

β-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*

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Cardiac hypertrophy often leads to heart failure and is associated with abnormal myocardial adrenergic signaling. This enlargement of myocardial mass can involve not only an increase in cardiomyocyte size, but increased proliferation of cardiac fibroblasts. A potential key player in the cardiac hypertrophic response is the ERK family of MAPKs. To gain mechanistic insight into adrenergic regulation of myocardial mitogenic signaling, we examined β-adrenergic receptor (β-AR) stimulation of ERK activation and DNA synthesis in cultured adult rat cardiac fibroblasts, including the involvement of tyrosine kinases in this signaling pathway. Addition of the β-AR agonist isoproterenol (ISO) to serum-starved cells induced DNA synthesis in a dose-dependent manner, and this was inhibited by selective inhibitors of the epidermal growth factor receptor (EGFR). Importantly and in agreement with the involvement of MAPKs and the EGFR in this response in cardiac fibroblasts, the EGFR inhibitor AG1478 attenuated ISO-induced ERK phosphorylation. Moreover, pretreatment with PP2, a selective inhibitor of the Src tyrosine kinase, attenuated both ISO-mediated EGFR phosphorylation and ERK activation. Furthermore, studies in these cardiac fibroblasts showed that phosphatidylinositol 3-kinase contributed to β-AR-mediated ERK activation, but not to EGFR activation. Finally, studies using selective inhibitors of matrix metalloproteases indicated that they and heparin-bound EGF shedding were involved in β-AR-induced ERK activation and subsequent DNA synthesis in cardiac fibroblasts. Because these cells primarily express the β2-AR subtype, our findings indicate that β2-AR-mediated EGFR transactivation of intracellular tyrosine kinase signaling pathways is the major signaling pathway responsible for the adrenergic stimulation of mitogenesis of cardiac fibroblasts.

Cardiac hypertrophy, generally thought to be an adaptive condition to normalize increased work loads placed on the heart, often becomes maladaptive, leading to significant ventricular dysfunction and heart failure (reviewed in Ref. 1). The myocardial enlargement associated with the hypertrophic response involves increased size and mass of terminally differentiated cardiomyocytes and may also involve proliferation of cardiac fibroblasts (2). Recent studies in cardiovascular systems have implicated G protein-coupled receptors (GPCRs) in the initiation of proliferation signals that play a critical role in the pathogenesis of pressure overload myocardial hypertrophy (3–5), neointimal hyperplasia of vascular smooth muscle cells (VSMCs) (6, 7), and proliferation of neonatal cardiac fibroblasts (8, 9). In cardiac myocytes and VSMCs, a number of different GPCR agonists, including norepinephrine (10), phenylephrine (10), isoproterenol (ISO) (9, 11), endothelin-1 (6), and angiotensin II (7, 8), can induce hypertrophic and proliferative responses concurrent with increased extracellular signal-regulated kinase (ERK) activation of the mitogen-activated protein kinase (MAPK) family. Accordingly, there has been considerable interest in the possibility that modulation of GPCR signaling pathways may have the potential to treat pathophysiological mechanisms that are fundamental to the progression of hypertrophy and heart failure (5, 12, 13).

The mechanisms involved in GPCR-stimulated mitogenic signaling have been the subject of intense investigation in recent years; and in several cell lines, it appears to involve the phosphorylation of receptor tyrosine kinases via a ligand-independent transactivation mechanism, which leads to MAPK activation and subsequent mitogenesis (6, 8, 14, 15). This can involve the epidermal growth factor receptor (EGFR), as the EGFR-specific inhibitor tyrphostin AG1478 or expression of a dominant-negative mutant of the EGFR blocks GPCR-mediated EGFR phosphorylation and ERK activation (14). This strongly suggests that GPCR-mediated “transactivation” of the receptor tyrosine kinase cascade is an obligatory step in the MAPK activation pathway (14, 15). In VSMCs and neonatal cardiac fibroblasts, stimulation of angiotensin II rapidly phosphorylates the EGFR and platelet-derived growth factor receptor and causes an increase in Shc-Grb2 complex formation (7, 8, 16).

