Molecular Pharmacology Fast Forward. Published on June 18, 2013 as doi:10.1124/mol.113.087312
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CONNECTIVE TISSUE GROWTH FACTOR / CCN2 ATTENUATES β-ADRENERGIC RECEPTOR RESPONSIVENESS AND CARDIOTOXICITY BY INDUCTION OF G PROTEIN-COUPLED RECEPTOR KINASE-5 (GRK5) IN CARDIOMYOCYTES

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Running title: CTGF-induced desensitization of β-ARs

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Number of text pages: 40

Number of tables: 0

Number of figures: 5

Number of references: 49

Word count Abstract: 245

Word count Introduction: 690

Word count Discussion: 1352

Non-standard abbreviations:

β-AR: β-adrenergic receptor

CHO: Chinese hamster ovary

CTGF/CCN2: Connective tissue growth factor

ERK: Extracellular signal-regulated kinase

GRK5: G protein-coupled receptor kinase-5

GRK2: G protein-coupled receptor kinase-2

IBMX: 3-isobutyl-1-methylxanthine

MEM: Minimum Essential Medium

NLC: non-transgenic littermate control

rec-hCTGF: recombinant human CTGF

PCR: Polymerase Chain Reaction

PVDF: Polyvinylidene difluoride

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ABSTRACT

Myocardial connective tissue growth factor (CTGF/CCN2) is induced in heart failure, a condition associated with diminution of β-adrenergic receptor (β-AR) responsiveness. Accordingly, we aimed to investigate whether CTGF could play a mechanistic role in regulation of β-AR responsiveness. Concentration-response curves of isoproterenol-stimulated cAMP generation in cardiomyocytes from transgenic mice with cardiac-restricted overexpression of CTGF (Tg-CTGF) or cardiomyocytes pretreated with recombinant human CTGF (rec-hCTGF) revealed marked reduction of both β_1-AR and β_2-AR responsiveness. Consistently, ventricular muscle strips from Tg-CTGF mice stimulated with isoproterenol displayed attenuation of maximal inotropic responses. However, no differences of maximal inotropic responses of myocardial fibres from Tg-CTGF mice and non-transgenic littermates (NLC) were discerned when stimulated with supramaximal concentrations of dibutyryl-cAMP, indicating preserved downstream responsiveness to cAMP. Congruent with a mechanism of desensitization of β-ARs, mRNA and protein levels of GRK5 were found isoform-selective upregulated in both cardiomyocytes from Tg-CTGF mice and cardiomyocytes exposed to rec-hCTGF. Corroborating a mechanism of GRK5 in CTGF-mediated control of β-AR sensitivity, CHO cells pretreated with rec-hCTGF displayed increased agonist- and biased ligand-stimulated β-arrestin-binding to β-ARs. Despite increased sensitivity of cardiomyocytes from GRK5-knockout mice (GRK5-KO) to β-adrenergic agonist, pretreatment of GRK5-KO cardiomyocytes with rec-hCTGF, as opposed to cardiomyocytes from wild type mice, did not alter β-AR responsiveness. Finally, Tg-CTGF mice subjected to chronic (14 days) exposure to isoproterenol, revealed blunted myocardial hypertrophy and

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preserved cardiac function versus NLC mice. In conclusion, this study uncovers a novel mechanism controlling β-AR responsiveness in cardiomyocytes involving CTGF-mediated regulation of GRK5.

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INTRODUCTION

Among the different G protein-coupled receptors expressed on cardiomyocytes, β-adrenergic receptors (β-ARs) appear to play a pivotal role in regulation of cardiac function both in the physiologic state as well as in heart failure. A common characteristic and critical pathophysiologic mechanism of heart failure is activation of the sympathetic nervous system and persistent stimulation of cardiac β-ARs with subsequent desensitization of β-AR responsiveness (Bristow et al., 1982; Brodde, 1993; Packer, 1988). It is well established that chronic hyperstimulation of myocardial β-ARs may exert deleterious actions in the heart, ultimately leading to pathologic remodeling and heart failure (Engelhardt et al., 1999; Hittinger et al., 1989). Conversely, the use of β-blockers improves cardiac function and decreases mortality in heart failure patients (Bristow, 2000; Waagstein et al., 1975). Desensitization of cardiac β-ARs upon persistent stimulation with agonist (epinephrine/norepinephrine) conceivably protects the heart against arrhythmias, hypertrophy and energy depletion (Lohse et al., 2003). Yet, desensitization of myocardial β-ARs also contributes to the reduced cardiac contractility associated with heart failure. Increased activities of G protein-coupled receptor kinases (GRKs) play a critical role in desensitization of cardiac β-AR signaling pathways in heart failure (Rockman et al., 2002). Cardiomyocyte GRK2 and GRK5, which are important regulators of β-ARs, are both upregulated in experimental and human heart failure (Dzimiri et al., 2004; Freedman et al., 1995; Vinge et al., 2001). Substantial evidence supports a pathophysiologic role of increased cardiomyocyte GRK2 activity in heart failure (Harding et al., 2001; Koch et al., 1995; Rockman et al., 1998; Raake et al., 2008). Despite the fact that GRK5 is well recognized to catalyse phosphorylation and

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desensitization of β-ARs on cardiomyocytes, the function of GRK5 in the heart is poorly understood (Freedman et al., 1995). Current evidence suggests that increased GRK5 activity in the failing heart may confer both protective and dysfunctional effects (Gold et al., 2012; Liggett et al., 2008). Contrary to GRK2, GRK5 has been shown to play a preeminent role in phosphorylation of β-ARs coding for β-arrestin binding to the receptor and generation of β-arrestin scaffolds for a wide range of signaling molecules, suggesting distinct functions of GRK5 in heart failure (Shenoy et al., 2006). Although GRKs may be putative targets for novel treatment of heart failure, their intracellular localization may pose potential difficulties in terms of pharmacologic intervention (Iaccarino and Koch, 2003). However, little knowledge is available on factors that contribute to regulation of myocardial GRK levels, in particular for GRK5, in heart failure (Penela et al., 2006). Thus, identification of such factors, which may provide strategies for indirect modulation of GRK activities in heart failure, warrants particular focus.

Connective tissue growth factor (CCN2/CTGF) is a 38 kDa cystein-rich protein and member of the CCN family (acronym for Cyr61, CTGF/Fisp-12, Nov). These matricellular proteins are secreted, heparin-binding and extracellular matrix (ECM) associated proteins involved in multiple cellular events including ECM production, cell adhesion, cell proliferation, or in some cell types apoptosis. CTGF is dramatically induced in heart failure in proportion with the severity of the disease (Ahmed et al., 2004; Chen et al., 2000; Ohnishi et al., 1998). Recent data from our laboratory demonstrate that transgenic mice with cardiac-restricted overexpression of CTGF (Tg-CTGF) display remarkable increase of tolerance towards ischemia/reperfusion injury (Ahmed et al.,

2011). Furthermore, Tg-CTGF mice subjected to chronic pressure overload by constriction of the abdominal aorta, a condition associated with robust neurohumoral activation, displayed attenuated left ventricular dilatation and preserved cardiac function compared with non-transgenic littermate control (NLC) mice (Gravning et al., 2013). Interestingly, analyses of both the myocardial and the cardiomyocyte transcriptomes of Tg-CTGF mice revealed upregulation of several genes and gene programs with reported cardioprotective properties (Ahmed et al., 2011; Moe et al., 2013). GRK5 mRNA levels were also among the more substantially upregulated transcripts in cardiac myocytes from Tg-CTGF mice. Thus, the principal aim of the present study was to investigate the functional consequences of CTGF-induced regulation of GRK5 activity in cardiomyocytes. Tg-CTGF and NLC mice, as well as primary cultures of adult cardiomyocytes stimulated in the presence or absence of recombinant human CTGF (rec-hCTGF), were used as principal tools to study the role of this matricellular protein in regulation of β-AR function.

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MATERIALS AND METHODS

All animal studies were performed in accordance with the current NIH Guide for the Care and Use of Laboratory Animals (2011) and were approved by institutional and national boards for laboratory animal research.

Reagents

Rec-hCTGF was purified from the cell culture medium of stably transfected HEK293 cells by EMP Genetech, Inc. (Ingolstadt, Germany), essentially as previously described (Ahmed et al., 2011).

Transgenic CTGF mice and GRK5-KO mice

Transgenic mice of C57BL/6 background, with cardiac-restricted overexpression of rat CTGF cDNA under control of the mouse α-myosin heavy chain (α-MHC) promoter, were used and are previously described (Ahmed et al., 2011). Homozygous GRK5 knockout mice (GRK5- KO/Grk5^{tm1Rjl}) were obtained from The Jackson Laboratory, Inc. (Maine, USA). The GRK5-KO was originally established in the laboratory of Professor Robert J. Lefkowitz (Duke University, Durham, NC) and was backcrossed to C57BL/6 for 12 generations before donation to The Jackson Laboratory (Gainetdinov et al., 1999). GRK5$^{+/+}$ littermates having both GRK5 alleles intact served as controls.

Isolation of cardiomyocytes and maintenance of primary cultures

Cardiomyocytes from Tg-CTGF, GRK5-KO and NLC hearts (male mice, 4-6 months of age) were isolated by enzymatic digestion following by retrograde perfusion with

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collagenase type II (Worthington Biochemical Corp., USA) and maintained in primary culture, essentially as previously described (O'Connell et al., 2007). Cardiomyocytes were pretreated or treated with recombinant human CTGF (200 nM) as indicated in the figure legends.

Assay of β-AR stimulated cAMP levels in isolated cardiomyocytes

For analysis of receptor-stimulated cAMP generation, cardiomyocytes from Tg-CTGF and NLC hearts or cardiomyocytes pretreated in the absence or presence of rec-hCTGF (200 nM; 48 hrs) were stimulated with increasing concentrations of (-)-isoproterenol in the presence of 3-isobutyl-1-methylxanthine (IBMX, phosphodiesterase inhibitor, Sigma-Aldrich; 0.1 mM). β_1- and β_2-adrenergic receptor subtype-specific efficacies were investigated by increasing concentrations of (-)-isoproterenol in the presence of the β_2-adrenergic receptor-selective antagonist ICI118551 (1- [2,3-dihydro-7-methyl-1*H*-inden-4-yl]oxy-3-[(1-methylethyl)amino]-2-butanol) (0.1 μM) or the β_1-arenergic receptor-selective antagonist CGP20712A (2-hydroxy-5- [2- [[2-hydroxy-3-[4-[1-metyl-4-(trifluorometyl)-1H-imidazol-2-yl]phenoxy]propyl]amino]ethoxy]-benzamine) (0.3 μM), respectively. In order to investigate the putative contribution from G_i-mediated inhibition of adenylyl cyclase in CTGF-engendered regulation of β-AR responsiveness, cells were pretreated in the absence or presence of 250ng/ml pertussis-toxin (PTX) (Alexis Biochemicals) for 14-16 hours, before stimulation with isoproterenol. To determine the efficacy of PTX treatment, control experiments with PTX treatment were performed in parallel wells with cardiomyocytes stimulated with isoproterenol in the absence or presence of carbachol (10 μM). Total cellular cAMP was measured by a

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radioimmunoassay ([^{125}I]-cAMP Flashplate assay, PerkinElmer Life and Analytical Sciences, Inc.) according to the manufacturer's instructions.

Analysis of contractility of isolated myocardial strips

Six months old, male Tg-CTGF and NLC mice were anesthetized using sodium pentobarbital (10 mg i.p.) and euthanized by excision of the hearts (n=6 in each group). The aorta was cannulated and the heart was subjected to retrograde perfusion with relaxing buffer (118.3 mM NaCl, 3.0 mM KCl, 0.5 mM $CaCl_2$, 4.0 mM $MgSO_4$, 2.4 mM KH_2PO_4, 24.9 mM $NaHCO_3$, 10.0 mM glucose, 2.2 mM mannitol) containing 20 mM 2,3-butanedione monoxime (BDM) and equilibrated with 95 % O_2/ 5% CO_2 to pH 7.4 at 31 ºC. Left ventricular muscle strips were ligated at each end, carefully excised and mounted in organ baths, and allowed to adapt for 20 min before BDM was washed out, Ca^{2+} was gradually increased to 1.8 mM, and Mg^{2+} lowered to 1.2 mM. The muscle strips were field-stimulated with alternating polarity at 1 Hz with impulses of 5 msec duration and current about 20 % above individual threshold (10-15 mA, determined in each experiment). The isometrically contracting muscles were stretched to the maximum of their length-tension curve. The force was recorded and analyzed as previously described (Andersen et al., 2004). After equilibration in the presence of prazosin (0.1 μM), isoproterenol was added directly to the organ baths at increasing concentrations until supramaximal concentration of agonist was obtained with respect to the inotropic response. Signal-averaged contraction-relaxation cycles were calculated for the different experimental periods and used to determine the inotropic response (expressed as percent increase of $(dF/dt)_{max}$ above basal) as a function of increasing concentrations of

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isoproterenol. For stimulation of ventricular muscle fiber contractility with the plasma membrane permeable cAMP analog dibutyryl-cAMP, a supramaximal concentration (10 mM) was employed based on previous experiments (Skomedal et al., 1981).

Assay of receptor-stimulated phosphoprotein activities in cardiomyocytes

Six hours before assay of receptor-generated responses, the cell culture medium was replaced with Minimum Essential Medium (MEM) containing the supplements as indicated previously, except insulin, transferrin and sodium selenite. The cells were subsequently stimulated with isoproterenol (15 min), in the presence of 0.1 mM IBMX. The assays were stopped at the indicated time points and harvested in sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 4% sodium dodecylsulphate (SDS), 1 mM sodium orthovanadate, 5 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride) for Western blot analysis.

Isolation of RNA, determination of myocardial gene expression levels and Western blot analysis

Cardiac tissues were snap-frozen in liquid nitrogen immediately after excision of the hearts. Isolation of total RNA and real-time quantitative PCR analysis was performed as previously described (Ahmed et al., 2011). Preparations of cell and tissue extracts for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis were performed as previously described (Ahmed et al., 2011). After electroblotting of the SDS-PAGE gels, the PVDF-membranes were blocked and incubated with anti-phospho-ERK1/2-specific IgG (anti-phosphothreonine-202/phosphotyrosine-204 ERK1/2, Cell Signaling Technology Inc.), anti-phosphoserine-16 phospholamban IgG (Upstate

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Biotechnology), anti-ERK1/2 IgG (Cell Signaling Technologies, Inc.) or anti-phospholamban IgG (Upstate Biotechnology) according to the manufacturer's instructions.

Analysis of GRK5 contents in cardiomyocytes from Tg-CTGF and NLC mice

Investigation of cardiomyocyte contents of GRK5 in NLC and Tg-CTGF mice was performed by immunoprecipitation of GRK5 from cardiomyocytes isolated from NLC and Tg-CTGF mice. The isolated cardiomyocytes were solubilised in RIPA-buffer (0.15M NaCl, 10mM Tris-HCl pH 7.3, 0.5% NP-40, 5mM EDTA, 0.2mM phenylmethanesulfonyl fluoride, 1μg/ml aprotinin, 1μg/ml pepstatin and 1μg/ml leupeptin), incubated for 30 min. at 4^{o}C and centrifuged for 10 min at 16000xg. GRK5 was immunoprecipitated from clarified cell lysate (1 mg total cellular protein) with 8 μg of anti-GRK 4-6 clone A16/17 (Upstate Biotechnology, Inc.) overnight at 4^{o}C. The immunocomplexes were captured with Protein A agarose after 2 h at 4^{o}C. Immune complexes were washed 2 times with PBS and subsequently resuspended in 2x loading buffer and subjected to SDS-PAGE and subsequent immunoblot analysis with anti-GRK5 IgG (H-64:sc-11396, Santa Cruz Biotechnology). Immunoreactivities were visualized by secondary HRP-conjugated anti-rabbit IgG and the LumiGLO Peroxidase Chemiluminescent Substrate Kit (KPL, Inc. USA). Positive control of GRK expression was extract from Sf9 cells infected with baculovirus encoding rat GRK5 (data not shown).

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Assay of β-arrestin binding to the β_2-adrenergic receptor

Assay of β-arrestin binding to the β_1-AR or β_2-AR was performed in genetically engineered CHO cells expressing complementary fragments of Lac Z fused to β-arrestin and β_1-AR or β_2-AR, respectively, as described by the manufacturer (PathHunter β-Arrestin Assay; DiscoveRx, Corp.). The cells were maintained in the presence of 5-bromo-2´-deoxyuridine to inhibit cell proliferation and treated in the presence or absence of rec-hCTGF (200 nM) for 48 hrs before ligand-stimulated assay of β-arrestin binding was performed as described by the manufacturer.

Cardiac responses to continuous infusion of isoproterenol

Six months old, male Tg-CTGF and NLC mice were randomized to continuous subcutaneous infusion of isoproterenol bitartrate (Sigma-Aldrich; 50 mg/kg/day) or vehicle for 14 days (n=9 mice in each treatment group) delivered by subcutaneously implanted micro-osmotic pumps (Alzet®, CA). Transthoracic echocardiography was performed before and 14 days after implantation of the micro-osmotic pumps.

Echocardiography

Transthoracic echocardiography was performed using the Vivid 7 System (GE Vingmed Ultrasound, Horten, Norway) and a 13 MHz linear array transducer as previously described (Ahmed et al., 2011).

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Radioligand binding assay of β-adrenergic receptor densities in myocardial membranes

The density of β-AR was measured in membrane particles prepared from homogenates of cardiac tissue from NLC and Tg-CTGF mice by a 96-well format radioligand binding assay using 0.03–0.07 nM [^{125}I]-(−)iodocyanopindolol (PerkinElmer) with or without 10 μM (-)propranolol in a binding buffer containing 50 mM Tris–HCl (pH 7.5 at RT), 1 mM EDTA, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM ascorbate, 0.1% BSA and 100 mM GTP. The plates were incubated at 21^{o}C for 90 min and harvested onto UniFilter-96 GF/C (Packard Instrument Co., Meriden, CT). Non-specific binding was defined as binding that was not inhibited by excess (-)propranolol. The total number of specific binding sites (B_{max}) was estimated by the equation $B_{max}=y(K_d+x)/x$, where y is the specific binding of [^{125}I]-(−)iodocyanopindolol, K_d the equilibrium dissociation constant (0.047 nM in LNCaP cells) and x is the concentration of free [^{125}I]-(−)iodocyanopindolol.

Morphometric analysis of histochemically stained tissue sections

Myocardial tissue sections were stained with Masson´s trichrome solution to assess myocardial fibrosis and morphology of hearts from NLC and Tg-CTGF mice after chronic isoproterenol infusion, as previously described (Ahmed et al., 2011).

Analysis of myocardial collagen content

Quantitative analysis of tissue contents of hydroxyproline was performed by HPLC using the AccQ-Flour reagent kit (Waters Corporation, Milford, MA, USA) as previously

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described (Ahmed et al., 2011). The relation of myocardial hydroxyproline contents to myocardial collagen has previously been reported (Laurent et al., 1981).

Statistical analysis

Data are presented as mean±SEM. Comparison between groups were made by two-tailed unpaired Student's *t* test. For multiple comparisons, 2-way ANOVA was performed. *P* values <0.05 were considered as statistically significant.

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RESULTS

CTGF attenuates β-adrenergic receptor responsiveness in cardiomyocytes

Concentration-response curves of isoproterenol-stimulated cAMP generation revealed substantial reduction of efficacy of isoproterenol in cardiomyocytes from Tg-CTGF mice versus those from NLC mice (Fig. 1A). Similar desensitization of β-ARs was recapitulated in cardiomyocytes from wild-type mice (C57BL/6) pretreated in the presence of rec-hCTGF (200 nM) for 48 hours before investigating isoproterenol-stimulated generation of cAMP (Fig. 1B). The concentration-response curves displayed attenuation of receptor-generated cAMP levels in myocytes pretreated with rec-hCTGF, demonstrating that cardiomyocytes are a direct target for this effect of CTGF on β-adrenergic responsiveness.

CTGF confers desensitization of both β_1 and β_2 adrenergic receptors

In order to investigate whether CTGF attenuates both β_1- and β_2-adrenergic receptor responsiveness or confers subtype-selective desensitization, cardiomyocytes isolated from Tg-CTGF mice and NLC mice were stimulated with increasing concentrations of isoproterenol in the presence of the β_2-adrenoceptor-selective antagonist ICI118551 (0.1 μM) (Fig. 1C) and the β_1-adrenoceptor-selective antagonist CGP20712A (0.3 μM), respectively (Fig. 1D). The concentration-effect curves demonstrate attenuation of both β_1 and β_2 receptor-generated cAMP levels in cardiomyocytes from Tg-CTGF.

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Functional consequences of attenuated β-adrenergic receptor responsiveness in Tg-CTGF mice

In order to investigate to what extent the blunted response to isoproterenol in cardiomyocytes from Tg-CTGF mice would be reflected in attenuated isoproterenol-stimulated contractility, concentration-response curves of isoproterenol-stimulated inotropic responses in isolated left ventricular muscle strips from Tg-CTGF mice versus NLC mice were performed. Consistent with the reduced efficacy of isoproterenol in β-AR-stimulated cAMP synthesis, the isolated muscle strips from Tg-CTGF mice also revealed blunted inotropic responses to isoproterenol (Fig. 1E). These blunted responses were not due to altered downstream responsiveness to cAMP, since muscle strips stimulated with supramaximal concentrations of dibutyryl-cAMP (10 mM), a membrane permeable cAMP analog, elicited similar inotropic response in muscle strips from both Tg-CTGF and NLC hearts (Fig. 1F).

Effects of CTGF on isoproterenol-stimulated phosphorylation of phospholamban

Consistent with desensitization of cardiac β-ARs in Tg-CTGF mice, as measured by impaired isoproterenol-stimulated cAMP generation, phosphorylation of phospholamban was attenuated in cardiomyocytes from Tg-CTGF mice upon isoproterenol stimulation, as compared to cardiomyocytes from NLC mice (Fig. 1G).

CTGF stimulates myocardial gene expression and tissue levels of GRK5

DNA microarray analysis of the myocardial transcriptome of Tg-CTGF mice versus non-transgenic littermate control (NLC) mice revealed robust upregulation of GRK5 mRNA

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levels in Tg-CTGF mice (Ahmed et al., 2011). Upregulation of GRK5 mRNA levels were verified by real-time quantitative PCR of RNA isolated from cardiomyocytes of Tg-CTGF mice versus that of NLC mice (Fig. 2A). This upregulation was selective for GRK5, as the mRNA levels of other GRK isoforms expressed in cardiomyocytes (GRK2, GRK3 and GRK6) were unaltered in cardiomyocytes from Tg-CTGF mice versus NLC mice. CTGF-mediated upregulation of GRK5 was also confirmed by immunoblot analysis of protein extracts of cardiomyocytes from Tg-CTGF mice versus NLC mice (Fig. 2B). Consistently, primary cultures of cardiomyocytes from wild-type mice (C57BL/6) stimulated with rec-hCTGF (200 nM) for 48 hours also displayed a robust upregulation of GRK5 mRNA levels (Fig. 2C).

Lack of GRK5 in cardiomyocytes prevents CTGF-engendered reduction of β-AR responsiveness to agonist

In order to corroborate the evidence that the blunted responses to isoproterenol in cardiomyocytes from Tg-CTGF mice were due to GRK5-catalyzed desensitization of β-ARs, we investigated β-AR responsiveness to isoproterenol in cardiomyocytes from GRK5-KO mice and from NLC mice pretreated in the absence or presence of rec-hCTGF (200 nM) for 48 hours. In cardiomyocytes from NLC mice, pretreatment with rec-hCTGF led to reduced β-AR responsiveness to subsequent stimulation with isoproterenol. In contrast, in cardiomyocytes from GRK5-KO mice, the elevated β-AR responsiveness was not reduced following pretreatment with rec-hCTGF (Fig. 2D).

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CTGF-induced GRK5 enhances agonist- and biased ligand-induced β-arrestin binding to βARs

Genetically engineered CHO cells expressing complementary fragments of Lac Z fused to the β_1- or β_2-adrenergic receptor and β-arrestin, respectively, were pretreated in the presence or absence of rec-hCTGF (200 nM; 48 hours) and subsequently stimulated with increasing concentrations of isoproterenol or carvedilol. Cells pretreated with rec-hCTGF displayed enhanced isoproterenol-stimulated binding of β-arrestin to both the β_1-AR and the β_2-AR compared to that of non-treated cells, as detected by complementation of Lac Z activity and generation of luminescent product (Fig. 3A-B). However, isoproterenol-stimulated binding of β-arrestin to the β_2-AR appeared to be enhanced more than that of β-arrestin binding to the β_1-AR subtype following pretreatment with rec-hCTGF. As shown in Fig. 3 C-D, this difference of binding of β-arrestin to the β-AR subtypes was even more prominent for the biased ligand carvedilol suggesting that CTGF-induced GRK5 enhances phosphorylation of the β_2-AR at sites that selectively enhances G protein-independent signaling from this receptor subtype. Shown in Fig. 3E are CHO cells treated with rec-hCTGF (200 nM; 48 hours) confirming increased GRK5 contents compared with non-treated controls.

Effects of CTGF on phosphoprotein activities of G protein-independent β-AR signaling

As GRK5 recently has been ascribed a crucial role in β-arrestin-mediated, G protein-independent signaling, we investigated if this pathway was activated in Tg-CTGF mice. As shown in Fig. 3F, cardiomyocytes from Tg-CTGF mice displayed enhanced

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isoproterenol-stimulated ERK-phosphorylation. This may indicate increased GRK5-catalyzed, β-arrestin-dependent activation of ERK, a pathway reported to confer cytoprotection of cardiomyocytes.

G_i does not contribute to the attenuated β-adrenergic receptor responsiveness in cardiomyocytes from Tg-CTGF mice

Additional experiments were performed in order to confirm that the reduced efficacy of isoproterenol in cardiomyocytes from Tg-CTGF mice was due to GRK5-catalyzed desensitization of β-ARs. First, cardiomyocytes from Tg-CTGF and NLC mice were pretreated with pertussis-toxin in order to investigate a putative contribution from G_i-mediated inhibition of adenylyl cyclase. Although pertussis toxin-mediated inactivation of G_i affected the efficacy of isoproterenol-stimulated cAMP synthesis, in particular in myocytes from NLC mice, pertussis toxin-sensitive G_i-activity did not explain the reduced efficacy of isoproterenol in cardiomyocytes from Tg-CTGF mice, i.e. pertussis toxin-mediated inactivation of G_i did not resensitize the responsiveness to isoproterenol (Fig. 4A). The effectiveness of the pertussis-toxin treatment was confirmed by the capacity of pertussis-toxin to abrogate the inhibitory effects of carbachol, a muscarinic cholinergic receptor agonist, on isoproterenol-stimulated cAMP generation in cardiomyocytes (Fig. 4B).

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Increased myocardial GRK5 protects from chronic isoproterenol-induced cardiotoxicity

Tg-CTGF mice and corresponding NLC mice were subjected to chronic exposure to isoproterenol (50 mg/kg/day) or vehicle (phosphate buffered saline) for 14 days, administered subcutaneously by micro-osmotic pumps as detailed in the Materials and Methods section. Radioligand binding studies of myocardial membranes from mice infused with vehicle revealed slightly higher β-AR densities in myocardial membranes from Tg-CTGF mice than in those from NLC mice (Fig. 5B). However, chronic administration of isoproterenol led to downregulation of myocardial β-ARs in both Tg-CTGF mice and non-transgenic control mice to an extent eliminating this difference (Fig. 5B). Thus, as compared with myocardial β-AR densities in mice infused with vehicle, downregulation of myocardial β-AR densities appeared to be greater in Tg-CTGF mice than in NLC mice consistent with increased myocardial GRK5 in Tg-CTGF mice promoting enhanced desensitization of β-ARs and subsequent downregulation of the receptor. Interestingly, isoproterenol elicited a significant increase of cardiac mass in NLC mice, whereas the hypertrophic response in Tg-CTGF mice was blunted (Fig. 5A). Chronic administration of isoproterenol also led to left ventricular dilatation and impaired systolic function in non-transgenic control mice, whereas left-ventricular dimensions and systolic function were preserved in Tg-CTGF mice (Fig. 5 C and D). Myocardial collagen contents increased significantly in both NLC mice and Tg-CTGF mice upon chronic exposure to isoproterenol (Fig. 5E). However, the relative fold increase of myocardial collagen content was significantly attenuated in Tg-CTGF as compared to NLC mice (1.68±0.1 vs. 1.95 ± 0.10, $P<0.05$).

Molecular Pharmacology Fast Forward. Published on June 18, 2013 as DOI: 10.1124/mol.113.087312
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DISCUSSION

In the present study we demonstrate that CTGF stimulates isoform-selective upregulation of GRK5 expression in cardiomyocytes. The increased GRK5 levels in cardiomyocytes were associated with desensitization of both β_1-AR and β_2-AR subtypes and attenuation of second messenger generation in response to stimulation with isoproterenol. This CTGF-engendered desensitization of β-AR responsiveness was abolished in cardiomyocytes from GRK5-KO mice. In congruence with these findings, isolated myocardial muscle strips from Tg-CTGF mice displayed reduced inotropic response to isoproterenol. On the other hand, dibutyryl-cAMP-stimulated contractility of myocardial fibers from Tg-CTGF vs. NLC mice revealed that the effector responses downstream of cAMP were unaltered. CTGF-stimulated increase of GRK5 levels also enhanced agonist- and biased ligand (carvedilol)-stimulated β-arrestin binding to β-ARs. The latter finding not only corroborates the evidence of receptor desensitization, but also demonstrates that the CTGF-stimulated increase of GRK5 may enhance β_2-AR-stimulated G protein-independent signaling. The cardiotoxic effects of chronic exposure to isoproterenol stimulation were blunted in Tg-CTGF mice. Thus, this study uncovers CTGF as a novel regulator of GRK5 levels in cardiomyocytes controlling the sensitivity and signaling specificity of β-ARs and ultimately attenuating the cardiotoxic effects of chronic exposure to β-adrenergic agonists.

GRK activities may be regulated both at the transcriptional and posttranslational levels. Although incidental reports on phosphorylation of GRKs exist, transcriptional regulation of GRK expression is the more thoroughly documented (Pitcher et al., 1998) The

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induction of cardiomyocyte GRK2 and GRK5 mRNA and protein levels in heart failure is well documented, both in animal models and in humans (Dzimiri et al., 2004; Freedman et al., 1995; Ungerer et al., 1993; Vinge et al., 2001). The expression of GRK2 is shown to increase upon β-AR stimulation and consistently to decrease in the presence of β-blockade, indicating that GRK2 levels are controlled by the sympathetic nervous system (Iaccarino et al., 1998). Although increase of myocardial GRK5 levels upon chronic adrenergic stimulation has also been reported, the mechanisms of GRK5 regulation are still poorly characterized (Oyama et al., 2005). However, a recent report demonstrated that GRK5 levels in cardiomyocytes are regulated by NF-κB transcriptional activity through binding of NF-κB to the promoter of GRK5 (Islam and Koch, 2012). NF-κB transcriptional activities are known to be activated by the PI3K/Akt signaling pathway (Lu et al., 2008), which is an important pathway for CTGF-mediated effects on cardiomyocytes, as recently reported from our laboratory (Ahmed et al., 2011; Lu et al., 2008).

An interesting difference between regulation of GRK2 and GRK5 activities deserves consideration. Translocation of GRK2 to the cytoplasmic surface of the plasma membrane is regulated by the PH-domain of GRK2 which binds $G_{\beta\gamma}$ and phosphatidyl-inositolbisphosphate residing in the plasma membrane (Penela et al., 2006). Translocation of GRK2 to the membrane by binding of $G_{\beta\gamma}$ makes translocation dependent on the activity of the G protein and subunit dissociation of G_{α} and $G_{\beta\gamma}$ subunits. GRK5, on the other hand, does not contain a carboxyl-terminal PH domain and

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its activities do not appear to be regulated by $G_{\beta\gamma}$. Thus, increased GRK5 activity may to a larger extent promote G protein-independent β-arrestin signaling as indicated in the present study.

The magnitude of increased myocardial GRK5 levels in Tg-CTGF mice is comparable with the induction of myocardial GRK5 in heart failure (2-3 fold elevation) (Vinge et al., 2001). Thus, the functional consequences of increased myocardial GRK5 in Tg-CTGF mice may be congruent with the consequences of increased myocardial GRK5 in heart failure. Furthermore, the recently reported single nucleotide polymorphism of the human GRK5 gene also resulted in a GRK5 isoform with enhanced GRK5 activity of similar magnitude as reported in this study (Liggett et al., 2008). Thus, the reduced mortality of chronic heart failure patients with the allele resulting in the more active GRK5 isoform demonstrates that even modest alterations of GRK5 activity may be of significant clinical importance. However, analysis of the myocardial transcriptome of Tg-CTGF mice versus NLC mice revealed upregulation of several genes and functional gene programs that may confer cardioprotection both towards ischemia/reperfusion injury and towards chronic pressure overload such as, for example, genes involved in scavenging of free oxygen radicals, anti-apoptosis, and unfolded protein response (Ahmed et al., 2011). Hence, the quantitative contribution to cardioprotection played by increased myocardial GRK5 levels in the Tg-CTGF mice has yet to be determined. Despite this limitation, GRK5 has several functions that conceivably could confer cardioprotective properties. Induction of GRK5 with subsequent phosphorylation and desensitization of cardiac β-ARs and AT_1 angiotensin receptor sensitivities might be one of the key mechanisms underlying these

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beneficial effects. Indeed, both β-ARs and AT_1 receptors are established targets in treatment of chronic heart failure (β-blockers and AT_1-receptor antagonists). Furthermore, recent findings show that GRK5 is among the GRK isoforms not only involved in desensitization of G protein-coupled receptors and uncoupling of G protein signaling, but may also catalyze β-arrestin-dependent, G protein-independent signaling (Noma et al., 2007). Indeed, GRK5 and GRK6 have been shown to selectively catalyze phosphorylation of serine residues of the β_2-adrenergic receptor promoting β-arrestin-mediated G protein-independent signaling. Certain β-blockers in clinical use (e.g. carvedilol) may act as biased ligands, blocking β-AR activation of G protein signaling, yet at the same time eliciting β-arrestin-dependent stimulation of other signaling pathways, like for example the ERK1/2 cascade through the scaffolding actions of β-arrestin (Kim et al., 2008; Wisler et al., 2007). ERK1/2 is reported to confer cardioprotective actions in the heart both in ischemia/reperfusion injury and in chronic heart failure (Adderley and Fitzgerald, 1999; Lips et al., 2004; Yue et al., 2000). G protein-independent activation of ERK1/2 is dependent on β-arrestin and GRK5, but is not affected by increased GRK2 (Shenoy et al., 2006). Thus, the increased GRK5 activities in cardiomyocytes pretreated with CTGF are a tantalizing finding, suggesting that the increased myocardial levels of CTGF associated with heart failure may enhance G protein-independent signaling through the β_2-adrenergic receptor. Indeed, the increased isoproterenol-stimulated ERK1/2 activities in cardiac myocytes from Tg-CTGF mice in the context of desensitization of β-ARs and reduced isoproterenol-stimulated cAMP generation, as shown in this study, indicates a shift towards enhanced G protein-

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independent signaling in cardiac myocytes from Tg-CTGF mice versus cardiac myocytes from NLC mice.

Several substrates other than G protein-coupled receptors have recently also been identified for GRK5 that could also convey cardioprotective properties. First, GRK5 has been shown to phosphorylate low-density lipoprotein receptor-related protein-6 of the Wnt pathway leading to increased Wnt-signaling and accumulation of downstream β-catenin (Chen et al., 2009). Secondly, p53 was recently identified as a novel substrate of GRK5 whereby GRK5-dependent phosphorylation of p53 at Thr-55 promotes ubiquitination and down-regulation of p53 and impaired p53-dependent apoptosis (Chen et al.). However, other functions attributed to GRK5 are not consistent with merely cardioprotective actions of GRK5 in the heart. For example, GRK5 contains a nuclear transport sequence suggesting nuclear functions of GRK5 independent of G protein-coupled receptor activation (Johnson et al., 2004). In this respect, GRK5 was shown to accumulate in the nucleus of cardiomyocytes during chronic pressure overload and act as a class II histone deacetylase kinase regulating gene transcription linked to myocardial hypertrophy (Martini et al., 2008). Indeed, cardiac-specific deletion of GRK5 was recently shown to protect from pathologic hypertrophy and heart failure following chronic aortic constriction in mice (Gold et al., 2012). The latter potentially maladaptive functions of GRK5 are in contrast to the reduced mortality among heart failure patients with single nucleotide polymorphisms of the GRK5 gene encoding a more active GRK5 variant, suggesting that increased GRK5 activities provide overall cardioprotective actions (Liggett et al., 2008). Yet, the latter findings were not replicated in a recent study,

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leaving the significance of the Gln41Leu polymorphisms unsettled (Kurnik et al., 2009). Thus, the precise role of GRK5 in the pathophysiology of heart failure remains to be resolved.

In conclusion, CTGF attenuates second messenger responses of the heart to β-AR agonists by mechanisms involving induction of cardiomyocyte GRK5 levels. By similar mechanisms CTGF also increases β-arrestin binding to β-ARs on cardiomyocytes, in particular β-arrestin binding to the β_2-AR subtype, enhancing G protein-independent signaling. This novel paracrine regulation of GRK5 activity in cardiomyocytes may contribute to the cardioprotective actions of CTGF and maintenance of cardiac function in heart failure.

Authorship Contributions

Participated in research design: Gravning, Ahmed, Edvardsen, Valen, Osnes, Skomedal and Attramadal.

Conducted experiments: Gravning, Ahmed, Qvigstad, Krobert, Moe, Hagelin and Sagave.

Performed data analysis: Gravning, Ahmed, Qvigstad, Krobert, Moe, Hagelin, Levy and Attramadal.

Wrote or contributed to writing of the manuscript: Gravning, Ahmed, Edvardsen, Krobert, Valen, Levy, Osnes, Skomedal and Attramadal.

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Molecular Pharmacology Fast Forward. Published on June 18, 2013 as DOI: 10.1124/mol.113.087312
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FOOTNOTES

This study was supported by grants from the South-Eastern Norway Regional Health Authority [Grants, 2009086, 2011006], The Norwegian Council on Cardiovascular Disease [Grant 6484], and The Norwegian Research Council [Grant 170463/V40].

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FIGURE LEGENDS

Figure 1. A-B. Semi-logarithmic plots demonstrating concentration-response curves of isoproterenol-stimulated cAMP generation in A) cardiomyocytes from Tg-CTGF mice (—■—) and NLC mice (--▲--) and in B) cardiomyocytes pretreated for 48 hours in the absence (--▲--) or presence (—■—) of recombinant human CTGF (200 nM). The data are mean ± SEM of three independent observations each assayed as triplicates. IBMX (0.1 mM) was added to the cell culture medium 10 min prior to stimulation. C-D. Semi-logarithmic plot demonstrating concentration-response curves of selective β_1-AR stimulated and selective β_2-AR stimulated cAMP generation in cardiomyocytes from Tg-CTGF mice (—■—) and NLC mice (--▲--). The cells were stimulated with increasing concentrations of isoproterenol in the presence of the β_2-adrenergic receptor-selective antagonist ICI118551 (C; selective β_1-AR stimulation) or increasing concentrations of isoproterenol in the presence of the β_1-adrenergic receptor-selective antagonist CGP20712A (D; selective β_2-AR stimulation). The data are mean ± SEM of three independent observations each assayed as triplicates. IBMX (0.1 mM) was added to the cell culture medium 10 min prior to stimulation E. Semi-logarithmic plot demonstrating concentration-response curve of isoproterenol-stimulated contractility of isolated left ventricular muscle strips from Tg-CTGF (—■—) and NLC mice (--▲--). Inotropic responses are expressed as increase in $(dF/dt)_{max}$ as percent above basal $(dF/dt)_{max}$ (non-stimulated). The data are mean ± SEM of independent observations from Tg-CTGF and NLC mice (n=6 of each). F. Maximal inotropic responses in isolated ventricular muscle strips from Tg-CTGF and NLC mice (n=6 of each) stimulated with a supramaximal

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concentration of dibutyryl-cAMP (db-cAMP, 10 mM). Data are presented as % increase of $(dF/dt)_{max}$ above the basal level prior to stimulation with db-cAMP (mean ± SEM). G. Photomicrograph of Western blot demonstrating isoproterenol-stimulated serine-16 phosphorylation of phospholamban (1 μM isoproterenol; 15 min stimulation). Data are as mean ± SEM (N =3 in each group). *P<0.05 vs. corresponding NLC group, $^{\dagger}P$<0.05 vs. corresponding basal control.

Figure 2. A. Real-time quantitative PCR of mRNA levels of GRK isoforms (GRK2, GRK3, GRK5, and GRK6) in adult cardiomyocytes from Tg-CTGF (n=6) and NLC mice (n=6), presented relative to 18S RNA levels in the cardiomyocyte samples. B. Immunoblot of GRK5 immunoprecipitated from extracts of cardiomyocytes from Tg-CTGF mice (n=3) and non-transgenic control mice (n=3) confirming upregulation of GRK5 in Tg-CTGF mice. The bar graph shows the optical densities of immunoreactive GRK5 on the immunoblot. C. Bar graph demonstrating GRK5 mRNA levels in isolated cardiomyocytes treated for 48 hours with or without recombinant human CTGF (200 nM). Results are expressed as mean ± SEM (N=3 in each group). D. Concentration-response curves of isoproterenol-stimulated cAMP generation in cardiomyocytes from GRK5-KO and NLC mice following pretreatment of the cardiomyocytes in the absence or presence of rec-hCTGF (200 nM) for 48 hours (--▲-- NLC, non-stimulated; —■— NLC + rec-hCTGF; --Δ-- GRK5-KO, non-stimulated; —□— GRK5-KO + rec-hCTGF). The data are mean±SEM of three independent experiments each assayed as triplicates. *P<0.05 vs. corresponding NLC group.

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Figure 3. Agonist (isoproterenol)- and biased ligand (carvedilol)- stimulated binding of β-arrestin to human β_1-AR or β_2-AR in CHO cells pretreated in the presence (—■—) or absence (--▲--) of recombinant human CTGF (200 nM) for 48 hours. Figure demonstrates semi-logarithmic plot of concentration–response curves of isoproterenol- (A-B) and carvedilol-stimulated (C-D) β-arrestin binding using the Lac Z complementation assay (complementary fragments of Lac Z fused to the β_1-AR or β_2-AR and βarrestin, respectively, in genetically engineered CHO cells) from of DiscoveRx Corp. (CA, USA). The data are mean ±SEM of three independent experiments each assayed as triplicates. E. Immunoblot of GRK5 immunoprecipitated from extracts of CHO cells confirming upregulation of GRK5 after stimulation with recombinant human CTGF (200 nM) for 48 hours. The bar graph shows the densitometric analysis of the immunoreactive GRK5 bands on the immunoblot. F. Western blot analysis of ERK phosphorylation (phospho-ERK [Thr202/Tyr 204]) in cardiomyocytes from Tg-CTGF versus NLC mice upon stimulation with isoproterenol (1 μM; 10 min exposure). Data are as mean ± SEM (N =3 in each group). **P*<0.05 vs. corresponding NLC group, †*P*<0.05 vs. corresponding basal control.

Figure 4. A. Concentration-response curves of isoproterenol-stimulated cAMP generation in cardiomyocytes from Tg-CTGF and NLC mice following pretreatment in the presence or absence of 250 ng/mL pertussis-toxin (PTX) overnight (—□— NLC PTX, —■— NLC, --Δ-- Tg-CTGF PTX, --▲-- Tg-CTGF). IBMX (0.1 mM) was added to the cell culture medium 10 min prior to stimulation with isoproterenol (1 μM). Data are mean±SEM of cAMP levels in cardiomyocytes from triplicate wells from Tg-CTGF

Molecular Pharmacology Fast Forward. Published on June 18, 2013 as DOI: 10.1124/mol.113.087312
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mice and non-transgenic control mice for each given concentration. B. The effectiveness of PTX-engendered inactivation of G_i was confirmed by assay of carbachol (10μM) inhibition of isoproterenol-stimulated cAMP generation following pretreatment of cardiomyocytes in the presence or absence of PTX. **P*<0.05 vs. corresponding control group

Figure 5. β-AR-induced cardiomyopathy in Tg-CTGF mice versus NLC mice, subjected to continuous treatment with isoproterenol (50 mg/kg/day s.c. via microosmotic pumps) for 14 days. Similar groups of Tg-CTGF and NLC mice that received saline were included as controls. A. Cardiac mass at end-point was measured immediately following termination of the treatment protocol. Cardiac mass is presented as heart weight (HW) relative to tibia length (TL). B. β-AR densities in membranes from myocardial tissue sampled immediately after termination of treatment protocol. β-AR densities were determined by radioligand binding assay, using [^{125}I]-iodocyanopindolol as detailed in Material and Methods. C and D. Left ventricular end-diastolic diameter (LVIDd) and fractional shortening (FS) determined by transthoracic echocardiography at study end-point. E. Representative photomicrographs of myocardial sections stained with Masson´s trichrome stain. Sections are from NLC and Tg-CTGF mice after 14 days of isoproterenol exposure. Histogram demonstrates myocardial collagen contents in vehicle- versus isoproterenol-treated NLC and Tg-CTGF mice determined by quantitative analysis of myocardial hydroxyproline contents. All data are presented as mean ± SEM of n=9 in each group. **P*<0.05 vs. corresponding NLC group, †*P*<0.05 vs. corresponding basal control.

Fig. 1

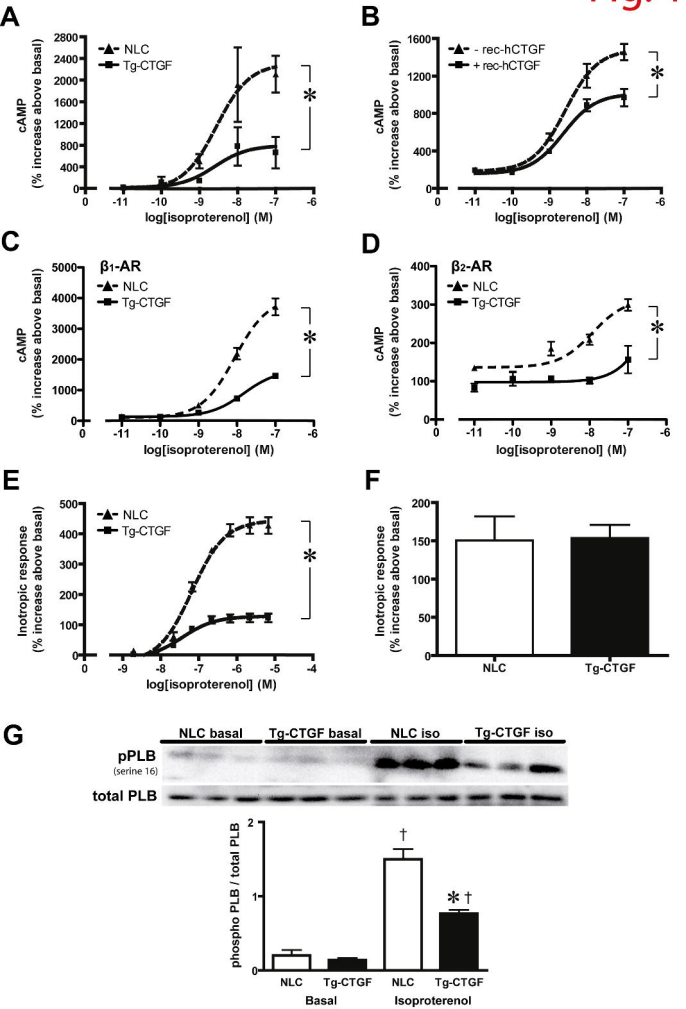

A
NLC
Tg-CTGF
cAMP
(% increase above basal)
log[isoproterenol] (M)
B
- rec-hCTGF
+ rec-hCTGF
cAMP
(% increase above basal)
log[isoproterenol] (M)
C
β1-AR
NLC
Tg-CTGF
cAMP
(% increase above basal)
log[isoproterenol] (M)
D
β2-AR
NLC
Tg-CTGF
cAMP
(% increase above basal)
log[isoproterenol] (M)
E
NLC
Tg-CTGF
Inotropic response
(% increase above basal)
log[isoproterenol] (M)
F
Inotropic response
(% increase above basal)
NLC
Tg-CTGF
G
NLC basal
Tg-CTGF basal
NLC iso
Tg-CTGF iso
pPLB
(serine 16)
total PLB
phospho PLB / total PLB
NLC
Tg-CTGF
NLC
Tg-CTGF
Basal
Isoproterenol

Fig. 2

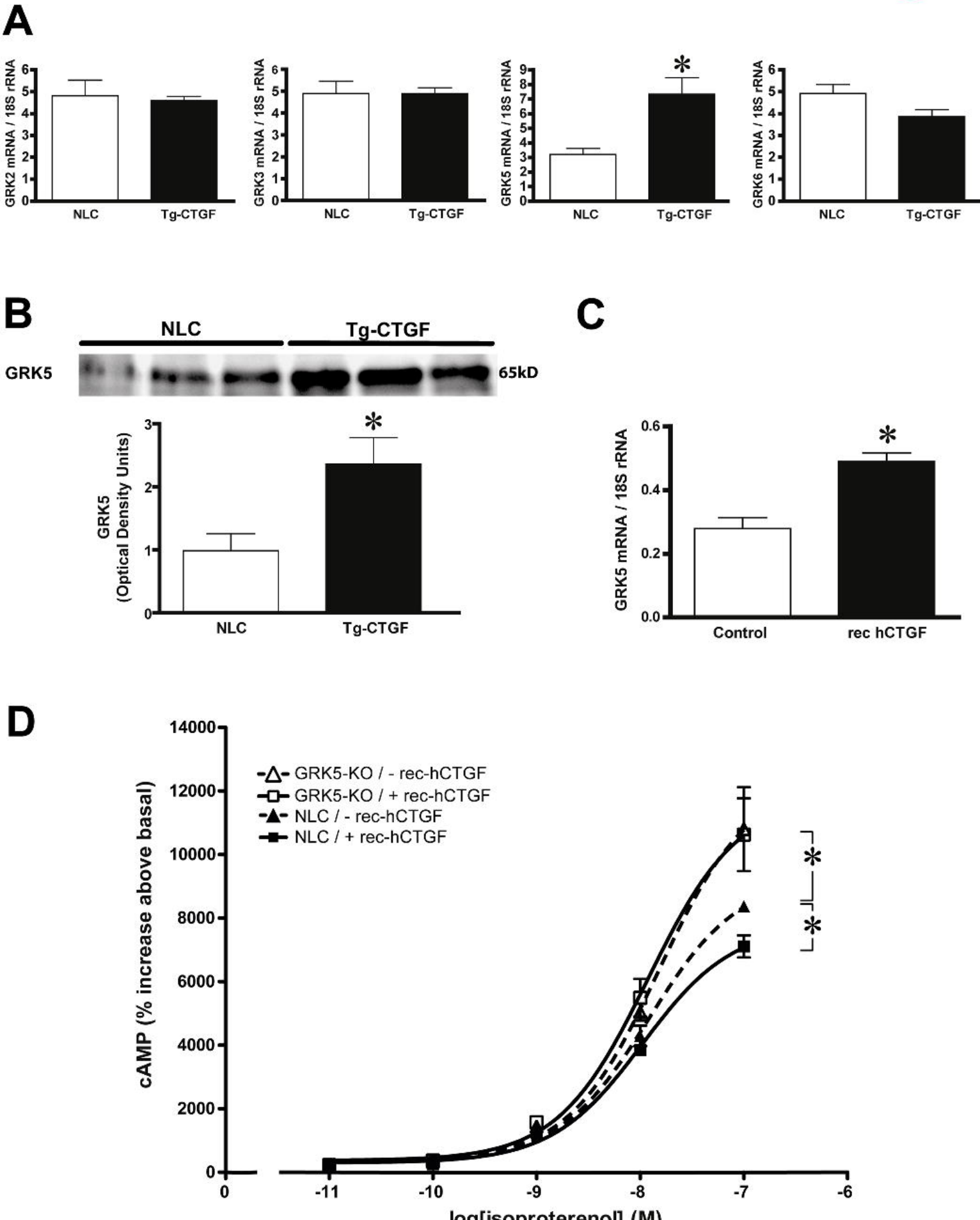

A
GRK2 mRNA / 18S rRNA
GRK3 mRNA / 18S rRNA
GRK5 mRNA / 18S rRNA
GRK6 mRNA / 18S rRNA
NLC
Tg-CTGF
B
NLC
Tg-CTGF
GRK5
65kD
GRK5 (Optical Density Units)
C
GRK5 mRNA / 18S rRNA
Control
rec hCTGF
D
GRK5-KO / - rec-hCTGF
GRK5-KO / + rec-hCTGF
NLC / - rec-hCTGF
NLC / + rec-hCTGF
cAMP (% increase above basal)
log[isoproterenol] (M)

Fig. 3

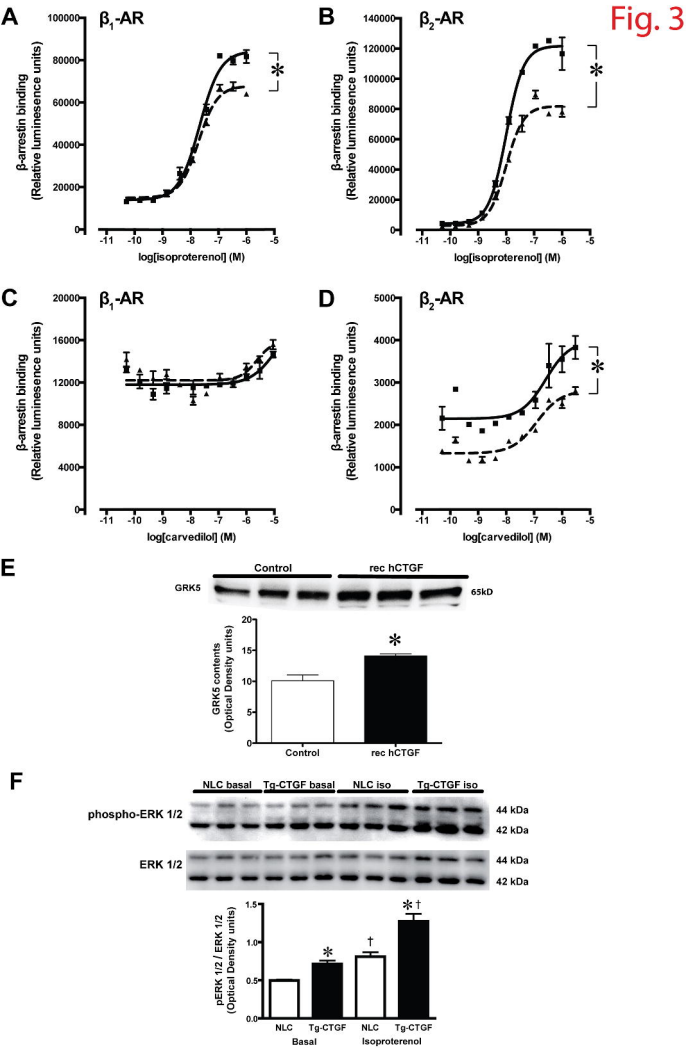
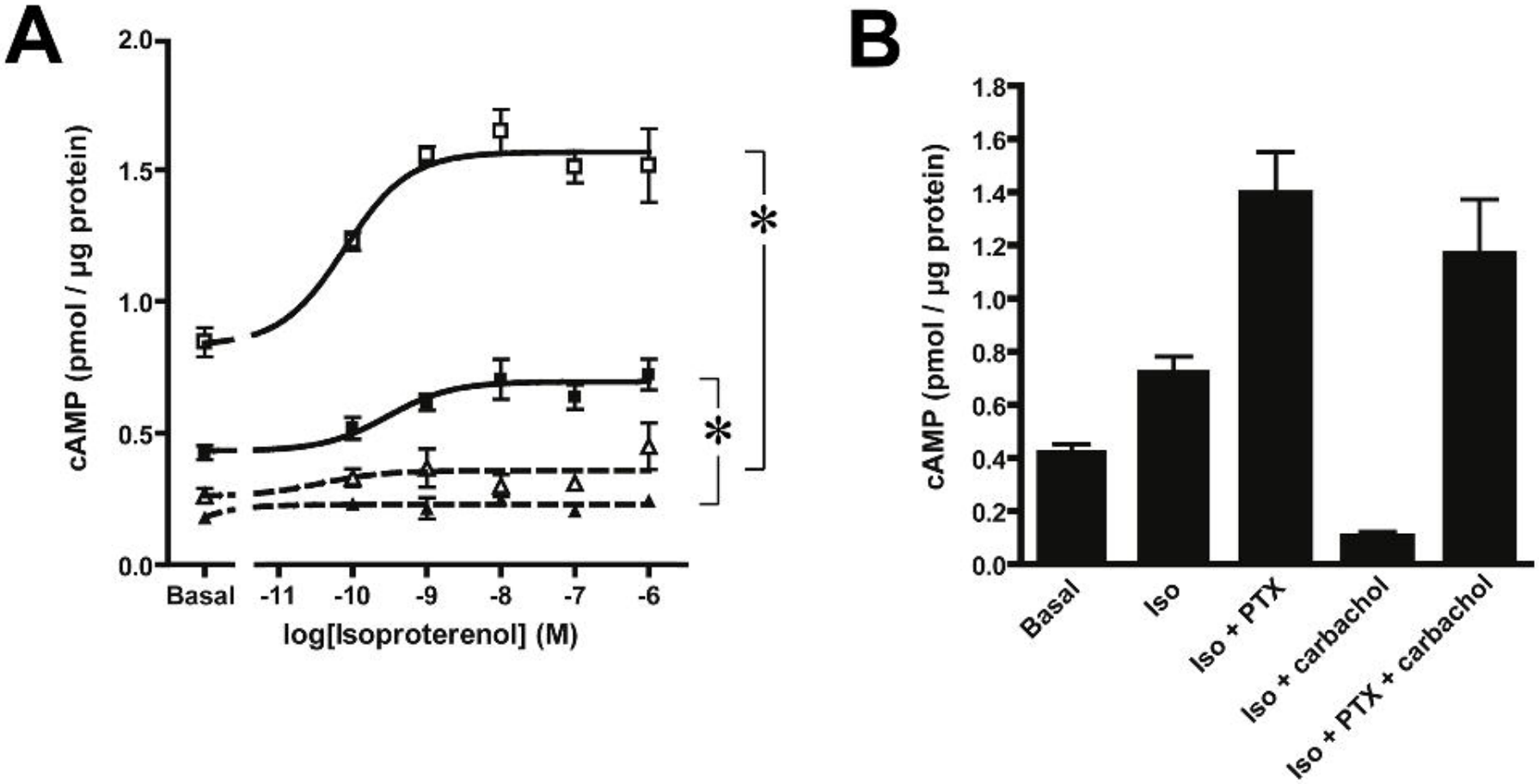
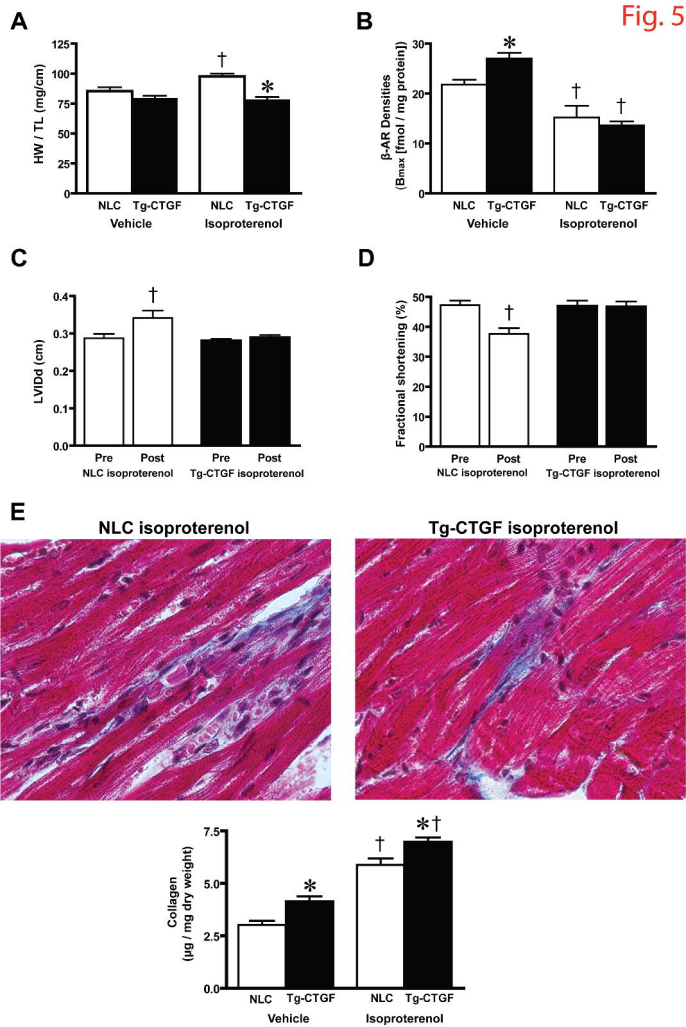

A
β1-AR
β-arrestin binding (Relative luminescence units)
log[isoproterenol] (M)
B
β2-AR
β-arrestin binding (Relative luminescence units)
log[isoproterenol] (M)
C
β1-AR
β-arrestin binding (Relative luminescence units)
log[carvedilol] (M)
D
β2-AR
β-arrestin binding (Relative luminescence units)
log[carvedilol] (M)
E
Control
rec hCTGF
GRK5
65kD
GRK5 contents (Optical Density units)
Control
rec hCTGF
F
NLC basal
Tg-CTGF basal
NLC iso
Tg-CTGF iso
phospho-ERK 1/2
44 kDa
42 kDa
ERK 1/2
44 kDa
42 kDa
pERK 1/2 / ERK 1/2 (Optical Density units)
NLC
Tg-CTGF
NLC
Tg-CTGF
Basal
Isoproterenol

Fig. 4


A
cAMP (pmol / µg protein)
2.0
1.5
1.0
0.5
0.0
Basal
-11
-10
-9
-8
-7
-6
log[Isoproterenol] (M)
*
*
B
cAMP (pmol / µg protein)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Basal
Iso
Iso + PTX
Iso + carbachol
Iso + PTX + carbachol


A
HW / TL (mg/cm)
125
100
75
50
25
0
†
*
NLC
Tg-CTGF
NLC
Tg-CTGF
Vehicle
Isoproterenol
B
β-AR Densities
(Bmax [fmol / mg protein])
30
20
10
0
*
†
†
NLC
Tg-CTGF
NLC
Tg-CTGF
Vehicle
Isoproterenol
C
LVIDd (cm)
0.4
0.3
0.2
0.1
0.0
†
Pre
Post
Pre
Post
NLC isoproterenol
Tg-CTGF isoproterenol
D
Fractional shortening (%)
50
40
30
20
10
0
†
Pre
Post
Pre
Post
NLC isoproterenol
Tg-CTGF isoproterenol
E
NLC isoproterenol
Tg-CTGF isoproterenol
Collagen
(µg / mg dry weight)
7.5
5.0
2.5
0.0
*
†
*†
NLC
Tg-CTGF
NLC
Tg-CTGF
Vehicle
Isoproterenol