Journal of Molecular and Cellular Cardiology 72 (2014) 39–51

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Original article

β-Adrenergic receptor-mediated transactivation of epidermal growth factor receptor decreases cardiomyocyte apoptosis through differential subcellular activation of ERK1/2 and Akt

Laurel A. Grisanti [a], Jennifer A. Talarico [c], Rhonda L. Carter [a], Justine E. Yu [a], Ashley A. Repas [a], Scott W. Radcliffe [c], Hoang-ai Tang [c], Catherine A. Makarewich [d], Steven R. Houser [d], Douglas G. Tilley [a,b,*]

[a] Center for Translational Medicine, Temple University School of Medicine, Philadelphia, PA 19140, USA
[b] Department of Pharmacology, Temple University School of Medicine, Philadelphia, PA 19140, USA
[c] Department of Pharmaceutical Sciences, Jefferson School of Pharmacy, Thomas Jefferson University, Philadelphia, PA 19107, USA
[d] Cardiovascular Research Center, Temple University School of Medicine, Philadelphia, PA 19140, USA

ARTICLE INFO

Article history:
Received 4 October 2013
Received in revised form 15 January 2014
Accepted 12 February 2014
Available online 22 February 2014

Keywords:
β-Adrenergic receptor
Cardiomyocyte
Epidermal growth factor receptor
Apoptosis

ABSTRACT

β-Adrenergic receptor (βAR)-mediated transactivation of epidermal growth factor receptor (EGFR) has been shown to relay pro-survival effects via unknown mechanisms. We hypothesized that acute βAR-mediated EGFR transactivation in the heart promotes differential subcellular activation of ERK1/2 and Akt, promoting cell survival through modulation of apoptosis. C57BL/6 mice underwent acute i.p. injection with isoproterenol (ISO) ± AG 1478 (EGFR antagonist) to assess the impact of βAR-mediated EGFR transactivation on the phosphorylation of ERK1/2 (P-ERK1/2) and Akt (P-Akt) in distinct cardiac subcellular fractions. Increased P-ERK1/2 and P-Akt were observed in cytosolic, plasma membrane and nuclear fractions following ISO stimulation. Whereas the P-ERK1/2 response was EGFR-sensitive in all fractions, the P-Akt response was EGFR-sensitive only in the plasma membrane and nucleus, results confirmed in primary rat neonatal cardiomyocytes (RNCM). βAR-mediated EGFR-transactivation also decreased apoptosis in serum-depleted RNCM, as measured via TUNEL as well as caspase 3 activity/cleavage, which were sensitive to the inhibition of either ERK1/2 (PD184352) or Akt (LY-294002) signaling. Caspase 3 activity/cleavage was also sensitive to the inhibition of transcription, which, with an increase in nuclear P-ERK1/2 and P-Akt in response to ISO, suggested that βAR-mediated EGFR transactivation may regulate apoptotic gene transcription. An Apoptosis PCR Array identified *tnfsf10* (TRAIL) to be altered by ISO in an EGFR-sensitive manner, results confirmed via RT-PCR and ELISA measurement of both membrane-bound and soluble cardiomyocyte TRAIL levels. βAR-mediated EGFR transactivation induces differential subcellular activation of ERK1/2 and Akt leading to increased cell survival through the modulation of caspase 3 activity and apoptotic gene expression in cardiomyocytes.

1. Introduction

β-Adrenergic receptors (βAR) are critical regulators of cardiac function both normally and pathologically during heart failure (HF) where increased catecholamine release leads to dysregulation of βAR signaling [1]. Chronic impairment of βAR signaling contributes to alterations in cardiac structure through increased apoptosis, hypertrophy and fibrosis and steadily decreased contractile function as HF progresses [2]. βAR are G protein-coupled receptors (GPCRs), stimulation of which promotes both Gs protein-dependent and -independent signaling effects via engagement of cAMP generation and GPCR kinase (GRK)/β-arrestin pathways, respectively. βAR primarily relay their effects through Gs protein-dependent activation of protein kinase A (PKA), which regulates a number of important processes, including cardiomyocyte contractility [1]. Additionally, it was recently demonstrated that chronic PKA-dependent βAR signaling is responsible for the cardiotoxic response to elevated catecholamines in a mouse model of HF [3]. Conversely, G protein-independent βAR signaling through β-arrestin-mediated epidermal growth factor receptor (EGFR) transactivation was demonstrated to increase pro-survival ERK1/2 activation and prevent apoptosis under conditions of chronic catecholamine stress [4]. While generalized β-blockade shields βAR from excess catecholamine

Abbreviations: ANOVA, analysis of variance; AR, adrenergic receptor; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; GPCR, G protein-coupled receptor; HF, heart failure; ISO, Isoproterenol; LDH, lactate dehydrogenase; RNCM, rat neonatal cardiomyocyte; RTK, receptor tyrosine kinase; SEM, standard error; TRAIL, TNF-related apoptosis inducing ligand; TUNEL, terminal deoxynucleotidyl transferase mediated dUTP nick end labeling.

* Corresponding author at: Department of Pharmacology and Center for Translational Medicine, Temple University School of Medicine, 945A MERB, 3500 N Broad St., Philadelphia, PA 19140, USA. Tel.: +1 215 707 9758; fax: +1 215 707 9890.
E-mail address: douglas.tilley@temple.edu (D.G. Tilley).

http://dx.doi.org/10.1016/j.yjmcc.2014.02.009
0022-2828/

and is associated with improvements in βAR responsiveness, cardiac function and HF patient mortality outcomes [5], these agents act to block both the maladaptive G protein-dependent and pro-survival G protein-independent signaling pathways. Selective engagement of pro-survival pathways, such as EGFR transactivation, in the absence of detrimental G protein-dependent pathways may provide a more refined approach to targeting βAR effects for treatment of HF [6]. Of note, a clinically used β-blocker, carvedilol, was found to be capable of inducing EGFR transactivation [7], though with a lower efficacy for activation of ERK1/2 signaling than a βAR agonist, suggesting that this paradigm could be therapeutically useful. Thus, we aim to elucidate the mechanisms by which βAR-dependent EGFR transactivation promotes survival signaling in the heart to provide further insight toward the rational development of therapeutics targeting this pathway.

βAR-dependent EGFR transactivation was associated with preserved cardiac function and reduced apoptosis in the aforementioned chronic catecholamine mouse model of HF, as well as in isolated cardiomyocytes [4,8]. While the proximal mechanism regulating βAR-mediated stimulation of EGFR has been defined [4], the distal events that actually relay EGFR-dependent pro-survival signaling in response to βAR stimulation remain to be determined. Direct stimulation of EGFR is known to enhance the activation of pro-survival pathways, such as those mediated by ERK1/2 and Akt, and impart anti-apoptotic effects in numerous cell lines [9–11]. Further, enhanced ERK1/2 and Akt activity is known to impart protection against ischemia–reperfusion [12], and several GPCRs have been shown capable of relaying ischemic preconditioning via EGFR transactivation [13–15]. βAR-mediated EGFR transactivation has also been shown to increase phosphorylated ERK1/2 (P-ERK1/2) and Akt (P-Akt) in both the heart and isolated cardiomyocytes, and has been shown to exert differential subcellular targeting of P-ERK1/2 in non-cardiac cells [8,16,17]. Neither the effect of βAR-mediated EGFR transactivation on subcellular activation of cardiac ERK1/2 and Akt nor the impact of such regulation on cardiomyocyte survival has been investigated. To begin to define the downstream mechanisms by which βAR-mediated EGFR transactivation promotes cardiac survival signaling, we tested the hypothesis that this pathway induces differential subcellular activation of ERK1/2 and Akt to modulate apoptosis. Through the investigation of βAR-mediated EGFR transactivation-dependent signaling in the whole heart and isolated cardiomyocytes, we demonstrate for the first time that this signaling paradigm indeed leads to differential subcellular activation of ERK1/2 and Akt, and is capable of promoting cardiomyocyte survival, in part, through the modulation of apoptotic gene expression.

2. Materials and methods

2.1. Mouse treatment

Wild-type, adult C57BL/6 mice were administered an intraperitoneal (IP) injection of EGFR antagonist (AG 1478, 10 mg/kg or gefitinib, 5 mg/kg; 10 min) or vehicle (0.1% DMSO) prior to IP injection of saline or ISO (1 mg/kg). Mice were euthanized 10 min or 1 h following ISO administration, hearts were excised and flash frozen in liquid nitrogen for use in biochemical assays. All animal procedures and experiments were carried out according to the National Institutes of Health Guidelines on the Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

2.2. Heart fractionation

Hearts were homogenized in lysis buffer (25 mM Tris pH 7.4, 5 mM EDTA, HALT protease inhibitor cocktail and phosphatase inhibitor cocktail) and incubated on ice for 15 min. Lysate was centrifuged at 3000 ×g for 5 min and the supernatant was spun at 20,000 ×g for 25 min. Supernatant was saved as the cytosolic fraction while the pellet was resuspended in RIPA and saved as the membrane fraction. The pellet from the first centrifugation was resuspended in hypotonic lysis buffer (10 mM HEPES–KOH pH 7.5, 10 mM KCl, 3 mM $MgCl_2$, 0.05% NP-40, 1 mM EDTA, HALT protease inhibitor cocktail and phosphatase inhibitor cocktail) and spun at 3000 ×g. After resuspending the pellet in hypotonic lysis buffer and centrifuging five times, the pellet was resuspended in 10 times pellet volume in Gel Shift Lysis Buffer (50 mM HEPES–KOH pH 7.9, 250 mM KCl, 0.1% NP-40, 0.1 mM EDTA pH 8, 0.1 mM EGTA pH 8, 10% glycerol, HALT protease inhibitor cocktail and phosphatase inhibitor cocktail) and incubated on ice for 30 min. Lysate was centrifuged at 13,200 ×g for 10 min and the supernatant was saved as the nuclear fraction.

2.3. Primary rat neonatal cardiomyocyte and fibroblast and adult feline ventricular myocyte isolation

Primary neonatal cell cultures were prepared from 1 to 2 days old Sprague Dawley rat pups (Harlan Laboratories; Indianapolis, IN) by enzymatic digestion. Hearts were excised and placed in a sterile ADS solution (116 mM NaCl, 20 mM HEPES, 80 μM Na_2HPO_4, 56 mM glucose, 5.4 mM KCl, 800 mM $MgSO_4$–$7H_2O$; pH 7.35). Blood and connective tissue were removed, ventricles were minced and subjected to five and 15 min enzymatic digestions using collagenase II (Worthington; Lakewood, NJ) and pancreatin. Rat neonatal cardiac fibroblasts (RNCF) and myocytes (RNCM) were separated via pre-plating for 2 h. Following isolation, RNCM were cultured overnight in F-10 media containing 10% horse serum, 5% fetal bovine system (FBS) and 1% PSF at 37 °C in a humidified incubator with 5% CO_2. The following day, media were replaced with F-10 media containing 5% FBS and 1% PSF. RNCF were cultured for 24 h in MEM containing 10% FBS and 1% PSF at 37 °C in a humidified incubator with 5% CO_2. After 24 h the media was replaced with 5% FBS-containing media.