Recently, it has been demonstrated that GPCR-mediated

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‡ The abbreviations used are: GPCRs, G protein-coupled receptors; VSMCs, vascular smooth muscle cells; ISO, isoproterenol; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; HB-EGF, heparin-bound epidermal growth factor; MMP, matrix metalloprotease; β-AR, β-adrenergic receptor; PI3K, phosphatidylinositol 3-kinase; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; PTX, pertussis toxin; DMEM, Dulbecco’s modified Eagle’s medium.

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transactivation of the EGFR involves release of a soluble EGFR ligand, heparin-bound EGFR (HB-EGF), from the cell surface, which can bind to and stimulate the phosphorylation of EGFRs (17, 18). HB-EGF is known to be a potent mitogen and chemotaxant for VSMCs in in vitro studies (19). Interestingly, the pharmacological drug KB-R7785 inhibits the shedding of HB-EGF and attenuates hypertrophic changes in cardiac cells (20). HB-EGF shedding is also sensitive to the nonselective matrix metalloprotease (MMP) inhibitor batimastat, indicating that, in some cell types, MMPs are involved in the GPCR-mediated EGFR transactivation pathway and subsequent ERK activation (17, 21, 22).

Although cardiac fibroblasts represent one of the major cardiac cell types and contribute to cardiac cell proliferation and hypertrophy in an autocrine/paracrine manner (23), few attempts have been made to characterize the GPCR-mediated growth of these cells. Of particular interest in cardiac fibroblasts is the β2-AR system, which is the major adrenergic receptor subtype in these cells (24–26). To understand the mechanisms of β2-AR-mediated cell proliferation of primary cultured adult rat cardiac fibroblasts, in this study, we examined the effects of selective receptor tyrosine kinase and other pharmacological inhibitors on ISO-induced DNA synthesis and ERK activation as an indicator of cell proliferation. Importantly, we demonstrate that activation of β2-ARs induces DNA synthesis in cardiac fibroblasts via ERK activation that is dependent on the EGFR. Moreover, other kinases appear to play a role in the proliferation of cardiac fibroblasts, including Src tyrosine kinase and phosphatidylinositol 3-kinase (PI3K). Finally, we have elucidated that the activation of MMP and HB-EGF shedding in cardiac fibroblasts are obligatory mechanisms in β2-AR-induced ERK activation and cell proliferation.

**EXPERIMENTAL PROCEDURES**

**Materials**—The EGFR-specific inhibitor tyrphostin AG1478, the Src-specific inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-\(\text{d}\)]pyrimidine (PP2), the MEK inhibitor PD98059, and the PI3K inhibitor wortmannin were obtained from Calbiochem. Bordetella pertussis toxin (PTX) was from List Biologicals. ISO, heparin, and platelet-deprived growth factor were purchased from Sigma. Human recombinant EGF was purchased from Roche Molecular Biochemicals. The MMP inhibitor BB2116 was kindly provided by Dr. Helen Mills (British Biotech). Sheep polyclonal anti-EGFR and monoclonal anti-phospho-EGFR (Tyr1173) antibodies used for immunoprecipitation and immunoblotting were from Upstate Biotechnology, Inc. Polyclonal anti-phospho-ERK antibody (Cell Signaling Technology) and polyclonal anti-ERK2 antibody (Santa Cruz Biotechnology) were used for ERK activation assay. A neutralizing antibody for human HB-EGF was obtained from R&D Systems. Anti-phosphotyrosine antibodies (PY20) were from BD Transduction Laboratories. [\(\text{\textsuperscript{3H}}\)]Thymidine was purchased from Amersham Biosciences.

**Cell Culture—**Fibroblasts were prepared by the methods of Meszaros et al. (26). Briefly, the ventricles of three hearts from adult male 300–350-g Sprague–Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, IN) were minced, pooled, and placed in a collagenase (Worthington)/protease (Sigma) digestion solution. Cells dissociated in the first treatment were discarded. After four digestions, cells were pooled, and debris and myocytes were removed by unit gravity sedimentation. Fibroblasts were isolated and suspended in DMEM (Invitrogen) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum. After a 60-min period of attachment to uncoated culture plates, cells that were weakly attached or unattached were rinsed free and discarded. For signaling assays, only early passage (≤ 5) cells were used. The purity of these cultures was >95% cardiac fibroblasts as measured by vimentin expression and negative by desmin (myocytes), smooth muscle α-actin (VSMCs), and von Willebrand factor (endothelial cells) as previously described (27).

