blue staining of the SDS-PAGE gel.

Fig. 2.

mTORC2 suppresses PE-stimulated CTGF expression: Adult feline CM cultured on laminin-coated dishes under serum and growth factor free conditions were infected with adenovirus expressing either shRictor or shRaptor for 24 h. Cells were then stimulated with PE (10 μ M) for 24 h. Cells were used for RNA extraction and the condition media were used for Western blot analyses as detailed under Materials and Methods. **(A)** RNA samples from control, β -gal and the shRictor expressing cells were processed for quantitative assessment of relative mRNA level by performing real time PCR using CTGF and GAPDH-specific primers. CTGF mRNA was normalized to GAPDH and the ratios from three independent experiments were summarized in the figure and represented as mean $\pm$ SEM. * p < 0.05 vs. Cont; @ p < 0.05 vs. β -gal; # p < 0.01 vs. shRictor; ^ p 0.01 vs. β -gal+PE. Middle panel shows a Western blot for the corresponding CTGF at protein level in conditioned media. In the bottom panel, equal loading of protein is shown by a nonspecific 42 kDa protein band (NS Protein) detected by commassie blue staining. **(B)** RNA samples from control, β -gal and shRaptor expressing cells were processed for quantitative assessment of relative mRNA level by performing real time PCR using CTGF and GAPDH-specific primers. CTGF mRNA was normalized to GAPDH and the ratios from three independent experiments were summarized in the figure and represented as mean $\pm$ SEM. * p < 0.05 vs. Cont; @ p < 0.05 vs. β -gal; # p < 0.01 vs. shRaptor; ^ p 0.01 vs. β -gal+PE. Middle panel shows a Western blot for the corresponding CTGF protein level in conditioned media. In the bottom panel, equal loading of protein is shown by a nonspecific 42 kDa protein band detected by commassie blue staining.

Fig. 3.

Loss of PI3K and Akt activities augments PE-stimulated CTGF expression: Adult feline CM cultured on laminin-coated dishes under serum and growth factor free conditions were either pretreated with low (10 μ M) and high (50 μ M) doses of LY294002 for 30 min or infected with adenovirus expressing dominant negative Akt (DN-Akt) for 24 h. Cells were then stimulated with PE (10 μ M) for 24 h. Cells were used for RNA extraction and the conditioned media were used for Western blot analyses as detailed under Materials and Methods. **(A)** RNA samples from control, and LY-294002 treated cells with $\pm$ PE were processed for quantitative assessment of relative mRNA level by performing real time PCR using CTGF and GAPDH-specific primers. CTGF mRNA was normalized to GAPDH and the ratios from three independent experiments were summarized in the figure and represented as mean $\pm$ SEM. * p < 0.01 vs. Cont; @ p < 0.01 vs. PE; # p < 0.01 vs. LY 10 μ M; ^ p < 0.01 vs. LY 50 μ M. Middle panel shows a Western blot for the corresponding CTGF at protein level in conditioned media. In the bottom panel, equal loading of protein is shown by a nonspecific 42 kDa protein band (NS Protein) detected by commassie blue staining. **(B)** RNA samples from control, β -gal and DN-Akt expressing cells were processed for quantitative assessment of relative mRNA level by performing real time PCR using CTGF and GAPDH-specific primers. CTGF mRNA was normalized to GAPDH and the ratios from three independent experiments were summarized in the figure and represented as mean $\pm$ SEM. * p < 0.01 vs. β -gal; @ p < 0.01 vs. β -gal+PE. Middle panel shows a Western blot for the corresponding CTGF protein level in conditioned media. In the bottom panel, equal loading of protein is shown by a nonspecific 42 kDa protein band detected by commassie blue staining.

Fig. 4.