Adult feline left ventricular myocytes were isolated as previously described [18,19]. Briefly, felines were anesthetized with sodium pentobarbital and hearts were rapidly excised, cannulated, and mounted on a constant flow Langendorff apparatus. Hearts were rinsed with a physiological Krebs–Henseleit buffer (KHB) and then retrogradely perfused with collagenase containing KHB. When the tissue softened, the left ventricle was isolated and gently minced, filtered, and equilibrated in KHB with 0.2 mmol/L $CaCl_2$, and 1% bovine serum albumin (BSA) at room temperature. Isolated myocytes were washed with serum-free culture medium (Medium 199, Sigma) supplemented with penicillin–streptomycin–glutamine (Gibco) and seeded in a clear-bottom black-walled 96-well plate (Greiner Bio-one) coated with laminin (BD Bioscience).

2.4. Cell lysis and fractionation

Following drug treatments, cells were rinsed with ice-cold PBS, collected and lysed in a buffer containing 20 mM Tris (pH 7.4), 137 mM NaCl, 10% glycerol, 1% NP-40, 10 mM NaF, 1 mM EDTA, HALT protease inhibitor cocktail and phosphatase inhibitor cocktail (Thermo Scientific; Rockford, IL). Protein quantities were estimated by Pierce 660 nM Protein Assay Reagent (Thermo Scientific). For fractionation, cells were rinsed with ice-cold PBS, collected and lysed using a Proteo Extract Subcellular Proteome Extraction Kit (Calbiochem; EMD Biosciences, La Jolla, CA). Collected cells were re-suspended in Extraction Buffer I and incubated for 10 min at 4 °C with gentle agitation. Cells were centrifuged at 1000 ×g for 10 min at 4 °C and the supernatant (cytosolic fraction) was collected. The remaining pellet was suspended in Extraction Buffer II and incubated at 4 °C with gentle agitation for 30 min. The sample was centrifuged at 6000 ×g for 10 min at 4 °C and the supernatant (membrane fraction) was collected. The pellet was re-suspended in Extraction Buffer III and incubated for 10 min at 4 °C with gentle agitation. The sample was centrifuged at 6800 ×g for 10 min at 4 °C. The supernatant (nuclear fraction) was collected.

2.5. Immunoblot analysis

Equal amounts of total cell lysates were resolved by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Odyssey Blocking Buffer (LI-COR Biosciences; Lincoln, NE) was used to prevent non-specific binding. Protein expression was measured by immunoblotting overnight at 4 °C with diluted antibodies against GAPDH (1:1000; Cell Signaling; Danvers, MA), E-catenin (1:1000; Invitrogen; Grand Island, NY), Lamin A/C (1:1000; Cell Signaling), phospho-CREB (1:1000; Cell Signaling), phospho-Akt Ser473 (1:1000; Cell Signaling), phsopho-Akt Thr308 (1:1000, Cell Signaling), total-Akt (1:1000; Cell Signaling), phospho-ERK1/2 (1:3000; Cell Signaling), total-ERK1/2 (1:5000; Cell Signaling) or cleaved caspase-3 (1:1000; Cell Signaling). After washing with TBS-T, membranes were incubated at room temperature for 60 min with the appropriate diluted secondary antibody (IRDye680 Donkey anti-rabbit IgG (H + L) at 1:20,000; IRDye800CW Goat anti-mouse IgG (H + L) at 1:10,000; LI-COR Biosciences). Bound antibody was detected using the LI-COR Biosciences Odyssey System (LI-COR Biosciences). For total tissue or cell lysate experiments, levels of P-ERK1/2 and P-Akt were normalized to T-ERK1/2 and T-Akt, respectively. Initial experiments in whole heart lysates measured the phosphorylation of Akt at both Ser473 and Thr308, but all subsequent experiments focused on Ser473. For all fractionation experiments, levels of phospho-ERK1/2, total-ERK1/2, phospho-Akt, total-Akt and phospho-CREB were normalized to loading controls appropriate to the subcellular fraction: GAPDH (cytosolic), E-catenin (plasma membrane) and Lamin A/C (nuclear). Cleaved caspase-3 levels were normalized to GAPDH.

2.6. Lactate dehydrogenase assay

RNCM cell death was measured using a lactate dehydrogenase (LDH) Colorimetric Assay Kit according to manufacturer's instructions (Abcam; Cambridge, MA). Release of LDH was determined in media from serum deprived RNCM treated with ISO 3 h in the presence or absence of AG 1478. Absorbance was measured by a spectrophotometer at 480 nm. LDH activity was interpolated from a standard curve and expressed as fold of control.

2.7. Caspase 3/7 activity assay

Caspase 3/7 activity was measured using a Caspase-Glo® 3/7 Assay according to the manufacturer's instructions (Promega; Madison, WI). In brief, RNCM were plated 20,000/well of a white walled 96-well plate. Following stabilization in culture and 24 h serum deprivation, cells were pretreated 10 min with antagonists prior to 3 h incubation with agonists. After treatment, 100 μL of Caspase-Glo® 3/7 Reagent was added to each well. Plates were incubated for 1 h prior to reading luminescence.

2.8. Cell viability assay

RNCM viability was assessed using a CellTiter 96® Non-Radioactive Cell Proliferation (MTT) Assay (Promega; Madison, WI). RNCM were plated 20,000/well in a 96-well plate. Following treatment, Dye Solution was added to the plate followed by the Solubilization/Stop Solution after a 3 h incubation at 37 °C. Absorbance was measure at 570 nm with a 750 nm reference wavelength.

2.9. TUNEL staining

An In Situ Cell Death Detection Kit, TMR Red (Roche Diagnostics; Mannheim, Germany) was used to measure RNCM apoptosis via terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL). RNCM were plated on coated glass coverslips and treated with 3 h with ISO with or without AG 1478. Cells were fixed with 4% paraformaldehyde pH 7.4 and permeabilized with 0.1% Triton-X. DNA strand breaks were labeled according to the manufacturer's instructions using tetra-methyl-rhodamine-dUTP and coverslips were mounted on glass slides using Prolong® Gold Antifade Reagent (Invitrogen). Cells were visualized at 20× magnification using a Nikon Eclipse microscope and the percentage of TUNEL-positive nuclei calculated in relation to the number of DAPI-stained nuclei.

2.10. Quantitative real-time PCR

mRNA was isolated from RNCM and RNCF using RNeasy Mini Kit (Qiagen) and from the whole heart using RNeasy Fibrous Tissue Mini Kit (Qiagen) following the manufacturer's protocol. cDNA was synthesized using a PCR array first strand-synthesis kit (SA Bioscience) and pooled from four individual experiments. RT-qPCR reactions were performed using RT^2 SYBR Green PCR Mastermix (SA Bioscience). A Rat Apoptosis RT^2 Profiler PCR Array (SA Bioscience; Frederick, MD) was used to measure changes in the expression of 84 genes involved in programmed cell death in RNCM according the manufacturer's instructions. RT-PCR was performed to validate and quantify changes in gene expression using total RNA which was isolated from individual RNCM, RNCF and whole heart mRNA. cDNA was synthesized using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems), and real-time PCR (RT-PCR) was performed with SYBR® Select Master Mix (Applied Biosystems). Real-time PCR was performed using primers listed in Supplemental Table 1 at an annealing temperature of 60.1 °C. Data from samples were analyzed in triplicate. All RT-PCR data were analyzed using the Applied Biosystems Comparative CT Method (ΔΔCT). Gene expression analysis was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.11. TRAIL enzyme-linked immunosorbent assay (ELISA)

Levels of membrane and soluble TRAIL were detected using a mouse TRAIL/TNFSF10 DuoSet ELISA kit (R&D Systems; Minneapolis, MN) according to manufacturer's instructions. Media from treated RNCM were concentrated 50× using Amicon® Ultra Centrifugal Filter Device (Millipore; Billerica, MA). Cells were lysed using RIPA buffer and an ELISA was performed according to the manufacturer's instructions. In brief, 96 well plates were coated overnight with Capture Antibody. Plates were blocked with Reagent Diluent and incubated with concentrated media or cellular lysate. Following incubations with the Detection Antibody and Streptavidin-HRP, a Substrate Solution was added and absorbance was measured at 450 nm with a 540 nm reference wavelength.

2.12. Statistical analysis

Data presented is expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by a Tukey's multiple comparisons test using Prism 5.0 software (GraphPad Software Inc.; San Diego, CA).

3. Results

3.1. βAR-mediated EGFR transactivation induces ERK1/2 and Akt activation in multiple subcellular compartments in the mouse heart in vivo

βAR-mediated EGFR transactivation has been shown to promote survival in a mouse model of HF [4]. The prosurvival kinases ERK1/2 and Akt are commonly activated following EGFR stimulation and could be responsible for mediating EGFR-dependent survival effects in response to βAR stimulation. To determine if both ERK1/2 and Akt signaling are regulated in the mouse heart via βAR-mediated EGFR transactivation, mice were given i.p. injections of the βAR agonist

isoproterenol in the presence or absence of the EGFR antagonist AG 1478, which shows high selectivity for the EGFR [20,21]. Immunoblot analysis of total heart lysates prepared from the animals showed that both ERK1/2 and Akt were significantly phosphorylated following ISO treatment in an AG 1478-senstive manner (Fig. 1A). Akt underwent ISO-mediated phosphorylation at both Ser473 (Fig. 1A) and Thr308 (Supplemental Fig. 1A), each of which was significantly inhibited by EGFR inhibition. Thus all subsequent Akt phosphorylation responses were assessed at Ser473. Together, these results confirm that βAR-mediated EGFR transactivation acutely promotes proximal survival signaling pathway activation in the heart in vivo.

Differences in the subcellular location of phosphorylated ERK1/2 and Akt (P-ERK1/2 and P-Akt) may be important in identifying the mechanisms of EGFR regulation and the outcomes of such signaling in the heart. To determine if βAR-mediated EGFR signaling leads to distinct patterns of ERK1/2 or Akt phosphorylation in different subcellular compartments, hearts from mice treated as described above were subjected to subcellular fractionation analysis. Cytosolic, plasma membrane and nuclear fractions were enriched and total and phosphorylated levels of ERK1/2 and Akt were assessed via immunoblotting. Both P-ERK1/2 and P-Akt (Fig. 1B) were significantly increased in cytosolic, membrane and nuclear fractions following ISO injection. AG 1478 pretreatment alone did not impact phosphorylation levels of either ERK1/2 or Akt, but significantly decreased the ISO-mediated P-ERK1/2 responses in all fractions. Interestingly, βAR-mediated Akt phosphorylation was sensitive to AG 1478 in the plasma membrane and nuclear fractions, but not the cytosolic fraction. To ensure that EGFR inhibition with AG 1478 did not block all βAR-mediated responses non-selectively, the nuclear fractions were analyzed for the phosphorylation of CREB, a transcription factor acutely regulated by canonical Gs-protein-dependent βAR signaling. CREB phosphorylation in response to ISO stimulation was not blocked by AG 1478 pretreatment (Supplemental Fig. 1B) demonstrating that EGFR-independent βAR signaling is not impaired by AG 1478. Of note, there were no significant differences in T-ERK1/2 or T-Akt levels in any of the fractions in response to the drug treatments. These results indicate differential phosphorylation of ERK1/2 and Akt occurs in specific subcellular compartments within the heart in response to βAR-mediated EGFR transactivation.

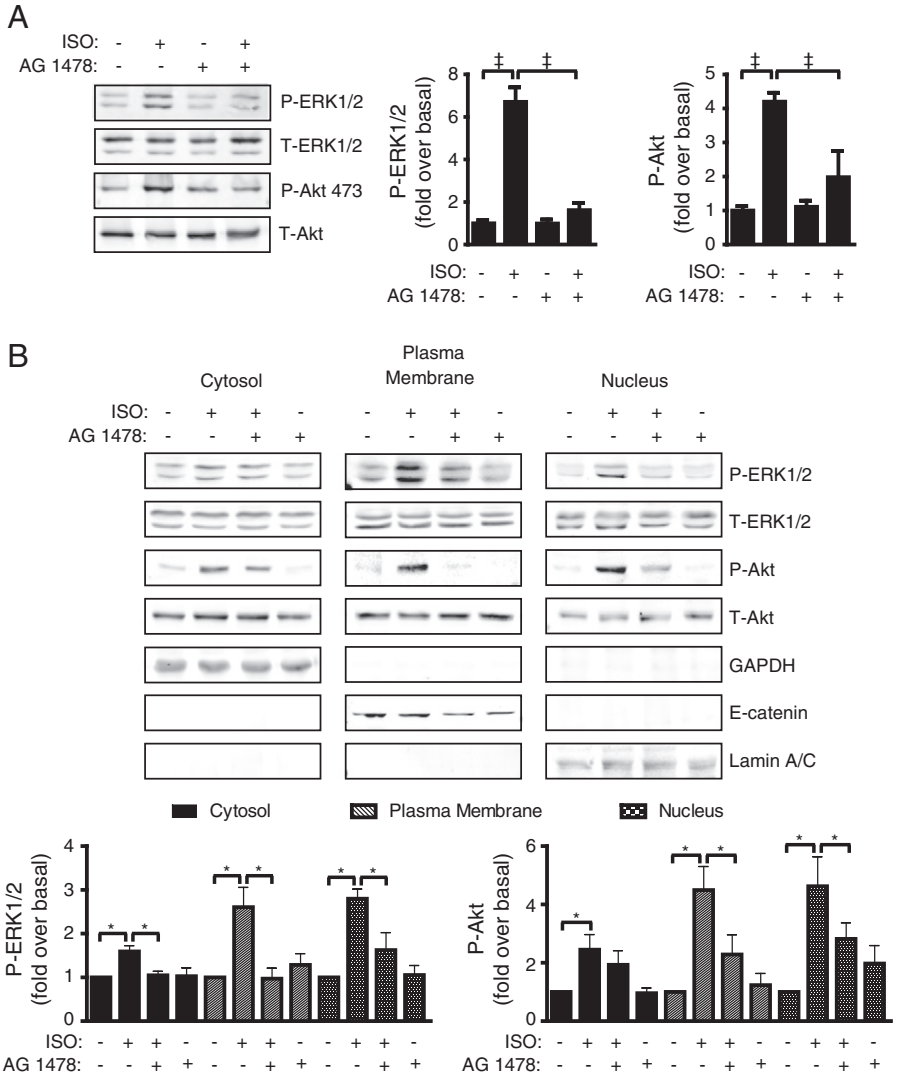

Fig. 1. βAR-mediated transactivation of EGFR activates ERK1/2 and Akt in the mouse heart. A, heart lysates from C57BL/6 mice injected with ISO (1 mg/kg) with or without AG 1478 (10 mg/kg) pretreatment were immunoblotted for P-ERK1/2, T-ERK1/2, P-Akt (Ser473) and T-Akt. ISO stimulation significantly increased P-ERK1/2 and P-Akt levels, which were significantly decreased by AG 1478 pretreatment. $n \geq 4$ independent experiments. ANOVA, $p < 0.001$. B, hearts from mice treated as in A underwent fractionation into cytosolic, plasma membrane and nuclear fractions with GAPDH, E-catenin and Lamin A/C serving as loading controls for each fraction, respectively. ISO induced significant increases in P-ERK1/2 and P-Akt levels in all three fractions. AG 1478 pretreatment blocked the P-ERK1/2 response in all fractions and the P-Akt response in the plasma membrane and nuclear fractions only. $n \geq 4$ independent experiments. ANOVA, $^*p < 0.05$.

3.2. βAR-mediated EGFR transactivation differentially impacts the subcellular activation and targeting of ERK1/2 and Akt in rat neonatal cardiomyocytes

In order to establish a model system to further characterize the mechanisms and impact of ERK1/2 and Akt signaling in response to ISO stimulation, we next performed a series of experiments using isolated RNCM. Initial experiments in total cell lysates revealed 10 μM ISO to be the most effective concentration to induce the greatest increase in both P-ERK1/2 and P-Akt levels (Supplemental Fig. 1C). Using this concentration of ISO, we next performed a timecourse analysis for P-ERK1/2 and P-Akt responses (Fig. 2A). Whereas ERK1/2 phosphorylation peaked early in these total RNCM lysates, Akt phosphorylation had a slower onset. To determine how these responses correspond to changes in P-ERK1/2 and P-Akt levels in the different subcellular compartments, RNCM treated in the same manner underwent fractionation (Fig. 2B). As was observed in the whole heart, P-ERK1/2 and P-Akt levels were significantly elevated in each of the cytosolic, plasma membrane and nuclear fractions. Additionally, at the 10 min timepoint, ISO treatment induced peak ERK1/2 and Akt phosphorylation responses in all fractions, thus this timepoint was used for subsequent experiments.

To next assess the sensitivity of ISO-mediated P-ERK1/2 and P-Akt responses to EGFR inhibition in RNCM, the cells were treated with ISO in the presence or absence of AG 1478. As observed in the whole heart, the ISO-induced P-ERK1/2 and P-Akt responses were blocked by pretreatment with AG 1478 (Fig. 3A). Importantly, AG 1478 pretreatment did not prevent the ability of ERK1/2 and Akt to respond to different stimuli, as treatment of RNCM with the insulin-like growth factor receptor ligand IGF increased ERK1/2 and Akt phosphorylation in the presence or absence of AG 1478 (Supplemental Fig. 1D). Similarly, receptor-independent direct activation of PKC, an upstream activator of the MEK1/2/ERK1/2 pathway, with phorbol myristate acetate (PMA) induced ERK1/2 phosphorylation that was insensitive to AG 1478. Since receptor internalization can play a role in relaying downstream signaling events, we also determined the impact of dynasore, an inhibitor of dynamin, on βAR-mediated EGFR transactivation. ISO-mediated phosphorylation of both ERK1/2 and Akt was abolished with dynasore pretreatment (Fig. 3B), suggesting that receptor internalization is an essential component of relaying βAR-mediated EGFR-dependent signaling in cardiomyocytes.

To determine the role of EGFR transactivation and internalization on the differential ERK1/2 and Akt phosphorylation responses in the subcellular compartments of RNCM, the cells were treated with ISO in the presence or absence of various pathway inhibitors prior to subcellular fractionation and immunoblot analysis (Figs. 4 and 5, full immunoblot examples are shown in Supplemental Fig. 2A). ISO-mediated phosphorylation of ERK1/2 was abrogated by EGFR inhibition with AG 1478 in each of the cytosolic, membrane and nuclear fractions. As observed in vivo, AG 1478 pretreatment blocked ISO-mediated Akt phosphorylation in the membrane and nuclear fractions, but had no effect on ISO-dependent increases in cytosolic P-Akt, suggestive of multiple mechanisms of βAR-dependent Akt activation in cardiomyocytes.

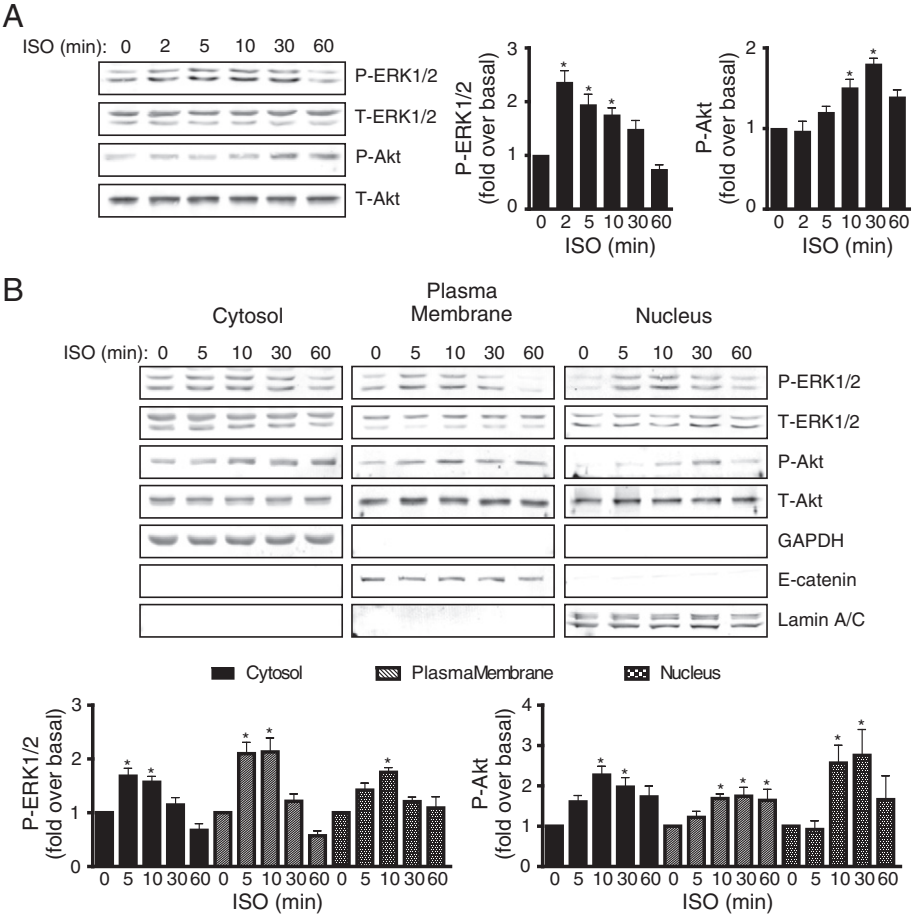

Fig. 2. Time-dependent activation of ERK1/2 and Akt by βAR stimulation. A, total cell lysates from RNCM stimulated 0–60 min with ISO (10 μM) were immunoblotted for P-ERK1/2, T-ERK1/2, P-Akt (Ser473) and T-Akt. ISO treatment significantly increased P-ERK1/2 levels following 2, 5 and 10 min treatment and P-Akt levels following 10 and 30 min treatment. $n \geq 4$ independent experiments. ANOVA, $^*p < 0.05$ versus 0 min. B, subcellular fractions from RNCM stimulated 0–60 min with ISO were immunoblotted as above. ISO treatment significantly increased P-ERK1/2 and P-Akt levels in each of the cytosolic, plasma membrane and nuclear fractions, with 10 min providing a common time of peak activation for all. $n \geq 3$ independent experiments. ANOVA, $^*p < 0.05$ versus 0 min control in corresponding fraction.

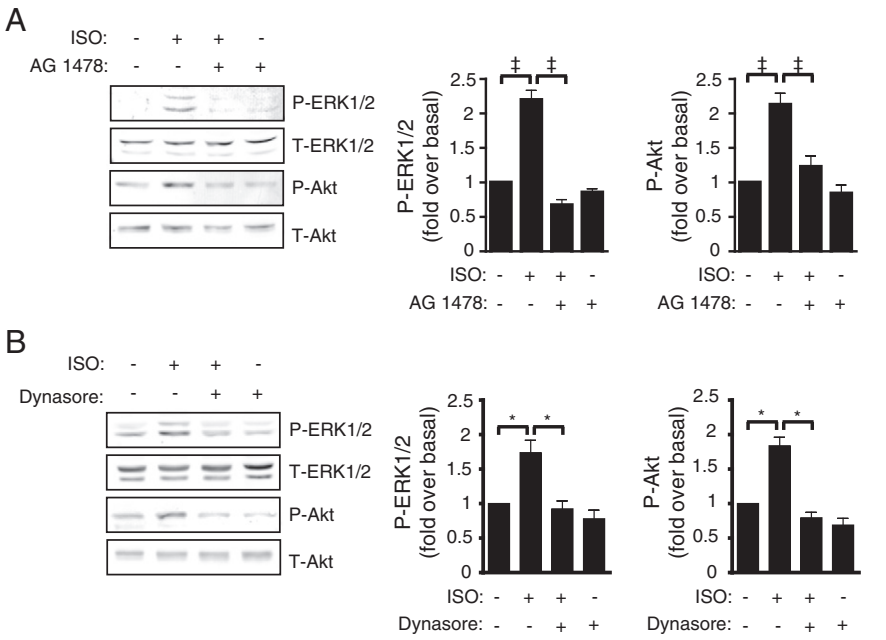

Fig. 3. βAR-mediated transactivation of EGFR activates ERK1/2 and Akt in RNCM. A, immunoblot analysis of total RNCM lysates show that ISO (10 μM, 10 min) significantly increased the phosphorylation of ERK1/2 and Akt (Ser473). AG 1478 pretreatment (10 μM, 10 min) completely blocked the P-ERK1/2 and P-Akt responses. n ≥ 6 independent experiments. ANOVA, $p < 0.001$. B, pretreatment of RNCM with dynasore (80 μM; 10 min) completely blocked the P-ERK1/2 and P-Akt responses to ISO treatment (10 μM; 10 min). n ≥ 4 independent experiments. ANOVA, $^{*}p < 0.05$. D, dynasore pretreatment (80 μM; 10 min) significantly reduced increases in P-Akt levels as a result of ISO treatment (10 μM; 10 min). No changes in P-Akt were observed with dynasore alone. n ≥ 4 independent experiments. ANOVA, $^{*}p < 0.05$.

To determine whether the ERK1/2 and Akt phosphorylation effects observed were due to classical upstream regulators, RNCM were treated with ISO in the presence or absence of the MEK1/2 inhibitor PD184352 or the PI3K inhibitor LY-294002, respectively. Pretreatment with PD184352 decreased ISO-mediated elevations in P-ERK1/2 in every fraction, whereas LY-294002 partially reduced ISO-mediated ERK1/2

Fig. 4. βAR-mediated EGFR transactivation directs differential subcellular localization of P-ERK1/2 and P-Akt in RNCM. RNCM were treated with ISO (10 μM; 10 min) with or without pretreatment with AG 1478 (1 μM; 10 min), PD184352 (10 μM; 10 min), LY-294002 (10 μM; 10 min) or dynasore (80 μM; 10 min) and underwent fractionation into cytosolic, plasma membrane and nuclear fractions. Immunoblot analysis demonstrates that the ISO-mediated increase in P-ERK1/2 is abrogated by AG 1478, PD184352 and dynasore in all fractions. The ISO-mediated increase in P-Akt (Ser473) is blocked by AG 1478 in the plasma membrane and nuclear fractions, and by LY-294002 and dynasore in all fractions. Non-phosphorylated (total) levels of ERK1/2 and Akt were not substantially altered by any of the drug treatments in any fraction, except that T-Akt was slightly reduced by the combination of ISO + AG 1478 in the plasma membrane fraction. Representative immunoblots of each condition are shown.

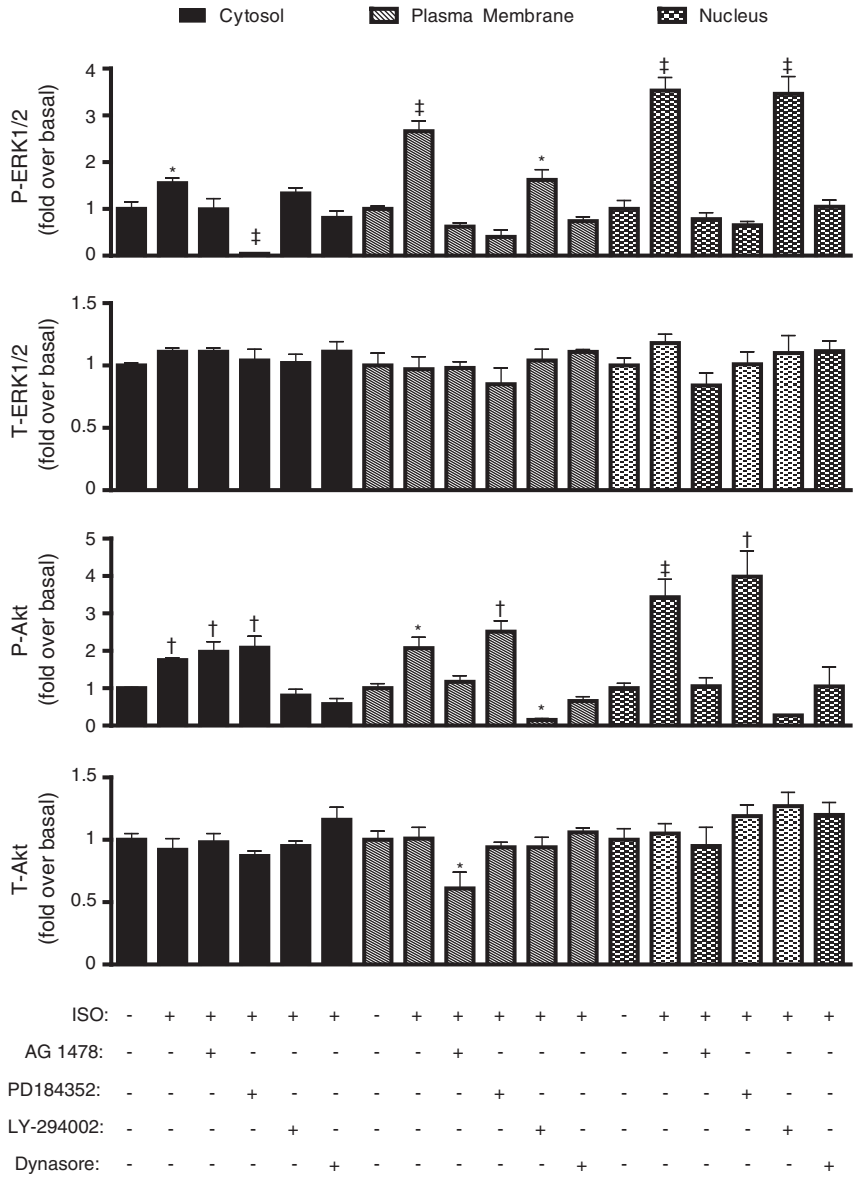

Fig. 5. Quantitation of the subcellular localization responses to βAR-mediated EGFR-transactivation. Histograms depict the quantitative results from Fig. 4 for P-ERK1/2, T-ERK1/2, P-Akt (Ser473) and T-Akt each normalized to the appropriate subcellular marker (GAPDH, E-catenin or Lamin A/C for cytosolic, plasma membrane and nuclear fractions, respectively). $n \geq 5$ independent experiments each. ANOVA, *$p < 0.05$, †$p < 0.01$, ‡$p < 0.001$ versus non-stimulated condition in corresponding fraction.

activation in the membrane fraction. However, elevations in nuclear and cytosolic P-ERK1/2 were unaffected by LY-294002 pretreatment. PD184352 pretreatment did not alter ISO-mediated elevations in P-Akt, while LY-294002 pretreatment abolished increases in the P-Akt response in all fractions. ISO-mediated phosphorylation of CREB was not altered by the inclusion of AG 1478, PD184352 or LY-294002 (Supplemental Fig. 2B). Of note, neither T-ERK1/2 nor T-Akt levels were altered between treatments in any of the fractions examined, with the exception that the combination of AG 1478 and ISO significantly decreased T-Akt levels at the membrane. Further, preventing receptor internalization with dynasore significantly attenuated ISO-mediated increases in both P-ERK1/2 and P-Akt in all fractions. Overall, these results demonstrate the importance of βAR-mediated EGFR transactivation on the regulation of ERK1/2 and Akt in different compartments in cardiomyocytes, confirm that the classical EGFR-MEK-ERK1/2 and EGFR-PI3K-Akt pathways are activated in these subcellular compartments in response to ISO stimulation and suggest that receptor internalization is a contributing component of the mechanisms leading to the activation of ERK1/2 and Akt throughout the cell.

3.3. βAR-mediated EGFR transactivation decreases apoptosis in RNCM

Since βAR-mediated EGFR transactivation has been shown to promote survival [4], and ERK1/2 and Akt are known regulators of apoptosis we next investigated the ability of βAR-mediated transactivation to modulate early apoptotic signaling events by assessing caspase activation. Serum deprivation is a well-characterized method for inducing apoptosis in various cell types including cardiomyocytes [22–26]. RNCM deprived of serum for 24 h showed an increase in caspase 3/7 activity (Fig. 6A) and cleavage (Fig. 6B) that was decreased by treatment with 10 μM ISO for 3 h. Concentration–response analysis demonstrated that under these conditions 10 μM ISO treatment produced a maximal decrease in caspase 3/7 activity that did not affect cellular viability (Supplemental Figs. 3A and B). To determine the contribution of βAR-mediated EGFR transactivation to this acute regulation of caspase activity, serum-deprived RNCM were treated with ISO in the presence or absence of AG 1478. The ISO-mediated decrease in caspase activity (Fig. 6C) and cleavage (Fig. 6D) was completely blocked by AG 1478 pretreatment. Importantly, we also observed under the same assay

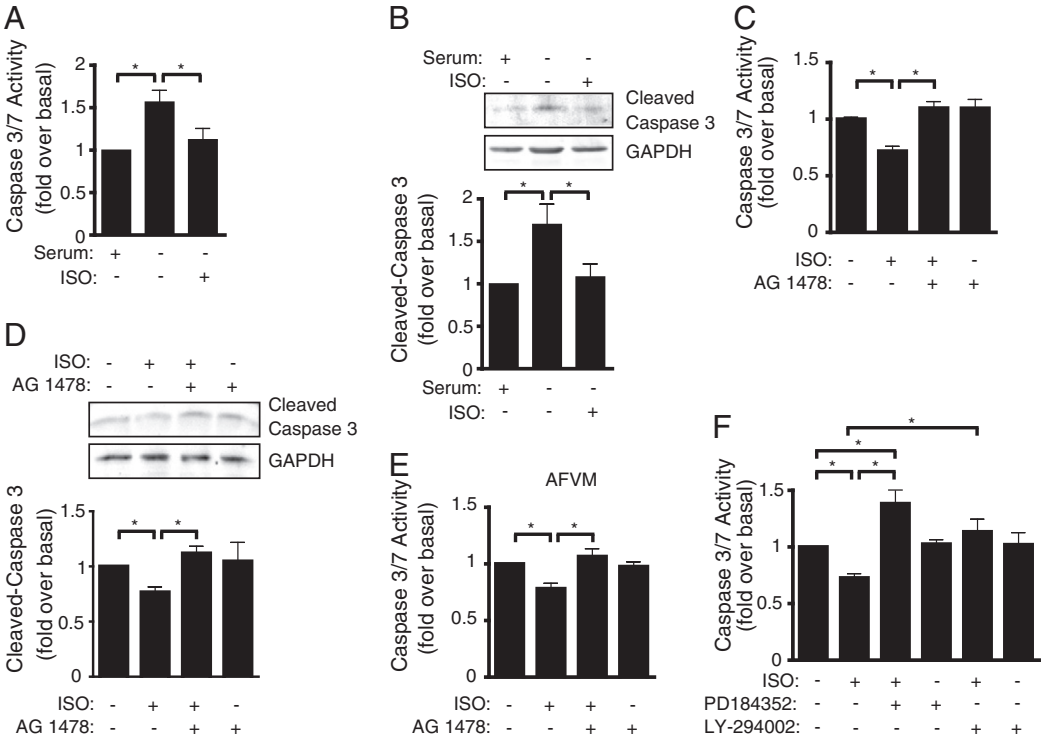

Fig. 6. βAR-mediated EGFR transactivation decreases apoptosis. Caspase 3/7 activity was measured by luciferase activity and caspase 3 cleavage by immunoblotting in RNCM with or without 24 h serum starvation. Serum starvation significantly increased caspase 3/7 activity (A) and caspase 3 cleavage (B) over RNCM cultured with serum. ISO treatment (10 μM for 3 h) decreased both caspase 3/7 activity and caspase 3 cleavage in response to serum starvation. n ≥ 4 independent experiments. ANOVA, *p < 0.05. Caspase 3/7 activity (C) and caspase 3 cleavage (D) were measured in serum-starved RNCM treated with ISO (10 μM for 3 h) with or without AG 1478 pretreatment (1 μM; 10 min). ISO significantly decreased both caspase 3/7 activity and caspase 3 cleavage which were prevented by pretreatment with AG 1478. n ≥ 5 independent experiments. ANOVA, *p < 0.05. E, under the same conditions described above, caspase 3/7 activity decreased in adult feline ventricular cardiomyocytes (AFVM) in response to ISO (10 μM for 3 h), which was blocked by AG 1478 pretreatment (1 μM; 10 min). n = 3 independent experiments. ANOVA, *p < 0.05. F, caspase 3/7 activity was decreased in RNCM treated with ISO (10 μM; 3 h), an effect blocked by pretreatment with PD184352 (10 μM; 10 min) and LY-294002 (10 μM; 10 min). n ≥ 4 independent experiments. ANOVA, *p < 0.05.

conditions that ISO decreased caspase 3/7 activity in primary isolated adult feline ventricular myocytes (AFVM) and that this effect was also blocked by AG 1478 (Fig. 6E). This suggests that the EGFR-dependent pro-survival effects of βAR stimulation in neonatal cardiomyocytes reflect that of adult cardiomyocytes. LDH activity was also measured in RNCM to assess generalized cell death and showed similar results wherein an ISO-mediated decrease in LDH activity was blocked by EGFR inhibition (Supplemental Fig. 3C). Additionally, inhibition of either ERK1/2 or Akt signaling pathways with PD184352 or LY-294002, respectively, abrogated ISO-mediated decreases in caspase 3/7 activity (Fig. 6F) as well as cleavage of caspase 3 (Supplemental Figs. 3D and E). In fact, while the inhibition of ERK1/2 signaling with PD184352 alone did not alter caspase 3/7 activity, PD184352 in the presence of ISO significantly enhanced caspase 3/7 activity over serum-deprived RNCM alone. This may indicate that βAR-mediated EGFR transactivation-dependent ERK1/2 signaling in particular may provide a crucial check against other pro-death βAR pathways such as that mediated via PKA [3].

While alterations in caspase activity and cleavage are indicative of changes in the initial steps toward apoptosis, they do not conclusively demonstrate the impact of βAR-mediated EGFR transactivation on the progression to cell death via apoptosis. Thus, a TUNEL assay was performed (Fig. 7, Supplemental Fig. 4A) using serum-depleted RNCM treated for 24 h with ISO in the presence or absence of AG 1478. ISO significantly decreased the number of TUNEL-positive nuclei in RNCM, which was abrogated in the presence of AG 1478, while RNCM cultured under normal conditions (+serum) had very few TUNEL positive nuclei. This demonstrates that acute initiation of pro-survival signaling via βAR-mediated EGFR transactivation does impact the long-term outcome on apoptosis in cardiomyocytes.

3.4. βAR-mediated EGFR transactivation regulates the expression and secretion of TNF-related apoptosis-inducing ligand (TRAIL) in RNCM

ERK1/2- and Akt-dependent modulation of various cytosolic- and plasma membrane-localized proteins has been shown to promote survival [27–30], but it is unclear how βAR-mediated EGFR transactivation-induced changes in nuclear signaling impact survival, though modulation of gene expression is an obvious candidate mechanism. To test whether changes in transcription and/or translation impact EGFR-sensitive survival effects in RNCM following βAR stimulation, we performed caspase 3/7 activity assays as described above in response to ISO stimulation in the presence or absence of actinomycin D (transcription inhibitor) or cycloheximide (translation inhibitor). Pretreatment of RNCM with either agent blocked the ability of ISO to decrease caspase 3/7 activity (Fig. 8A) and cleaved caspase 3 levels (Supplemental Fig. 4B), suggesting that βAR-mediated EGFR transactivation-induced alterations in transcription and/or translation are sufficient to promote survival signaling.

Since the inhibition of transcription can prevent βAR-mediated EGFR transactivation-dependent survival, we postulated that this pathway may regulate changes in the expression of apoptotic genes. To test this, we isolated total RNA from RNCM that underwent stimulation with ISO for 3 h in the presence or absence of AG 1478, generated cDNA and performed RT-qPCR. To ensure that ISO stimulation induced a predicted gene expression response, we initially measured the expression levels of *Nr4a1*, a gene known to be regulated by canonical Gs-protein-dependent βAR signaling [31]. As expected, ISO induced a significant increase in *Nr4a1* expression that was not blocked by AG 1478 pretreatment (Fig. 8B). Next, we subjected the cDNA to RT-qPCR using a RT^2 PCR apoptotic gene profiler array. Of the 84 genes included in the array

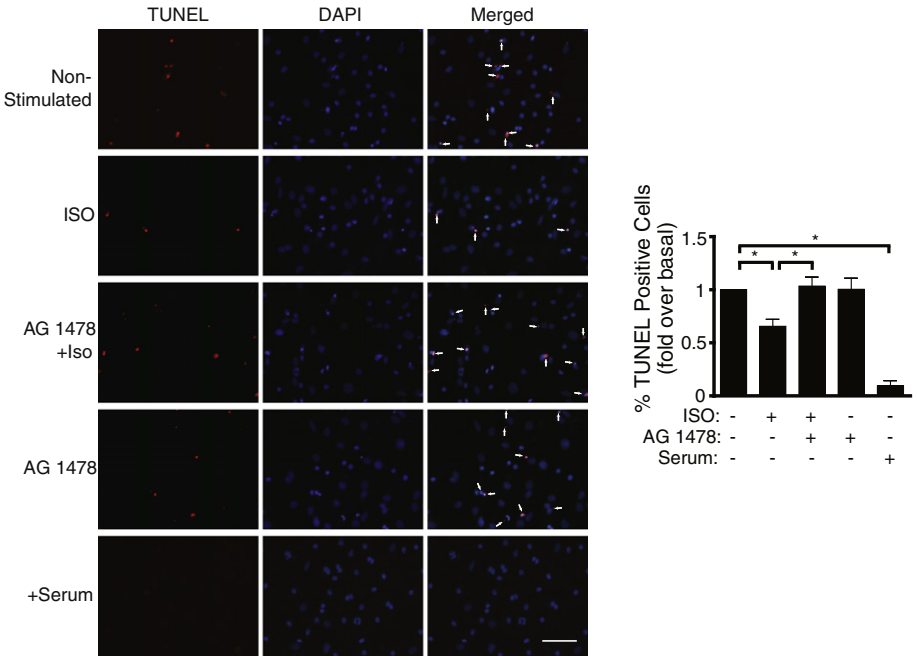

Fig. 7. βAR-mediated EGFR transactivation alters RNCM apoptosis. TUNEL analysis was performed to identify DNA fragmentation in serum-depleted RNCM following ISO treatment (10 μM; 24 h) with or without AG 1478 pretreatment (1 μM; 10 min). Serum depletion significantly enhanced TUNEL staining compared to non-starved controls. ISO significantly decreased the number of TUNEL-positive cells under the serum-depleted condition, which was prevented by pretreatment with AG 1478. n ≥ 4 independent experiments. ANOVA, *p < 0.05. Scale bar is 50 μm.

(Supplemental Table 2), 12 were indicated to be altered in a manner dependent upon βAR-mediated EGFR transactivation, while the remaining genes were either unaffected by treatment, sensitive to ISO alone or the ISO + AG 1478 responses were not different from AG 1478 alone. Validation of array results using RT-qPCR confirmed *tnfsf10* as being significantly decreased following 3 h ISO treatment. The inhibition of EGFR prevented ISO-mediated decreases in *tnfsf10* expression and significantly elevated levels above basal levels (Fig. 8C). This indicates that that *tnfsf10* expression can be negatively or positively regulated in response to βAR stimulation in cardiomyocytes through different mechanisms, with EGFR transactivation providing a dominant negative regulation of its expression. These data were confirmed in the whole heart of adult mice where an ISO-dependent increase in *Nr4a1* expression was independent of EGFR (Supplemental Fig. 4C), but an ISO-induced reduction in *tnfsf10* expression was blocked by EGFR inhibition (Supplemental Fig. 4D). Since βAR-mediated EGFR transactivation also occurs in cardiac fibroblasts, we tested the effect of ISO and AG 1478 on RNCF. Although ISO induced a larger increase in *Nr4a1* expression in RNCF than in RNCM, which was partially sensitive to AG 1478 pretreatment (Supplemental Fig. 4E), ISO did not significantly impact *tnfsf10* expression despite a trend toward increased expression within all treatment groups (Supplemental Fig. 4 F). Thus, the EGFR transactivation-dependent regulation of *tnfsf10* in response to βAR stimulation occurs specifically in cardiomyocytes.

Tnfsf10 encodes for TRAIL (TNF-related apoptosis-inducing ligand), a protein known to modulate apoptosis through its extracellular actions [32]. We further validated our findings by measuring the levels of membrane-bound and soluble TRAIL (sTRAIL) via ELISA from the membrane fractions of RNCM and the culture media, respectively, following ISO ± AG 1478 treatment. ELISA results showed a decrease in membrane-bound TRAIL in RNCM membrane lysates and reduced sTRAIL in media from ISO-treated cells (Fig. 8D). AG 1478 pretreatment prevented ISO-induced decreases in membrane TRAIL while significantly increasing sTRAIL levels over media from non-treated cells. The inhibition of ERK1/2 signaling using PD184352 or Akt with LY-294002 also abolished ISO-mediated decreases in membrane bound and sTRAIL (Figs. 8E and F) and significantly elevated membrane-bound TRAIL levels above non-treated levels. Although TRAIL has a known role in apoptosis regulation, its reported impact on cell death effects varies in different cells and tissues [33,34] and in relation to cardiomyocytes has only been shown in one study to enhance stretch-induced apoptosis [35]. Therefore, to determine the impact of enhanced TRAIL signaling in our model, we tested the effect of exogenously-added TRAIL on caspase 3/7 activity in serum-deprived RNCM. The addition of TRAIL significantly increased caspase 3/7 activity in RNCM following 3 h treatment, an effect that was completely blocked with the co-administration of an anti-TRAIL antibody, but not by normal IgG (Fig. 8G), demonstrating that increased TRAIL signaling directly leads to the activation of early apoptosis events in cardiomyocytes. Altogether, these results indicate that βAR-mediated EGFR transactivation directs differential subcellular ERK1/2 and Akt activation, with a strong influence on nuclear signaling events, including the negative regulation of pro-apoptotic TRAIL expression, to ultimately promote cardiomyocyte survival.

4. Discussion

βAR are crucial regulators of cardiac function both normally and pathologically during HF where their signaling becomes altered, contributing to detrimental increases in apoptosis, fibrosis and hypertrophy [1]. However, it has been shown that β1AR-mediated EGFR transactivation relays a pro-survival effect in a mouse model of HF [4], though the distal mechanisms by which this process exerts this effect are not known. βAR-dependent EGFR transactivation activates ERK1/2 signaling, which has been shown in numerous cell types to regulate several facets of survival signaling [4,8,16,17]. Akt signaling is also enhanced by EGFR activation to promote survival, though the impact of βAR stimulation on this branch of EGFR signaling has not been studied extensively [8]. Aside from the general activation of EGFR signaling, the subcellular targeting of these effects could regulate their impact on cell survival processes, and indeed, differential EGFR activation and ERK1/2 targeting responses to distinct ligands have been shown to occur [16,36]. Both ERK1/2 and Akt have diverse functions in the heart

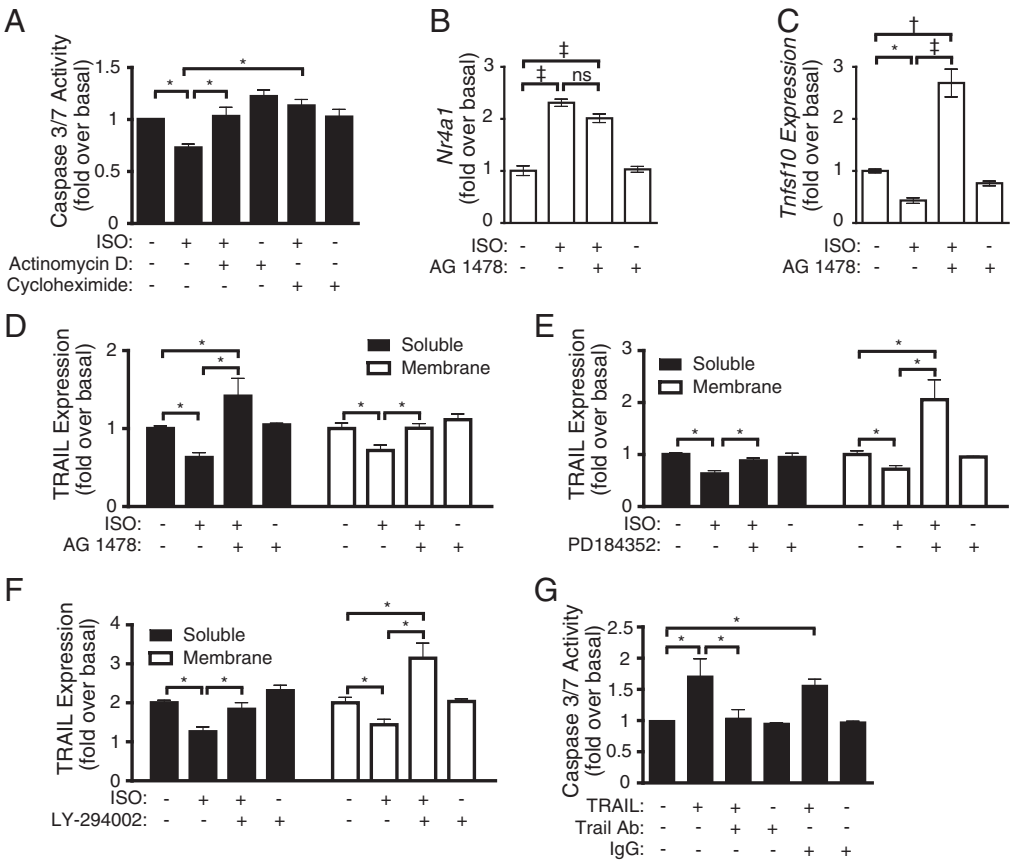

Fig. 8. βAR-mediated EGFR transactivation regulates the expression and secretion of TRAIL in RNCM. A, caspase 3/7 activity was measured by luciferase assay in serum-depleted RNCM treated with ISO (10 μM) for 3 h with or without actinomycin D (5 μg/mL) or cycloheximide (10 μg/mL) pretreatment (1 h). ISO significantly decreased caspase 3/7 activity which was prevented with both actinomycin D and cycloheximide pretreatment. n ≥ 5 from independent experiments. ANOVA, *p < 0.05. mRNA expression of *Nr4a1* (B) and *Tnfsf10* (C) was assessed by RT-qPCR from RNCM treated with ISO (10 μM, 3 h) in the presence or absence of AG 1478 (1 μM, 10 min pretreatment). *Nr4a1* was significantly increased with ISO, which was unaffected by AG 1478 pretreatment. *Tnfsf10* expression was reduced by ISO and significantly increased by the combination of ISO and AG 1478. n ≥ 3 independent experiments each. ANOVA, *p < 0.05, p < 0.001. TRAIL expression in concentrated media and cellular lysates from RNCM treated 3 h with ISO (10 μM) with or without pretreatment with (D) AG 1478 (1 μM; 10 min), (E) PD184352 (1 μM; 10 min) or (F) LY-294002 (1 μM; 10 min) was assessed by ELISA. ISO significantly decreased soluble TRAIL in the media and membrane TRAIL in the lysate of RNCM. Pretreatment with AG 1478, PD184352 or LY-294002 each prevented ISO-mediated decreases in both soluble and membrane TRAIL, in some cases significantly enhancing TRAIL expression in conjunction with ISO. n ≥ 5 independent experiments each. ANOVA, *p < 0.05. G, TRAIL (4 ng/mL; 3 h) significantly increased caspase 3/7 activity which was blocked by pretreatment with a TRAIL antibody (1:200; 1 h pretreatment). IgG (1:200; 1 h pretreatment) had no effect on TRAIL-mediated increases in caspase 3/7 activity. n ≥ 4 independent experiments. ANOVA, *p < 0.05.

including regulating cellular proliferation, differentiation, transcription and death [1,36–39]. Activation of ERK1/2 and Akt leads to the phosphorylation of a variety of cytosolic and nuclear targets which influence cell death and survival [27,28,38,40]. The subcellular distribution of downstream effectors of GPCRs and RTKs has been investigated for some time, revealing a wide range of receptor- and cell model-dependent responses [41–44]. Indeed, our own previous work in HEK 293 cells detailed the cytosolic retention of ERK1/2 by βAR-dependent EGFR transactivation [16] however in this study we show that the nuclear ERK1/2 and Akt undergo significant phosphorylation via this pathway, underscoring the impact of cell-type effects on EGFR signaling. That the same pattern of ERK1/2 and Akt phosphorylation was attained in the whole heart in vivo confirms that this is the physiologically relevant response to cardiac βAR-mediated EGFR transactivation. Interestingly, we did not see evidence for significant subcellular shuttling of non-phosphorylated ERK1/2 and Akt, suggesting that βAR-mediated EGFR transactivation results in rapid activation of distinct pools of ERK1/2 and Akt throughout the cardiomyocyte.

The location of receptor signaling is important for determining the physiological outcome [45–47]. Activated receptors can signal from the plasma membrane or from endocytic vesicles, which determines its interactions with signaling networks [48–50]. A major regulatory mechanism for directing EGFR signaling occurs via the binding of regulatory proteins to the receptor [51–60]. Different ligands produce distinct EGFR tyrosine phosphorylation patterns, which influence recruitment of signaling effectors [36]. βAR-mediated transactivation of EGFR may produce a unique EGFR phosphorylation response and has been shown to promote distinctive downstream responses [16]. Some studies show that the majority of EGFR signaling occurs through pathways initiated at receptors located at the plasma membrane while others require receptor internalization for full activation [61–63]. Rapid internalization kinetics of βAR and EGFR have been reported and discussed by others [45,50,64], as has the co-internalization of βAR with EGFR following catecholamine stimulation [16], suggesting that the subcellular phosphorylation of ERK1/2 and Akt in response to βAR stimulation could be mediated via EGFR trafficking. We used dynasore, a dynamin inhibitor that prevents receptor internalization, to determine the importance of receptor internalization in ERK1/2 and Akt activation with ISO-mediated EGFR transactivation. Dynamin inhibition prevented ERK1/2 and Akt phosphorylation in total RNCM lysates demonstrating the need for receptor internalization in this process. Furthermore, fractionation of RNCM revealed that receptor internalization is crucial for the activation of ERK1/2 and Akt throughout the cell. This suggests that while the receptors are activated at the membrane, the majority of signaling may occur from endocytic vesicles. Both βAR and EGFR are known to be present and functional on the nuclear membrane

and thought to alter gene expression [64–68]. Our data would suggest that although the receptors may be expressed at the nucleus, the plasma membrane-localized population is predominantly responsible for promoting EGFR-dependent ERK1/2 and Akt activation throughout the cardiomyocyte, including within the nucleus, in response to βAR stimulation.

Cell death is important in the pathogenesis of myocardial infarction and HF, where moderate increases in apoptosis are sustained [69,70]. To begin to elucidate the pro-survival mechanisms that have been observed with βAR-mediated EGFR transactivation, we examined the effects of acute ISO stimulation on cell death and apoptosis. We observed a decrease in LDH release from RNCM treated with ISO, which was prevented with EGFR inhibition, demonstrating the ability of βAR-mediated EGFR transactivation to protect cells from death. Furthermore, similar decreases were observed in caspase 3/7 activity following ISO treatment in both adult and neonatal cardiomyocytes, suggesting that the inhibition of cell death observed with ISO stimulation occurs in large part through decreases in apoptosis. It is important to note that in this study ISO alone did not induce apoptosis, however caspase 3 activation was monitored acutely while other studies observing pro-apoptotic effects of ISO in cardiomyocytes examined later timepoints [71–74]. βAR signaling through G protein-dependent mechanisms promotes cell death [3], and has been shown to contribute to apoptosis via ER stress [72], while G protein-independent βAR-mediated EGFR signaling promotes cell survival [4,8,17]. It is possible that the pro-survival effects of EGFR transactivation are rapid while pro-apoptotic βAR signaling dominates chronically. Additionally, EGFR-independent activation of cytosolic Akt or transactivation of other receptor tyrosine kinases could contribute to survival signaling [8,75]. Considering that acute βAR-mediating signaling has also been demonstrated to promote ischemic preconditioning [76,77], though without an exploration of a role for EGFR transactivation, understanding the balance between pro- and anti-survival βAR signaling events both acutely and chronically will be important in determining their potential therapeutic benefit.

To begin to investigate the linkage between nuclear signaling events and decreased apoptosis mediated by βAR-dependent EGFR transactivation, an RT^2 Profiler Array was used to identify changes in gene transcription of 84 apoptosis-related genes. Of the potential targets identified, TRAIL (Tnfsf10, Apo2L, or CD253) was identified to be decreased in response to ISO in RNCM, an effect that was blocked by AG 1478 treatment and validated not only via real time PCR, but also via ELISA. The impact of βAR-mediated EGFR transactivation on TRAIL mRNA expression was similar in the adult mouse heart, but absent in isolated cardiac fibroblasts, indicating that this effect occurs specifically in the cardiomyocyte population. Since β1AR expression in cardiomyocytes predominates, while β2AR expression predominates in cardiac fibroblasts, these results also suggest that catecholamine-dependent regulation of TRAIL occurs primarily via β1AR-mediated EGFR transactivation. In humans, TRAIL signals through two death receptors, TRAIL-R1 and -R2, as well as decoy receptors that do not induce cell death, while mice only have one death receptor and several decoy receptors [78]. Binding of TRAIL to a death receptor induces apoptosis through the formation of a death-inducing signaling complex and caspase activation [34]. While TRAIL is known to induce apoptosis in cancerous cells, non-tumor cells are often resistant to TRAIL-mediated cell death due to the competition of decoy receptors for TRAIL binding. While mRNA and protein expression for TRAIL, two of its pro-apoptotic receptors, TRAIL-R1 and TRAIL-R2, and a decoy receptor, TRAIL-R3, have been reported in human and primate cardiomyocytes [79]; the function of TRAIL in the heart is virtually unknown. Low levels of soluble TRAIL in the serum of patients with acute coronary syndrome and post-myocardial infarction have been reported [80–82] and in advanced HF and cardiovascular disease, decreased TRAIL levels are associated with worsened prognosis and increased mortality suggesting a protective role for TRAIL in the heart [81,83,84]. However, serum TRAIL levels were enhanced in chronic Chagas cardiomyopathy patients, correlating with decreased left ventricular ejection fraction and left ventricular diastolic dimension and increased B-type natriuretic peptide [85]. We are aware of only one study that reported a role for TRAIL in the regulation of apoptosis in cardiomyocytes, wherein the authors found that although TRAIL did not alter apoptosis alone, it did enhance cell death induced by mechanical stretch of cardiomyocytes [35]. Here, we show in our model of RNCM serum-deprivation that TRAIL significantly increases caspase 3/7 activity, thereby suggesting that the changes in TRAIL expression mediated via βAR-dependent EGFR transactivation in cardiomyocytes may provide an important contribution to the promotion of cardiac survival that has been observed in response to this signaling paradigm.

We have identified differences in the subcellular activation of ERK1/2 and Akt in the heart in response to βAR-mediated EGFR transactivation. These changes in ERK1/2 and Akt activation lead to decreased cardiomyocyte apoptosis, in part, through changes in apoptotic gene transcription, including decreased expression of the pro-apoptotic factor TRAIL. While βAR-dependent EGFR transactivation has been previously shown to promote cardiac fibroblast proliferation [86], to our knowledge, this is the first study to demonstrate EGFR-dependent regulation of βAR-mediated gene expression in cardiomyocytes. G protein-dependent βAR signaling through PKA has been shown to promote cardiotoxicity [6], while alternate βAR signaling pathways, including via EGFR transactivation, have been demonstrated to promote cardiac survival [4]. Thus, these data not only begin to explain the mechanisms by which βAR-mediated EGFR transactivation promotes survival in cardiomyocytes, but also establishes a paradigm by which this process can mediate acute changes in cardiac gene expression. Whether gene expression changes in response to EGFR transactivation persist in response to βAR stimulation remains to be tested, but will be an important concept to determine the therapeutic potential of this signaling mechanism in the context of HF.

Sources of funding

This work was supported by the National Institutes of Health (HL-105414 to DGT; HL-33921 to SRH), the WW Smith Charitable Trust (H1206 to DGT), an AHA postdoctoral fellowship (to LAG) and an AHA predoctoral fellowship (to CAM).

Disclosures

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2014.02.009.

References

[1] Tilley DG. G protein-dependent and G protein-independent signaling pathways and their impact on cardiac function. Circ Res 2011;109:217–30.
[2] Houser SR, Margulies KB, Murphy AM, Spinale FG, Francis GS, Prabhu SD, et al. Animal models of heart failure: a scientific statement from the American Heart Association. Circ Res 2012;111:131–50.
[3] Zhang X, Szeto C, Gao E, Tang M, Jin J, Fu Q, et al. Cardiotoxic and cardioprotective features of chronic beta-adrenergic signaling. Circ Res 2013;112:498–509.
[4] Noma T, Lemaire A, Naga Prasad SV, Barki-Harrington L, Tilley DG, Chen J, et al. Beta-arrestin-mediated beta1-adrenergic receptor transactivation of the EGFR confers cardioprotection. J Clin Invest 2007;117:2445–58.
[5] Kubon C, Mistry NB, Grundvold I, Halvorsen S, Kjeldsen SE, Westheim AS. The role of beta-blockers in the treatment of chronic heart failure. Trends Pharmacol Sci 2011;32:206–12.
[6] Violin JD, Soergel DG, Boerrigter G, Burnett Jr JC, Lark MW. GPCR biased ligands as novel heart failure therapeutics. Trends Cardiovasc Med 2013;23(7):242–9.
[7] Kim IM, Tilley DG, Chen J, Salazar NC, Whalen EJ, Violin JD, et al. Beta-blockers alprenolol and carvedilol stimulate beta-arrestin-mediated EGFR transactivation. Proc Natl Acad Sci U S A 2008;105:14555–60.

[8] Chen H, Ma N, Xia J, Liu J, Xu Z. Beta2-adrenergic receptor-induced transactivation of epidermal growth factor receptor and platelet-derived growth factor receptor via Src kinase promotes rat cardiomyocyte survival. Cell Biol Int 2012;36:237–44.
[9] Wang Y, Pennock S, Chen X, Wang Z. Endosomal signaling of epidermal growth factor receptor stimulates signal transduction pathways leading to cell survival. Mol Cell Biol 2002;22:7279–90.
[10] She QB, Solit DB, Ye Q, O'Reilly KE, Lobo J, Rosen N. The BAD protein integrates survival signaling by EGFR/MAPK and PI3K/Akt kinase pathways in PTEN-deficient tumor cells. Cancer cell 2005;8:287–97.
[11] Ramljak D, Coticchia CM, Nishanian TG, Saji M, Ringel MD, Conzen SD, et al. Epidermal growth factor inhibition of c-Myc-mediated apoptosis through Akt and Erk involves Bcl-xL upregulation in mammary epithelial cells. Exp Cell Res 2003;287:397–410.
[12] Hausenloy DJ, Tsang A, Mocanu MM, Yellon DM. Ischemic preconditioning protects by activating prosurvival kinases at reperfusion. Am J Physiol Heart Circ Physiol 2005;288:H971–6.
[13] Forster K, Kuno A, Solenkova N, Felix SB, Krieg T. The delta-opioid receptor agonist DADLE at reperfusion protects the heart through activation of pro-survival kinases via EGF receptor transactivation. Am J Physiol Heart Circ Physiol 2007;293:H1604–8.
[14] Krieg T, Cui L, Qin Q, Cohen MV, Downey JM. Mitochondrial ROS generation following acetylcholine-induced EGF receptor transactivation requires metalloproteinase cleavage of proHB-EGF. J Mol Cell Cardiol 2004;36:435–43.
[15] Methner C, Donat U, Felix SB, Krieg T. Cardioprotection of bradykinin at reperfusion involves transactivation of the epidermal growth factor receptor via matrix metalloproteinase-8. Acta Physiol 2009;197:265–71.
[16] Tilley DG, Kim IM, Patel PA, Violin JD, Rockman HA. Beta-arrestin mediates beta1-adrenergic receptor-epidermal growth factor receptor interaction and downstream signaling. J Biol Chem 2009;284:20375–86.
[17] Maudsley S, Pierce KL, Zamah AM, Miller WE, Ahn S, Daaka Y, et al. The beta(2)-adrenergic receptor mediates extracellular signal-regulated kinase activation via assembly of a multi-receptor complex with the epidermal growth factor receptor. J Biol Chem 2000;275:9572–80.
[18] Makarewich CA, Correll RN, Gao H, Zhang H, Yang B, Berretta RM, et al. A caveolae-targeted L-type Ca(2)+ channel antagonist inhibits hypertrophic signaling without reducing cardiac contractility. Circ Res 2012;110:669–74.
[19] Silver LH, Hemwall EL, Marino TA, Houser SR. Isolation and morphology of calcium-tolerant feline ventricular myocytes. Am J Physiol 1983;245:H891–6.
[20] Levitzki A, Gazit A. Tyrosine kinase inhibition: an approach to drug development. Science 1995;267:1782–8.
[21] Norris RP, Freudzon M, Nikolaev VO, Jaffe LA. Epidermal growth factor receptor kinase activity is required for gap junction closure and for part of the decrease in ovarian follicle cGMP in response to LH. Reproduction 2010;140:655–62.
[22] Kulkarni GV, McCulloch CA. Serum deprivation induces apoptotic cell death in a subset of Balb/c 3T3 fibroblasts. J Cell Sci 1994;107(Pt 5):1169–79.
[23] Braun F, Bertin-Ciftci J, Gallouet AS, Millour J, Juin P. Serum-nutrient starvation induces cell death mediated by Bax and Puma that is counteracted by p21 and unmasked by Bcl-x(L) inhibition. PLoS One 2011;6:e23577.
[24] Kuzman JA, Gerdes AM, Kobayashi S, Liang Q. Thyroid hormone activates Akt and prevents serum starvation-induced cell death in neonatal rat cardiomyocytes. J Mol Cell Cardiol 2005;39:841–4.
[25] Zhu H, McElwee-Witmer S, Perrone M, Clark KL, Zilberstein A. Phenylephrine protects neonatal rat cardiomyocytes from hypoxia and serum deprivation-induced apoptosis. Cell Death Differ 2000;7:773–84.
[26] Wu CF, Bishopric NH, Pratt RE. Atrial natriuretic peptide induces apoptosis in neonatal rat cardiac myocytes. J Biol Chem 1997;272:14860–6.
[27] Datta SR, Ranger AM, Lin MZ, Sturgill JF, Ma YC, Cowan CW, et al. Survival factor-mediated BAD phosphorylation raises the mitochondrial threshold for apoptosis. Dev Cell 2002;3:631–43.
[28] Gardai SJ, Hildeman DA, Frankel SK, Whitlock BB, Frasch SC, Borregaard N, et al. Phosphorylation of Bax Ser184 by Akt regulates its activity and apoptosis in neutrophils. J Biol Chem 2004;279:21085–95.
[29] Kim AH, Khursigara G, Sun X, Franke TF, Chao MV. Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1. Mol Cell Biol 2001;21:893–901.
[30] Yeh JH, Hsu SC, Han SH, Lai MZ. Mitogen-activated protein kinase kinase antagonized fas-associated death domain protein-mediated apoptosis by induced FLICE-inhibitory protein expression. J Exp Med 1998;188:1795–802.
[31] Soker T, Godecke A. Expression of the murine Nr4a1 gene is controlled by three distinct genomic loci. Gene 2013;512:517–20.
[32] Wajant H, Moosmayer D, Wuest T, Bartke T, Gerlach E, Schonherr U, et al. Differential activation of TRAIL-R1 and -2 by soluble and membrane TRAIL allows selective surface antigen-directed activation of TRAIL-R2 by a soluble TRAIL derivative. Oncogene 2001;20:4101–6.
[33] Di Pietro R, Zauli G. Emerging non-apoptotic functions of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/Apo2L. J Cell Physiol 2004;201:331–40.
[34] Bodmer JL, Holler N, Reynard S, Vinciguerra P, Schneider P, Juo P, et al. TRAIL receptor-2 signals apoptosis through FADD and caspase-8. Nat Cell Biol 2000;2:241–3.
[35] Liao X, Wang X, Gu Y, Chen Q, Chen LY. Involvement of death receptor signaling in mechanical stretch-induced cardiomyocyte apoptosis. Life Sci 2005;77:160–74.
[36] McCole DF, Truong A, Bunz M, Barrett KE. Consequences of direct versus indirect activation of epidermal growth factor receptor in intestinal epithelial cells are dictated by protein-tyrosine phosphatase 1B. J Biol Chem 2007;282:13303–15.
[37] Kehat I, Molkentin JD. Extracellular signal-regulated kinase 1/2 (ERK1/2) signaling in cardiac hypertrophy. Ann N Y Acad Sci 2010;1188:96–102.
[38] Baines CP, Molkentin JD. STRESS signaling pathways that modulate cardiac myocyte apoptosis. J Mol Cell Cardiol 2005;38:47–62.
[39] Tucka J, Bennett M, Littlewood T. Cell death and survival signalling in the cardiovascular system. Front Biosci 2012;17:248–61.
[40] Yoon S, Seger R. The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. Growth Factors 2006;24:21–44.
[41] Murphy LO, Blenis J. MAPK signal specificity: the right place at the right time. Trends Biochem Sci 2006;31:268–75.
[42] Kondoh K, Torii S, Nishida E. Control of MAP kinase signaling to the nucleus. Chromosoma 2005;114:86–91.
[43] Mebratu Y, Tesfaigzi Y. How ERK1/2 activation controls cell proliferation and cell death: Is subcellular localization the answer? Cell Cycle 2009;8:1168–75.
[44] Roskoski Jr R. ERK1/2 MAP kinases: structure, function, and regulation. Pharmacol Res 2012;66:105–43.
[45] von Zastrow M, Sorkin A. Signaling on the endocytic pathway. Curr Opin Cell Biol 2007;19:436–45.
[46] Miaczynska M, Pelkmans L, Zerial M. Not just a sink: endosomes in control of signal transduction. Curr Opin Cell Biol 2004;16:400–6.
[47] Sadowski L, Pilecka I, Miaczynska M. Signaling from endosomes: location makes a difference. Exp Cell Res 2009;315:1601–9.
[48] Haugh JM, Huang AC, Wiley HS, Wells A, Lauffenburger DA. Internalized epidermal growth factor receptors participate in the activation of p21(ras) in fibroblasts. J Biol Chem 1999;274:34350–60.
[49] Burke P, Schooler K, Wiley HS. Regulation of epidermal growth factor receptor signaling by endocytosis and intracellular trafficking. Mol Biol Cell 2001;12:1897–910.
[50] Sorkin A. Internalization of the epidermal growth factor receptor: role in signalling. Biochem Soc Trans 2001;29:480–4.
[51] Stern KA, Place TL, Lill NL. EGF and amphiregulin differentially regulate Cbl recruitment to endosomes and EGF receptor fate. Biochem J 2008;410:585–94.
[52] Rozakis-Adcock M, McGlade J, Mbamalu G, Pelicci G, Daly R, Li W, et al. Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. Nature 1992;360:689–92.
[53] Fazioli F, Minichiello L, Matoska V, Castagnino P, Miki T, Wong WT, et al. Eps8, a substrate for the epidermal growth factor receptor kinase, enhances EGF-dependent mitogenic signals. EMBO J 1993;12:3799–808.
[54] Margolis B, Li N, Koch A, Mohammadi M, Hurwitz DR, Zilberstein A, et al. The tyrosine phosphorylated carboxyterminus of the EGF receptor is a binding site for GAP and PLC-gamma. EMBO J 1990;9:4375–80.
[55] Anderson D, Koch CA, Grey L, Ellis C, Moran MF, Pawson T. Binding of SH2 domains of phospholipase C gamma 1, GAP, and Src to activated growth factor receptors. Science 1990;250:979–82.
[56] Thien CB, Langdon WY. EGF receptor binding and transformation by v-cbl is ablated by the introduction of a loss-of-function mutation from the *Caenorhabditis elegans* sli-1 gene. Oncogene 1997;14:2239–49.
[57] Levkowitz G, Waterman H, Ettenberg SA, Katz M, Tsygankov AY, Alroy I, et al. Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. Mol Cell 1999;4:1029–40.
[58] Levkowitz G, Waterman H, Zamir E, Kam Z, Oved S, Langdon WY, et al. c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. Genes Dev 1998;12:3663–74.
[59] Lill NL, Douillard P, Awwad RA, Ota S, Lupher Jr ML, Miyake S, et al. The evolutionarily conserved N-terminal region of Cbl is sufficient to enhance down-regulation of the epidermal growth factor receptor. J Biol Chem 2000;275:367–77.
[60] Muthuswamy SK, Gilman M, Brugge JS. Controlled dimerization of ErbB receptors provides evidence for differential signaling by homo- and heterodimers. Mol Cell Biol 1999;19:6845–57.
[61] Sousa LP, Lax I, Shen H, Ferguson SM, De Camilli P, Schlessinger J. Suppression of EGFR endocytosis by dynamin depletion reveals that EGFR signaling occurs primarily at the plasma membrane. Proc Natl Acad Sci U S A 2012;109:4419–24.
[62] Brankatschk B, Wichert SP, Johnson SD, Schaad O, Rossner MJ, Gruenberg J. Regulation of the EGF transcriptional response by endocytic sorting. Sci Signal 2012;5:ra21.
[63] Vieira AV, Lamaze C, Schmid SL. Control of EGF receptor signaling by clathrin-mediated endocytosis. Science 1996;274:2086–9.
[64] Madshus IH, Stang E. Internalization and intracellular sorting of the EGF receptor: a model for understanding the mechanisms of receptor trafficking. J Cell Sci 2009;122:3433–9.
[65] Wakshull EM, Wharton W. Stabilized complexes of epidermal growth factor and its receptor on the cell surface stimulate RNA synthesis but not mitogenesis. Proc Natl Acad Sci U S A 1985;82:8513–7.
[66] Boivin B, Lavoie C, Vaniotis G, Baragli A, Villeneuve LR, Ethier N, et al. Functional beta-adrenergic receptor signalling on nuclear membranes in adult rat and mouse ventricular cardiomyocytes. Cardiovasc Res 2006;71:69–78.
[67] Vaniotis G, Del Duca D, Trieu P, Rohlicek CV, Hebert TE, Allen BG. Nuclear beta-adrenergic receptors modulate gene expression in adult rat heart. Cell Signal 2011;23:89–98.
[68] Lin SY, Makino K, Xia W, Matin A, Wen Y, Kwong KY, et al. Nuclear localization of EGF receptor and its potential new role as a transcription factor. Nat Cell Biol 2001;3:802–8.
[69] Olivetti G, Abbi R, Quaini F, Kajstura J, Cheng W, Nitahara JA, et al. Apoptosis in the failing human heart. N Engl J Med 1997;336:1131–41.
[70] Saraste A, Pulkki K, Kallajoki M, Heikkila P, Laine P, Mattila S, et al. Cardiomyocyte apoptosis and progression of heart failure to transplantation. Eur J Clin Invest 1999;29:380–6.
[71] Zaugg M, Xu W, Lucchinetti E, Shafiq SA, Jamali NZ, Siddiqui MA. Beta-adrenergic receptor subtypes differentially affect apoptosis in adult rat ventricular myocytes. Circulation 2000;102:344–50.
[72] Dalal S, Foster CR, Das BC, Singh M, Singh K. Beta-adrenergic receptor stimulation induces endoplasmic reticulum stress in adult cardiac myocytes: role in apoptosis. Mol Cell Biochem 2012;364:59–70.

[73] Wang W, Zhang H, Gao H, Kubo H, Berretta RM, Chen X, et al. {beta}1-Adrenergic receptor activation induces mouse cardiac myocyte death through both L-type calcium channel-dependent and -independent pathways. Am J Physiol Heart Circ Physiol 2010;299:H322–31.
[74] Communal C, Singh K, Pimentel DR, Colucci WS. Norepinephrine stimulates apoptosis in adult rat ventricular myocytes by activation of the beta-adrenergic pathway. Circulation 1998;98:1329–34.
[75] Murray DR, Mummidi S, Valente AJ, Yoshida T, Somanna NK, Delafontaine P, et al. Beta2 adrenergic activation induces the expression of IL-18 binding protein, a potent inhibitor of isoproterenol induced cardiomyocyte hypertrophy in vitro and myocardial hypertrophy in vivo. J Mol Cell Cardiol 2012;52:206–18.
[76] Salie R, Moolman JA, Lochner A. The role of beta-adrenergic receptors in the cardioprotective effects of beta-preconditioning (betaPC). Cardiovasc Drugs Ther 2011;25:31–46.
[77] Salie R, Moolman JA, Lochner A. The mechanism of beta-adrenergic preconditioning: roles for adenosine and ROS during triggering and mediation. Basic Res Cardiol 2012;107:281.
[78] Schneider P, Olson D, Tardivel A, Browning B, Lugovskoy A, Gong D, et al. Identification of a new murine tumor necrosis factor receptor locus that contains two novel murine receptors for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). J Biol Chem 2003;278:5444–54.
[79] Spierings DC, de Vries EG, Vellenga E, van den Heuvel FA, Koornstra JJ, Wesseling J, et al. Tissue distribution of the death ligand TRAIL and its receptors. J Histochem Cytochem 2004;52:821–31.
[80] Osmancik P, Teringova E, Tousek P, Paulu P, Widimsky P. Prognostic value of TNF-related apoptosis inducing ligand (TRAIL) in acute coronary syndrome patients. PLoS One 2013;8:e53860.
[81] Secchiero P, Corallini F, Beltrami AP, Ceconi C, Bonasia V, Di Chiara A, et al. An imbalanced OPG/TRAIL ratio is associated to severe acute myocardial infarction. Atherosclerosis 2010;210:274–7.
[82] Secchiero P, Corallini F, Ceconi C, Parrinello G, Volpato S, Ferrari R, et al. Potential prognostic significance of decreased serum levels of TRAIL after acute myocardial infarction. PLoS One 2009;4:e4442.
[83] Volpato S, Ferrucci L, Secchiero P, Corallini F, Zuliani G, Fellin R, et al. Association of tumor necrosis factor-related apoptosis-inducing ligand with total and cardiovascular mortality in older adults. Atherosclerosis 2011;215:452–8.
[84] Niessner A, Hohensinner PJ, Rychli K, Neuhold S, Zorn G, Richter B, et al. Prognostic value of apoptosis markers in advanced heart failure patients. Eur Heart J 2009;30:789–96.
[85] Lula JF, Rocha MO, Nunes Mdo C, Ribeiro AL, Teixeira MM, Bahia MT, et al. Plasma concentrations of tumour necrosis factor-alpha, tumour necrosis factor-related apoptosis-inducing ligand, and FasLigand/CD95L in patients with Chagas cardiomyopathy correlate with left ventricular dysfunction. Eur J Heart Fail 2009;11:825–31.
[86] Kim J, Eckhart AD, Eguchi S, Koch WJ. Beta-adrenergic receptor-mediated DNA synthesis in cardiac fibroblasts is dependent on transactivation of the epidermal growth factor receptor and subsequent activation of extracellular signal-regulated kinases. J Biol Chem 2002;277:32116–23.