**Immunoprecipitation and Western Blotting—**Immunoprecipitation was performed by a modification of the methods of Daub et al. (15). Cells were serum-starved in DMEM with 0.1% bovine serum albumin for 72 h. Stimulation of serum-starved passage 1 cardiac fibroblasts in 150-mm dishes with appropriate agonists was done at 37 °C for 2 min. After stimulation, monolayers were washed once with ice-cold phosphate-buffered saline and lysed in buffer containing 50 μl HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin. The EGFR was immunoprecipitated using 4 μl of anti-EGFR antibody plus 25 μl of 50% slurry of Protein G Plus/Protein A-agarose and agitated overnight at 4 °C. Western blotting was performed to detect phosphorysine residues of the EGFR with antibody PY20, which detects phosphorylated tyrosine residues. The phosphorylated EGFR was then visualized by enzyme-linked chemiluminescence (ECL, Amersham Biosciences) and quantified by scanning laser densitometry.

**DNA Synthesis—**DNA synthesis was determined as incorporation of [\(\text{\textsuperscript{3H}}\)]thymidine into trichloroacetic acid-insoluble material as described (28). Cells were seeded at an initial density of 20,000 cells/well in 12-well plates and grown to subconfluence (1 day). Cells were rendered quiescent by 72 h in serum-free DMEM supplemented with 0.1% bovine serum albumin and stimulated by addition of fresh medium containing the agonists and/or inhibitors. [\(\text{\textsuperscript{3H}}\)]Thymidine (0.5 Ci/ml) was added to the medium at the time of stimulation. Cells were washed twice with phosphate-buffered saline and three times with 0.5% trichloroacetic acid, and then cells were lysed with 1 N NaOH. Extent of cell growth was determined by counting incorporation of [\(\text{\textsuperscript{3H}}\)]thymidine into nascent DNA strands.

**Statistical Analysis—**Data were presented as means ± S.E. An unpaired Student’s t test was used to compare in vitro ERK activation and EGFR phosphorylation. A repeated-measurements analysis of variance was used to evaluate the effect of treatment on [\(\text{\textsuperscript{3H}}\)]thymidine incorporation. Typically, all assays were repeated five to six times.

**RESULTS**

**ERK and the EGFR Regulate β-AR-induced DNA Synthesis in Adult Rat Cardiac Fibroblasts—**Treatment with catecholamines not only increases heart rate and contractile activity in the myocardium, but also induces hypertrophic responses. To investigate the contribution of the β-AR signaling pathway to cardiac cell proliferation, we studied ISO-mediated DNA synthesis in adult rat cardiac fibroblasts as an indicator of cell proliferation. Cardiac fibroblasts contain almost exclusively β2-ARs (24–26); and thus, in our assays, we measured primarily β2-AR responses. As shown in Fig. 1A, ISO increased [\(\text{\textsuperscript{3H}}\)]thymidine incorporation in a dose-dependent manner, with a maximum increase achieved with 1 μM (1.6 ± 0.72-fold over control (n = 5); p < 0.01). To test the requirement of MAPKs and the EGFR in β-AR and EGF-induced DNA synthesis, cardiac fibroblasts were pretreated with pharmacological inhibitors of MEK (PD98059) and of the EGFR (AG1478), followed by stimulation with 1 μM ISO or 10 ng/ml EGF. In control cells without any inhibitors (vehicle only), ISO and EGF significantly increased DNA synthesis in cardiac fibroblasts (Fig. 1B). Pretreatment with PD98059 resulted in a 75% inhibition (n = 5; p < 0.05) and AG1478 a 50% inhibition (n = 5; p < 0.05) for both ISO- and EGF-induced DNA synthesis in cardiac fibroblasts compared with vehicle (MeSO4)-treated controls (Fig. 1B). Although both inhibitors attenuated virtually all activation over basal levels to ISO and EGF, PD98059 caused an even more profound lowering of basal DNA synthesis (Fig. 1B).