PKC δ is required for agonist-stimulated CTGF expression and mTOR-mediated negative regulation: Adult feline CM cultured on laminin-coated dishes under serum and growth factor free conditions were infected with adenovirus expressing β -gal, wild type WT-PKC δ and DN-PKC δ for 24 h. Cells were then stimulated with PE (10 μ M) for 24 h and the condition media were used for Western blot analyses as detailed under Materials and Methods. (A) Cells were used for RNA extraction and processed for quantitative assessment of relative mRNA level by performing real time PCR using CTGF and GAPDH-specific primers. CTGF mRNA was normalized to GAPDH and the ratios from three independent experiments were summarized in the figure and represented as mean $\pm$ SEM. * p < 0.01 vs. β -gal; @ p < 0.01 vs. WT-PKC δ ; & p < 0.01 vs. β -gal+PE; # p < 0.01 vs. DN-PKC δ ; ^ p < 0.01 vs. β -gal+PE. Middle panel shows a Western blot for the corresponding CTGF protein level in conditioned media. In the bottom panel, equal loading of protein is shown by a nonspecific 42 kDa protein band detected by commassie blue staining. (B) Cells were extracted with Triton X-100 buffer and the protein samples were processed for Western blot analyses with anti-phospho Ser473 Akt and anti-Akt antibodies. Graph shows summary data for the ratio of phospho-Akt to total Akt which was taken as 100 for β -gal untreated controls. * p < 0.01 vs. β -gal; \$ p < 0.05 vs. β -gal; @ p < 0.01 vs. WT-PKC δ ; & p < 0.01 vs. β -gal+PE; # p < 0.05 vs. DN-PKC δ ; ^ p < 0.01 vs. β -gal+PE. Lower panel shows representative Western blot data for phospho and total Akt.

Fig. 5.

Role of Smad3 phosphorylation on CTGF expression: (A) Adult feline CM cultured on laminin-coated dishes under serum and growth factor free conditions were pretreated with low (10 μ M) and high (50 μ M) doses of LY294002 for 30 min. Cells were then stimulated with PE (10 μ M) for 24 h. Cells were extracted with Triton X-100 buffer and the protein samples were processed for Western blot analyses with anti-phospho Ser423/425 Smad3 antibody and anti-Smad3 regular antibody. Graph shows summary data for the ratio of phospho-Smad3 to total Smad3 which was taken as 100 for untreated controls. * $p < 0.01$ vs.

Control; $^{\wedge}p < 0.01$ vs. PE. **(B)** Adult feline CM cultured on laminin-coated dishes under serum and growth factor free conditions were pretreated with rapamycin (5 nM) or torin1 (100 nM) for 30 min. Cells were then stimulated with PE (10 μ M) for 24 h. Cells were extracted with Triton X-100 buffer and the protein samples were processed for Western blot analyses with anti-phospho Ser423/425 Smad3 and anti-Smad3 antibodies. Graph shows summary data for the ratio of phospho-Smad3 to total Smad3 which was taken as 100 for untreated controls. $*p < 0.01$ vs. Control; $^{\wedge}p < 0.01$ vs. PE or rapamycin + PE. **(C)** Adult feline CM cultured on laminin-coated dishes under serum and growth factor free conditions were infected with adenovirus expressing β -gal, WT-PKC $_{\delta}$ and DN-PKC $_{\delta}$ for 24 h. Cells were extracted with Triton X-100 buffer and the protein samples were processed for Western blot analyses with anti-phospho Ser423/425 Smad3 and anti-Smad3 (total) antibodies. **(D)** Adult feline CM cultured on laminin-coated dishes under serum and growth factor free conditions were added $\pm$ 5 μ M SIS3 prior to infection with β -gal or shRictor expressing adenovirus. After 24 h infection, cells were stimulated with PE (10 μ M) for 24 h. The media were collected and the cells were extracted with Triton X-100 buffer. *(Upper panels)* The cell extracts were processed for Western blot analyses with anti-phospho Ser423/425 Smad3 and anti-Smad3 antibodies. *(Lower panels)* Conditioned media from the above experiments were collected and concentrated for Western blot analysis with anti-CTGF antibody as detailed in the Materials and Methods. Equal loading of protein is shown by a nonspecific (NS) protein band detected by commassie blue staining. Results were confirmed with one additional experiment.

Fig. 6. Schematic showing mTOR-mediated negative regulation of PE stimulated CTGF expression: Hypertrophic agonist PE stimulates PKC_δ and contributes to mTOR activation via IRS phosphorylation and PI3K activation in addition to its direct effect on mTOR. While the PKC_δ mediated PI3K/Akt/mTOR axis suppresses agonist stimulated CTGF expression, PKC_δ independently favors PE-stimulated CTGF expression. In hypertrophying

myocardium, loss of the negative regulation by PI3K/Akt/mTOR axis is expected to result in excessive CTGF secretion that might contribute to the development of cardiac fibrosis.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript