**Protease-Activated Receptor-1–Mediated DNA Synthesis in Cardiac Fibroblast Is via Epidermal Growth Factor Receptor Transactivation**

Distinct PAR-1 Signaling Pathways in Cardiac Fibroblasts and Cardiomyocytes

Abdelkarim Sabri, Jacob Short, Jianfen Guo, Susan F. Steinberg

**Abstract**—Proteases elaborated by inflammatory cells in the heart would be expected to drive cardiac fibroblasts to proliferate, but protease-activated receptor (PAR) function in cardiac fibroblasts has never been considered. This study demonstrates that PAR-1 is the only known PAR family member functionally expressed by cardiac fibroblasts and that PAR-1 activation by thrombin leads to increased DNA synthesis in cardiac fibroblasts. The increase in DNA synthesis induced by PAR-1 substantially exceeds the effects of other G protein–coupled receptor agonists in this cell type. PAR-1 stimulates phosphoinositide hydrolysis and mobilizes intracellular calcium via pertussis toxin (PTX)-sensitive and PTX-insensitive pathways. Activation of PAR-1 leads to an increase in Src, Fyn, and epidermal growth factor receptor (EGFR) phosphorylation, with EGFR receptor transactivation by Src family kinases the major mechanism for PAR-1–dependent activation of extracellular signal–regulated kinase, p38-mitogen-activated protein kinase, and protein kinase B. Activation of PAR-1 also leads to an increase in DNA synthesis. PAR-1 signaling is highly contextual in nature, inasmuch as PAR-1 activates extracellular signal–regulated kinase and only weakly stimulates protein kinase B via a pathway that does not involve EGFR transactivation in cardiomyocytes. PAR-1 responses in cardiac fibroblasts and cardiomyocytes are predicted to contribute importantly to remodeling during cardiac injury and/or inflammation.

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**Key Words:** thrombin ■ protease-activated receptors ■ cardiac fibroblasts ■ epidermal growth factor receptors ■ signal transduction

Cardiomyocytes occupy as much as 75% of cardiac mass but constitute only about one third of the total cell number in the heart. The remaining noncardiomyocytes consist mainly of interstitial cardiac fibroblasts, which provide structural support for cardiomyocytes, regulate extracellular matrix, and are the source of paracrine growth factors. Fibroblast proliferation and synthesis of matrix is essential for scar formation at sites of myocardial infarction. However, replacement of necrotic myofibrils by noncontractile fibrotic scar tissue disrupts the transmission of electrical impulses. Extensive fibroblast-induced adverse structural remodeling also occurs at distal noninfarcted segments of the ventricle, where it leads to diastolic stiffness and ultimately contributes to mechanical failure.

Cardiac fibroblast growth and fibrillar collagen synthesis is highly regulated by humoral and mechanical stimuli. Angiotensin II (Ang II) and endothelin have been implicated as important mediators of interstitial remodeling in the context of hypertension, coronary heart disease, myocarditis, and congestive heart failure. Ang II acts via a G protein–coupled receptor (GPCR) to stimulate a spectrum of biochemical signals that culminate in the expression of growth-associated nuclear protooncogenes and mitogenesis. Initial studies implicated G, protein βγ subunits, Src family tyrosine kinases, tyrosine phosphorylation of the adapter protein Shc, and the formation of Shc-Grb2 complexes in the pathway, leading to extracellular signal–regulated kinase (ERK) activation by Ang II. This model was subsequently extended to include an obligatory role for epidermal growth factor (EGF) receptor (EGFR) transactivation in Ang II–dependent stimulation of ERK and induction of DNA synthesis. Of note, Ang II also activates ERK in cardiomyocytes, but this reportedly occurs via a distinct pathway involving protein kinase C (PKC), not tyrosine kinases. The other GPCR reported to be mitogenic for cardiac fibroblasts is the endothelin receptor; although EGFR transactivation has been implicated in endothelin receptor activation of ERK in heterologous expression systems, the pathway for endothelin-dependent mitogenesis in...
cardiac fibroblasts involves PKC.4,5 These results emphasize the highly contextual nature of GPCR responses and the need for experiments that define GPCR signaling in clinically relevant targets, such as cardiac fibroblasts and cardiomyocytes.

Thrombin is a serine protease that is generated at sites of vascular injury. Thrombin has well-characterized functions in hemostasis. Thrombin also plays a very important role in wound healing as a result of the direct proteolytic activation of a family of GPCRs that influence cell shape, growth, and differentiation. The prototypical receptor for thrombin, protease-activated receptor (PAR)-1, is activated by cleavage of its extracellular N-terminus to expose a new N-terminal sequence that binds intramolecularly and serves as a tethered ligand.6 PAR-1 activation is also accomplished nonproteolytically by synthetic peptides that mimic its tethered ligand sequence (SFLLRN). PAR-3 and PAR-4 (newer PAR family members) are also activated by thrombin, whereas serine proteases with different specificity (trypsin or mast cell lysytically by synthetic peptides that mimic its tethered ligand sequence that binds intramolecularly and serves as a tethered ligand.6 PAR-1 activation is also accomplished nonproteolytically by synthetic peptides that mimic its tethered ligand sequence (SFLLRN). PAR-3 and PAR-4 (newer PAR family members) are also activated by thrombin, whereas serine proteases with different specificity (trypsin or mast cell trypsate, but not thrombin) activate PAR-2.6 Thrombin-proteases with different specificity (trypsin or mast cell lysytically by synthetic peptides that mimic its tethered ligand sequence that binds intramolecularly and serves as a tethered ligand.6 PAR-1 activation is also accomplished nonproteolytically by synthetic peptides that mimic its tethered ligand sequence (SFLLRN). PAR-3 and PAR-4 (newer PAR family members) are also activated by thrombin, whereas serine proteases with different specificity (trypsin or mast cell trypsate, but not thrombin) activate PAR-2.6 Thrombin-dependent cleavage of PAR-1 leads to fibroblast, vascular smooth muscle cell, and endothelial cell proliferation. PAR-1 elicits a spectrum of acute signaling responses (phosphoinositide hydrolysis, calcium mobilization, PKC activation, ERK activation, and c-fos expression), which are required for protein synthesis, but thrombin-dependent mitogenesis requires protein tyrosine phosphorylation.7,8 Initial studies identified PAR-1-dependent induction of various autocrine/paracrine mitogens whose actions at receptors with tyrosine kinase activity support progression through the cell cycle.9,10 The focus has shifted more recently to tyrosine phosphorylation of EGFR family members, with EGFR transactivation implicated in PAR-1 activation of ERK in heterologous expression systems.11 Of note, EGFR transactivation by native PAR-1 (ie, under conditions that avoid promiscuous interactions that are due to receptor overexpression) has never been reported, and there is, as yet, no evidence that EGFR kinase activity is required for mitogenic signaling by PAR-1.

Cardiomyocytes express PAR-1, which activates a spectrum of biochemical signals (phosphoinositide hydrolysis, mobilization of intracellular calcium, activation of ERK, and, to a lesser extent, c-Jun N-terminal kinase, p38-mitogen-activated protein kinase [p38-MAPK], and protein kinase B [Akt]) that culminate in acute changes in contractile performance and more chronic changes in gene expression, sarcomeric organization, and cardiomyocyte morphology.12–15 PAR-1 activation by thrombin (or proteases elaborated by inflammatory cells in the border zone of an infarct, a region that displays prominent cellular and matrix remodeling) would be expected to drive cardiac fibroblasts to proliferate during myocardial ischemia and/or infarction. However, a role for thrombin-dependent activation of PAR-1 in cardiac fibroblasts has never been considered. This study is the first to identify PAR-1 signaling in cardiac fibroblasts; important differences in the signaling cascades emanating from PAR-1 in cardiac fibroblasts and cardiomyocytes are also identified. These PAR-1–dependent responses likely contribute to pathophysiologically important remodeling in areas of cardiac inflammation.

**Materials and Methods**

Cultures of neonatal rat ventricular myocytes and cardiac fibroblasts were prepared, and assays of inositol phosphate (IP) accumulation were performed according to methods described previously.14 Immunoblotting was according to previously published methods or manufacturer’s instructions with antibodies for total or phosphorylated (activated) signaling proteins from the following sources: phosho-ERK1/2, total and phosho-p38-MAPK, and total and phosho-Akt were from Cell Signaling Technology; ERK1/2 and EGFR were from Santa Cruz Biotechnology. Each panel in each figure (Figures 3 through 6 and online Figures 1, 3, and 4, which can be accessed in the online data supplement at http://www.circresaha.org) represents results from a single gel exposed for a uniform duration, with bands detected by enhanced chemiluminescence and quantified by laser scanning densitometry.

Src kinase activity was assayed on lysates from cells extracted for 10 minutes on ice in extraction buffer (50 mmol/L HEPES, pH 7.4, 1 mmol/L EGTA, 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 10 mmol/L sodium orthovannadate, 10 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 10 µg/mL leupeptin, 10 µg/mL aprotinin, and 0.5 mmol/L phenylmethylsulfonyl fluoride). c-Src, Fyn, or Yes was immunoprecipitated from 550 µg lysates (precleared with protein G Sepharose). Immunoprecipitates were successively washed with extraction buffer, buffer A (20 mmol/L Tris [pH 7.4], 0.5 mmol/L LiCl, and 1 mmol/L EDTA), and buffer B (20 mmol/L Tris [pH 7.4], 10 mmol/L MnCl, and 1 mmol/L EDTA), followed by incubation with 30 µmol/L kinase buffer containing 20 mmol/L Tris (pH 7.4), 10 mmol/L MnCl, 1 mmol/L EGTA, 5 mmol/L MgCl, 5 µmol/L cold ATP, and 10 µCi [32P]ATP with 1 µg/mL enolase as substrate for 15 minutes at 20°C. Reactions were stopped with sample buffer, and proteins were separated by SDS-PAGE (10% gel); quantification was with a PhosphoImager (Molecular Dynamics). Precleared lysates (700 µg) were also subjected to immunoprecipitation using anti-EGFR antibodies and immunoblotting with anti-EGFR and anti-phosphotyrosine antibodies.

DNA synthesis was monitored as [3H]thymidine incorporation. Cells were growth-arrested by incubation in serum-free medium for 24 hours and then stimulated with the indicated agonist for 24 hours (with [3H]thymidine, 1 µCi/mL supplementing the culture medium for the final 4 hours). [3H]Thymidine in trichloroacetic acid (10%)–precipitated fractions was counted in a liquid scintillation counter.

Cytosolic calcium was measured photometrically in fura 2–loaded cardiac fibroblasts according to methods published previously.15 Cells were loaded with the acetoxymethyl ester (AM) form of fura 2 by incubation with 3 µmol/L fura 2-AM and 1.5 µL of 25% (w/v) in dimethyl sulfoxide) Pluronic F-127 (BASF Wyandotte Corp) for 20 minutes at 37°C. Cells were rinsed and maintained for at least 15 minutes at room temperature to ensure deesterification of the dye. Intracellular fura 2 fluorescence was monitored with a device (Photon Technologies, Inc) that alternately illuminates the cells with 340- and 380-nm light while measuring emission at 520 nm from a single cell within a group of cells (sampling rate 100 Hz). Cytosolic free calcium was calculated from the fura 2 fluorescence ratio at the two excitation wavelengths, with fluorescence calibrated to calcium concentration at the end of each experiment with ionomycin (10 µmol/L, maximal fluorescence) followed by MnCl2 (10 µmol/L, minimal fluorescence).

**Results**

**PAR-1 Activates PLC and Mobilizes Calcium via PTX-Sensitive and PTX-Insensitive Pathways in Cardiac Fibroblasts**

Figure 1A shows that SFLLRN (agonist peptide based on the tethered ligand sequence of PAR-1) and thrombin induce dose-dependent increases in phospholipase C (PLC) activity in cardiac fibroblasts. SFLLRN has stimulatory properties at both PAR-1 and PAR-2, and thrombin is an agonist for both...
PAR-1 and (at high concentrations) PAR-4. Therefore, initial studies considered whether responses to SFLLRN and thrombin might report the combined actions of PAR-1 and other PAR family members. Figure 1B shows that SLIGRL (PAR-2 agonist peptide) induces substantial PLC activation in cardiomyocytes but not in cardiac fibroblasts. In the context of our previous study, which identified PAR-2 transcripts in cardiomyocytes but not in cardiac fibroblasts, these results suggest that PAR-2 does not substantially contribute to SFLLRN responses in cardiac fibroblasts. A significant contribution from PAR-4 is also unlikely, because the modified PAR-4 agonist peptide AYPGKF (=10-fold more potent than the PAR-4 tethered ligand sequence GYPGKF at activating PAR-4) activates PLC in cardiomyocytes but does not detectably stimulate PLC in cardiac fibroblasts (Figure 1B). The EC50 for thrombin-dependent activation of PLC is ~0.3 nmol/L (Figure 1A), which corresponds well with thrombin concentrations typically required to activate PAR-1. Collectively, these results identify PAR-1 as the dominant receptor mediating the actions of thrombin in cardiac fibroblasts.

SFLLRN promotes the rapid and transient accumulation of inositol polyphosphates (IP3 and IP2), which is followed by more sustained IP1 accumulation (Figure 1C). Both the rapid elevations in IP2/IP3 levels and the more sustained accumulation of IP1 are markedly attenuated in cells pretreated with PTX according to a protocol that is associated with complete ADP-ribosylation (inactivation) of Gαi proteins (Figure 1D). Thrombin-dependent activation of PLC displays similar kinetics and PTX sensitivity (data not shown). This identifies PTX-sensitive and PTX-insensitive pathways for PLC activation by PAR-1 in cardiac fibroblasts. The PAR-1–dependent increase in IP1 is associated with the predicted increase in intracellular calcium, which is also attenuated (both in amplitude and duration) in PTX-pretreated cardiac fibroblasts (Figure 2; see online Table 1, which can be accessed in the data supplement at http://www.circresaha.org).

**PAR-1 Activates ERK, p38-MAPK, and Akt via a PTX-Sensitive Pathway and EGFR Transactivation in Cardiac Fibroblasts**

MAPK protein phosphorylation cascades integrate input from GPCRs and tyrosine kinase receptors and figure prominently as downstream targets of PAR-1 signaling in many cell types. Figure 3 shows that PAR-1 activation with SFLLRN or thrombin induces robust ERK and p38-MAPK activation in cardiac fibroblasts, as detected by immunoblot analysis with antibodies that specifically recognize the phosphorylated PAR-1 and (at high concentrations) PAR-4. Therefore, initial studies considered whether responses to SFLLRN and thrombin might report the combined actions of PAR-1 and other PAR family members. Figure 1B shows that SLIGRL (PAR-2 agonist peptide) induces substantial PLC activation in cardiomyocytes but not in cardiac fibroblasts. In the context of our previous study, which identified PAR-2 transcripts in cardiomyocytes but not in cardiac fibroblasts, these results suggest that PAR-2 does not substantially contribute to SFLLRN responses in cardiac fibroblasts. A significant contribution from PAR-4 is also unlikely, because the modified PAR-4 agonist peptide AYPGKF (=10-fold more potent than the PAR-4 tethered ligand sequence GYPGKF at activating PAR-4) activates PLC in cardiomyocytes but does not detectably stimulate PLC in cardiac fibroblasts (Figure 1B). The EC50 for thrombin-dependent activation of PLC is ~0.3 nmol/L (Figure 1A), which corresponds well with thrombin concentrations typically required to activate PAR-1. Collectively, these results identify PAR-1 as the dominant receptor mediating the actions of thrombin in cardiac fibroblasts.

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**Figure 1.** PAR-1 activates PLC in cardiac fibroblasts through PTX-sensitive (+PTX) and PTX-insensitive (−PTX) pathways. A through C, Stimulation with the indicated concentrations of SFLLRN or thrombin for 10 minutes (IP2) or 2 minutes (for IP3 and IP1) (A), SLIGRL (300 μmol/L) or AYPGKF (500 μmol/L) for 10 minutes (B), or SFLLRN (100 μmol/L) for the indicated intervals in cultures pretreated with vehicle or PTX (100 ng/mL) for 24 hours (C). For each panel, results are expressed as counts per minute (CPM) over basal (mean±SEM, n=3 or 4 separate cultures). D, In vitro PTX-dependent [32P]ADP-ribosylation of membranes from vehicle (−) or PTX-treated cardiac fibroblast cultures according to methods published previously.

**Figure 2.** PAR-1 increases intracellular calcium via PTX-sensitive and PTX-insensitive pathways. Shown are representative fura 2 recordings of intracellular calcium in a control and PTX-treated cardiac fibroblast before and during bolus stimulation with SFLLRN (100 μmol/L final concentration).
forms of these enzymes (or the antibody that recognizes total ERK protein, where phosphorylation/activation is associated with reduced mobility of the protein). ERK activation by SFLLRN and thrombin is rapid and transient; the magnitude with reduced mobility of the protein). ERK phosphorylation (pERK) measurements

Figure 3. PAR-1 activates ERK1/2 and p38-MAPK cascades via pathways that require $G_{i/o}$ and EGFR tyrosine kinase activity in cardiac fibroblasts. Cells were serum-starved for 24 hours with vehicle or PTX (100 ng/mL) as indicated. Pretreatment was with vehicle or AG1478 (2 μmol/L, 45 minutes), followed by incubations for 5 minutes in the absence or presence of thrombin (1 nmol/L) or SFLLRN (100 μmol/L). Cell lysates were subjected to SDS-PAGE and Western blotting with anti–phospho-ERK1/2 or anti–phospho-p38 MAPK antibodies. Blots were stripped and subjected to immunoblot analysis with total ERK1/2 or p38-MAPK to normalize for minor variations in protein loading. A, Representative autoradiograms. Prefix P indicates phospho. B, Quantification of ERK phosphorylation (pERK) measurements (n=3; measurements of p38-MAPK activation yielded similar results [data not shown]).