**ISO-induced Phosphorylation of ERK Is Attenuated by AG1478 and PTX—**One of the earliest downstream targets of mitogenic signaling are MAPKs, in particular the ERKs. Because ISO-induced DNA synthesis was attenuated by pretreatment using a MEK inhibitor in cardiac fibroblasts (Fig. 1B), we investigated the contribution of β-AR stimulation to ERK activation. Equal amounts of cell lysates from adult rat cardiac fibroblasts were used to detect activated ERK using anti-phos-
pho-ERK antibodies. ISO-induced phosphorylation of ERK and maximum activity were achieved at 5 min (data not shown). Phosphorylation of ERK was increased after addition of 10 \( \mu \)M ISO and 10 ng/ml EGF by 2.42 \( \pm \) 0.23-fold and 14.21 \( \pm \) 0.91-fold over basal levels (\( n = 7; p < 0.01 \)) (Fig. 2, A and B). We next tried to determine whether \( \beta \)-AR-mediated ERK activation is dependent on EGFR kinase activity. In the presence of 250 nM AG1478, both ISO- and EGF-mediated ERK activation was significantly inhibited by 70 and 87\% (\( n = 7; p < 0.05 \)), respectively (Fig. 2, A and B). Compared with pretreatment with AG1478, a pharmacological inhibitor of the platelet-derived growth factor receptor (AG1295) did not inhibit ISO- or EGF-induced ERK activation (Fig. 2B). These data suggest that ERK activation in response to ISO requires the tyrosine kinase activity of the EGFR, but not the platelet-derived growth factor receptor.

Although the \( \beta \)-AR primarily couples to the heterotrimeric G protein \( G_s \), classically activating adenyl cyclase and leading to the activation of cAMP-dependent protein kinase, the \( \beta \)-AR has also been shown to couple to PTX-sensitive \( G_i/G_o \) proteins (11, 29). To determine whether any dual \( G/G_i \) coupling is involved in \( \beta \)-AR-mediated ERK activation in cardiac fibroblasts, we examined the effect of PTX on ISO-induced ERK activation. Cells were pretreated with 100 ng/ml PTX overnight and then stimulated with 10 \( \mu \)M ISO for 5 min. As shown in Fig. 2C, PTX did partially suppress ISO-induced ERK activation in cardiac fibroblasts by 36\% (\( n = 5; p < 0.05 \)). Appropriately, PTX treatment did not affect EGF-induced ERK activation. Therefore, our data suggest that \( \beta \)-AR-mediated ERK activation in rat cardiac fibroblasts does involve significant coupling to \( G_i \) proteins.
ISO-induced DNA synthesis was inhibited by PP2. DNA synthesis in cardiac fibroblasts in the presence of ISO versus agonist was significantly decreased by 39% compared with MeSO.

Cardiac fibroblasts were grown to confluence and serum-deprived for 72 h before stimulation with 10 μM ISO and 10 ng/ml EGF for 2 min in the presence of MeSO (DMSO) control cultures and CND) versus PP2. Immunoprecipitation (IP) and immunoblot (IB) analyses were performed as described under “Experimental Procedures.” B, data are expressed as means ± S.E. of fold change in EGFR phosphorylation relative to the MeSO (DMSO) control cultures (n = 7). *, p < 0.05 for increased EGFR activity with agonist versus control; **, p < 0.05 for an inhibitory effect of PP2 versus ISO stimulation. C, shown is the inhibition of ISO-induced ERK phosphorylation by PP2. Cardiac fibroblasts were grown to confluence and serum-deprived for 72 h before stimulation with 10 μM ISO and 10 ng/ml EGF for 5 min in the presence of MeSO and 1 μM and 5 μM PP2. Western blot analysis was performed to examine phosphorylation of ERK. D, fold increase over basal ERK activity is presented as means ± S.E. (n = 7; *, p < 0.01 for an inhibitory effect of PP2 versus ISO stimulation). E, ISO-induced DNA synthesis was inhibited by PP2. DNA synthesis in cardiac fibroblasts in the presence of 1 μM and 5 μM PP2 was determined (see “Experimental Procedures”). [3H]Thymidine incorporation during 48 h of 1 μM ISO and 10 ng/ml EGF treatment was measured (n = 5, determined in triplicate). *, p < 0.01 for inhibition of PP2 versus ISO stimulation. CN, control; P-ERK, phosphorylated ERK.

**Src Regulates ISO-induced EGFR Phosphorylation, ERK Activation, and Subsequent DNA Synthesis**—In addition to EGFR activation, other tyrosine kinases such as Src can be activated following GPCR (and β2-AR) stimulation (30). To examine the role of Src in β-AR-mediated signaling in cardiac fibroblasts, ISO-induced phosphorylation of the EGFR and ERK was measured in the presence of PP2, a specific inhibitor of Src. The effect of PP2 on EGFR phosphorylation, detected using an anti-phosphotyrosine antibody after EGFR immunoprecipitation from cardiac fibroblasts, is shown in Fig. 3A. Stimulation of β-ARs induced a 2.05 ± 0.31-fold increase in EGFR phosphorylation compared with control (CN; medium alone) conditions (n = 7; p < 0.05). ISO-induced EGFR phosphorylation was significantly decreased by 39% compared with MeSO treatment following addition of 5 μM PP2 (n = 7; p < 0.05) (Fig. 3, A and B). Interestingly, PP2 treatment did not affect EGF-induced EGFR phosphorylation (Fig. 3, A and B), establishing that Src tyrosine kinase is an upstream activator of the EGFR after β-AR stimulation in rat cardiac fibroblasts.

To determine whether Src tyrosine kinase is specifically involved in β-AR-mediated ERK activation as well as EGFR phosphorylation, we determined whether PP2 addition affects phosphorylated ERK levels in cardiac fibroblast lysates. Fig. 3 (C and D) demonstrates that treatment with 1 and 5 μM PP2 blocked ISO-stimulated ERK phosphorylation by 38 and 80% (n = 7; p < 0.01), respectively, which is significantly lower than native ISO stimulation. In the case of EGF-induced ERK phosphorylation, 5 μM PP2 slightly attenuated ERK activation (Fig. 3, C and D). We also examined the role of Src in ISO-induced DNA synthesis. The results shown in Fig. 3E reveal that DNA synthesis was also decreased in the presence of PP2, concordant with the ERK phosphorylation data. Taken together, these results clearly demonstrate that Src tyrosine kinase is involved in the β2-AR-mediated transactivation of the EGFR, ERK phosphorylation, and ISO-mediated DNA synthesis in adult rat cardiac fibroblasts.

**P13K Is Involved Only in β2-AR-mediated ERK Activation and Subsequent DNA Synthesis, but Not in EGFR Phosphorylation**—Colombo et al. (9) previously demonstrated that ISO can stimulate ERK and PI3K activities in neonatal rat cardiac fibroblasts. Thus, we wanted to investigate PI3K activity in β-AR-mediated mitogenic signaling in adult cardiac fibroblasts.
**Fig. 4.** PI3K is involved only in β2-AR-induced ERK activation and subsequent DNA synthesis, but not in EGFR phosphorylation. A, shown is ISO-induced EGFR phosphorylation and ERK activation in the presence of wortmannin. Passage 1 cardiac fibroblasts were serum-starved for 72 h. Tyrosine phosphorylation was stimulated upon treatment with 10 μM ISO and 10 ng/ml EGF for 2 min in the presence of a Me2SO vehicle control and 1 μM wortmannin. Western blot analysis was performed as described under “Experimental Procedures.” B, data are expressed as means ± S.E. of fold change in EGFR phosphorylation relative to the Me2SO (DMSO) control (n = 5). C, data are expressed as means ± S.E. of fold change in ERK activation relative to the Me2SO control (n = 5; *, p < 0.05 for an inhibitory effect of wortmannin versus ISO and EGF stimulation). D, ISO-induced DNA synthesis was inhibited by wortmannin. DNA synthesis in cardiac fibroblasts in the presence of 1 μM wortmannin was determined (see “Experimental Procedures”). [3H]Thymidine incorporation during 48 h of 1 μM ISO and 10 ng/ml EGF treatment was measured (n = 5, determined in triplicate). *, p < 0.05 for inhibition of wortmannin versus ISO stimulation. CN, control; P-EGFR, phosphorylated EGFR; P-ERK, phosphorylated ERK.

and to determine whether PI3K is involved in β2-AR-mediated EGFR transactivation and subsequent ERK-mediated cell proliferation. To determine the requirement of PI3K in this signaling pathway, serum-starved cardiac fibroblasts were incubated with 1 μM wortmannin, a pharmacological inhibitor of PI3K, prior to ISO or EGF addition. Protein immunoblotting for the phosphorylated EGFR showed that wortmannin did not affect ISO- or EGF-mediated EGFR phosphorylation (Fig. 4, A and B). However, 1 μM wortmannin partially, but significantly, attenuated ISO-induced ERK activity by 40% compared with vehicle pretreatment (n = 5; p < 0.05) (Fig. 4, A and C). In addition, to elucidate the signaling pathways necessary for PI3K to exert its mitogenic effect following β-AR stimulation, we analyzed DNA synthesis in the presence of wortmannin or Me2SO (vehicle). As shown in Fig. 4D, wortmannin attenuated only ISO-induced DNA synthesis in adult rat cardiac fibroblasts and not that stimulated by EGF. Interestingly, the baseline activity was also inhibited (Fig. 4D), suggesting that PI3K is required for basal DNA synthesis in adult rat cardiac fibroblasts.

**MMP Inhibition of ISO-induced ERK Activation**—Recent studies have indicated that the transactivation of the EGFR by several GPCRs, including β2-ARs, requires pro-HB-EGF processing that can be blocked by a synthetic MMP inhibitor, batimastat (17, 21, 31). To determine whether this cascade is involved in cardiac fibroblast proliferation, we used the MMP-specific inhibitor BB2116 and measured β2-AR-mediated ERK activation. As shown in Fig. 5 (A and B), BB2116 inhibited ISO-induced ERK activation (n = 5; p < 0.01), but not that induced by EGF addition. In addition, BB2116 pretreatment also attenuated ISO-induced DNA synthesis in adult rat cardiac fibroblasts (n = 4; p < 0.05) (Fig. 5C).

A **Neutralizing Antibody for HB-EGF Blocks ISO-induced ERK Activation**—Because ISO-induced ERK activation and DNA synthesis were markedly inhibited by BB2116 in adult rat cardiac fibroblasts (Fig. 5), we wanted to assess whether this specifically involved the release of EGFR ligands. To do this, we used a neutralizing antibody for HB-EGF, one of the ligands for the EGFR. The results shown in Fig. 6 (A and B) show that the antibody for HB-EGF strongly blocked ISO-induced phosphorylation of ERK, but had no effect on EGF-induced phosphorylation of ERK. We next examined the effects of heparin on β-AR-mediated ERK activation because cleaved HB-EGF associates with the heparin sulfate proteoglycan matrix, preventing the immediate release of the mature growth factor into the conditioned medium. Heparin treatment markedly attenuated ISO-induced ERK activation (Fig. 6C). These results supply direct evidence that ISO-induced shedding of HB-EGF mediates β-AR-mediated ERK activation, and the processing of HB-EGF as a cell-associated ligand by MMP appears to be an essential component for ERK activation and subsequent DNA synthesis following ISO stimulation in adult rat cardiac fibroblasts.
DISCUSSION

Cardiac fibroblasts are an important component of the heart, as they comprise as much as two-thirds of cell population in cardiac tissue (26, 32, 33). Unlike cardiac myocytes, cardiac fibroblasts can proliferate even in the adult heart as well as produce extracellular matrix proteins such as fibronectin and collagen (33). Fibroblasts also produce other putative growth factors that may act as autocrine and/or paracrine modulators of cell function and growth within the heart (23). Therefore, understanding which hormones and signaling pathways control proliferation and synthetic capacities of cardiac fibroblasts is necessary to assess their contribution to cardiac remodeling, fibrosis, and hypertrophy. Importantly, the β-AR signaling system remains the most powerful means by which heart rate and contractility are physiologically regulated and maintained and, as our current data point out, may also be important for cardiac fibroblast signaling and activation. The β-AR system in cardiac fibroblasts consists almost exclusively of the β2-AR subtype; and thus, catecholamine stimulation of the heart proceeds via β1- and β2-ARs in cardiomyocytes, but primarily via β2-ARs in cardiac fibroblasts (24–26). Importantly, other GPCRs such as those for angiotensin II and endothelin that can alter proliferation and collagen deposition in cardiac fibroblasts have been studied, but mitogenic signaling induced by catecholamines through the β2-AR system has not been extensively studied in adult cardiac fibroblasts, which was the target of this study.

In this study, we have demonstrated that the β-AR agonist ISO stimulates DNA synthesis via ERK in adult rat cardiac fibroblasts. Our data show that the β2-AR agonist ISO stimulates DNA synthesis via ERK in adult rat cardiac fibroblasts. Importantly, other GPCRs such as those for angiotensin II and endothelin that can alter proliferation and collagen deposition in cardiac fibroblasts have been studied, but mitogenic signaling induced by catecholamines through the β2-AR system has not been extensively studied in adult cardiac fibroblasts, which was the target of this study.
fibroblasts. An additional major finding of this study is that ISO-stimulated ERK activation and subsequent DNA synthesis in adult rat cardiac fibroblasts require three different kinase activities, EGFR, Src, and PI3K. Src is involved in both ERK transactivation and direct ERK activation; however, β2-AR-dependent PI3K activation is not involved in EGFR transactivation. Moreover, studies using a neutralizing antibody for HB-EGF and a MMP-specific inhibitor suggest that processing of HB-EGF via MMP activity is required for the β2-AR-mediated EGFR transactivation in cardiac fibroblasts. As depicted in Fig. 7, these current data provide novel insight into the major players involved in the proliferative signaling that takes place in response to catecholamine activation of the β2-AR system in adult cardiac fibroblasts.

An increase in DNA synthesis is common in cardiac hypertrophy; and although the terminally differentiated cardiac myocyte does not proliferate, mitogenic signaling may contribute to cell proliferation of non-myocytes (i.e. fibroblasts) in the heart. GPCR-mediated ERK activation has been demonstrated to play an important role in cell hypertrophy and proliferation in various cardiac cells, including neonatal and adult rat ventricular myocytes (10, 11, 34, 35), VSMCs (6, 7), and cardiac fibroblasts (8, 24). Interestingly, a previous study in neonatal rat cardiac fibroblasts demonstrated that β2-AR-mediated stimulation of protein synthesis does not require ERK activity (9); however, for DNA synthesis in adult rat cardiac fibroblasts, we have found that ERK inhibition by PD98059, a MEK inhibitor, significantly attenuates ISO-induced mitogenic signaling. This is an example of discrepancies commonly found for GPCR signaling in the neonatal versus adult cardiac system, indicating that hormonal responsiveness and downstream effectors are disparate at different stages of development. Thus, identifying signaling components and proliferative mechanisms in each cardiac cell type and developmental stage is essential for a more complete understanding of the effects of hormones on cardiac function.

An area of recent interest in β2-AR and GPCR mitogenic signaling is transactivation of the EGFR (14, 15, 17, 31). We have found that, in adult rat cardiac fibroblasts, the tyrosine kinase activity of EGFR is clearly required for β2-AR-mediated ERK activation and subsequent DNA synthesis. Moreover, β2-AR activation in adult cardiac fibroblasts induces tyrosine phosphorylation of the EGFR presumably through multiple inputs (Fig. 7). Importantly, the trigger for this β2-AR-mediated EGFR transactivation in adult rat cardiac fibroblasts appears to involve Gβγ heterotrimeric G proteins, as PTX attenuated ISO-stimulated EGFR and ERK activation as well as DNA synthesis. Thus, as in cardiomyocytes, the β2-AR in cardiac fibroblasts can couple to both Gα and Gβγ proteins. Interestingly, in cardiomyocytes, dual β2-AR coupling to PTX-sensitive G proteins may be involved in novel regulation of calcium signaling and contractile function (36).

Our current data also show that the Src tyrosine kinase is involved in β2-AR-mediated mitogenic signaling in rat cardiac fibroblasts, which is consistent with other known GPCR signaling cascades (37). Src activity precedes EGFR transactivation, as both EGFR phosphorylation and ERK activity were attenuated in response to ISO in adult cardiac fibroblasts using the Src-specific inhibitor PP2. Importantly, GPCR-induced association of Src with the transactivated EGFR has been demonstrated previously in other cell types (38, 39). Moreover, in VSMCs, Src appears to be necessary for angiotensin II-mediated ERK activation (38). Thus, in adult cardiac fibroblasts, Src could be a link that connects the transactivated EGFR to ERK because it can be recruited by the β-arrestin-mediated internalization of desensitized β2-AR that is seen after ligand binding (40). The precise role of Src in transactivation remains to be determined, although β-arrestin-dependent recruitment of Src to the β2-AR may provide the initial mechanism for recruiting Src to the plasma membrane. Nevertheless, our present findings demonstrate that Src is a major regulatory protein involved in cardiac fibroblast proliferation after ISO stimulation by both an EGFR-dependent and Src-dependent EGFR-independent manner.

Although we did not specifically address the issue, the involvement of PTX-sensitive G proteins in β2-AR-mediated mitogenic signaling in cardiac fibroblasts suggests that G protein βγ-subunits (Gβγ) may be involved because Gαi primarily activates ERK in many cell types via Gβγ (41, 42). Accordingly, we speculated that Gβγ-mediated src might also play a role in β2-AR-mediated mitogenic signaling in these cardiac fibroblasts. One such kinase, PI3K, can regulate a number of cellular processes, including protein synthesis; cytoskeletal reorganization; cell growth; and suppression of apoptosis in numerous cell types, including neonatal and adult rat cardiomyocytes (43, 44). Moreover, β2-AR-dependent PI3K activity may be involved in cardiac hypertrophy (4). Consistent with this, it has recently been shown that ISO-induced protein synthesis in neonatal rat cardiac fibroblasts is concomitant with increased PI3K activity (9). In the present study, PI3K appeared to be required for β2-AR-mediated ERK activation, which plays a major role in DNA synthesis in adult rat cardiac fibroblasts. However, EGFR activation following ISO stimulation was independent of PI3K activity (9). In the present study, PI3K activity is also required for the ISO mitogenic response. Thus, β2-AR/PI3K/ERK signaling can occur independently of EGFR activity (Fig. 7).

Importantly, transactivation of the EGFR by GPCRs is an important mechanism that helps explain mechanistic questions regarding cell growth promotion by GPCRs (14, 15). Recently, the MMP-dependent generation of HB-EGF has been reported to mediate EGFR transactivation (17, 18, 21) and ERK activation (22, 31). The potential importance of MMP activity and “shedding” extends to cardiovascular cells, as thrombin and angiotensin II induce HB-EGF-dependent EGFR phosphorylation via MMPs in VSMCs (31). Moreover, the MMP
inhibitor BB94 inhibits VSMC migration and DNA synthesis following arterial injury (19, 45). In the present study, we used the MMP inhibitor BB2116 as well as a neutralizing antibody for HB-EGF to demonstrate attenuation of β-AR-mediated ERK activation and DNA synthesis in adult rat cardiac fibroblasts. These data implicate MMP activity and HB-EGF shedding as critical components of fibroblast proliferation in response to catecholamine stimulation.

Interestingly, HB-EGF itself has previously been implicated in heart diseases, as its transcriptional regulation has been linked to cardiac hypertrophy (46) and heart failure.2 Moreover, the enhanced expression of HB-EGF mRNA and protein in the left ventricle of spontaneously hypertensive rats suggests that this growth factor may play an important role during the early development of hypertrophy and cardiac fibrosis (47). Interestingly, our present data suggest that these phenotypic changes of hypertrophy and fibrosis might be through the actions of HB-EGF in cardiac fibroblasts because we have shown that MMP-dependent processing of EGFR ligands such as HB-EGF and the transactivation of EGFR are important components of mitogenic signaling in fibroblasts. Overall, the extent to which cardiac fibroblast activation and proliferation play in myocardial hypertrophy and failure is a source of debate; however, our data demonstrate that potent β-AR/receptor tyrosine kinase mitogenic signaling pathways are active in these cells. Therefore, because catecholamines are elevated in hypertrophy and heart failure, these signaling mechanisms in cardiac fibroblasts are no doubt being activated and may represent potential novel targets for the therapeutic treatment of heart disease.

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