Figure 4. PAR-1–dependent stimulation of ERK and Akt requires EGFR kinase activity in cardiac fibroblasts but not in cardiomyocytes. Cardiac fibroblasts or cardiomyocytes were treated with thrombin (Thr, 1 nmol/L) or EGF (100 nmol/L) for 5 minutes without or with AG1478 (2 μmol/L, starting 45 minutes before stimulation). Western blotting was with the anti–phospho-ERK1/2 (p-ERK) or anti–phospho-Akt (p-Akt) antibodies, with gels representative of results obtained in 3 separate experiments. Immunoblotting for total Akt was performed on stripped blots to normalize for minor variations in protein loading.

tify a stringent requirement for EGFR kinase activity in the PAR-1 signaling pathway. Figure 3 shows that SFLLRN- and thrombin-dependent activation of ERK and p38-MAPK is largely abrogated by combined treatment with PTX and AG1478. Hence, PAR-1 activates ERK and p38-MAPK via two distinct pathways: one pathway involves $G_i$ proteins, whereas the other pathway requires EGFR tyrosine kinase activity.

PLC enzymes are classified according to differences in their structure and modes of activation; PLCβ isoforms are activated by heterotrimeric G proteins ($G_o$ and $G_i$), whereas PLCγ isoforms are activated by tyrosine phosphorylation. Figure 1C shows that SFLLRN-dependent IP accumulation is markedly attenuated by PTX; however, substantial residual PLC activation by SFLLRN remains in PTX-treated cultures. If the mechanism for this residual PLC activation involved the transactivated EGFR (as a scaffold to recruit PLCγ), it would be blocked by AG1478. However, SFLLRN-dependent accumulation of IP1/IP3 (at 2 minutes) or IP3 (at 30 minutes) is fully preserved in cells treated with AG1478 (see data in online supplement), suggesting that EGFR transactivation functionally lies downstream from PAR-1–dependent activation of PLC.

Akt, also known as protein kinase B, has been implicated in metabolic responses, cell cycle control leading to proliferation, and protection from apoptosis. Akt activation is prominent in response to tyrosine kinase growth factor receptor stimulation, for which full kinase activation requires phosphorylation at Thr308 in the activation loop and Ser473 in a C-terminal domain. Some GPCRs are reported to activate Akt, but the mechanism(s) remains disputed. Figure 4 shows that PAR-1 markedly activates Akt in cardiac fibroblasts, whereas only a modest level of Akt activation by PAR-1 is variably detected in cardiomyocytes. The robust increase in Akt (and ERK) phosphorylation induced by thrombin in cardiac fibroblasts is highly sensitive to inhibition by AG1478; in contrast, the modest thrombin-induced increase in Akt phosphorylation in cardiomyocytes is relatively refractory to the inhibitory effects of AG1478. PAR-1 induces a robust increase in ERK in cardiomyocytes, but this is also via
a mechanism that differs from the activation pathway in cardiac fibroblasts; it too is relatively refractory to inhibition by AG1478. These results indicate that PAR-1 signaling pathways vary according to cell context; EGFR transactivation figures prominently in PAR-1 signaling in cardiac fibroblasts, but EGFR transactivation contributes little to PAR-1 signaling in cardiomyocytes.

In cardiac fibroblasts, Akt activation by PAR-1 agonists is attenuated slightly by PTX pretreatment, inhibited more strongly by AG1478, and abrogated in their combined presence (Figure 5A). These results identify PAR-1 signaling pathways vary according to cell context; EGFR transactivation figures prominently in PAR-1 signaling in cardiac fibroblasts, but EGFR transactivation contributes little to PAR-1 signaling in cardiomyocytes.

PAR-1 Activation by Thrombin or SFLLRN Increases DNA Synthesis

Ang II and endothelin-1 are widely recognized GPCR agonists that promote cardiac fibroblast proliferation. Figure 7
shows that the PAR-1 agonist peptide SFLLRN and thrombin increase DNA synthesis in cardiac fibroblasts and that these responses are substantially greater than the responses to endothelin-1 and Ang II (included as positive controls at maximally stimulatory concentrations in the assays). The PAR-1-dependent increase in DNA synthesis is attenuated by PTX; it is inhibited even more strongly by the EGFR antagonist AG1478 (either alone or combined with PTX, Figure 7B). In contrast, there is no inhibition with the equimolar platelet-derived growth factor receptor antagonist AG1295. Collectively, these results identify potent effects of PAR-1 to increase DNA synthesis via an EGFR transactivation pathway in cardiac fibroblasts.

**Discussion**

The present study constitutes the first report of PAR-1 actions in cardiac fibroblasts. The results support the following novel observations: (1) PAR-1 is the only known PAR family member functionally expressed by cardiac fibroblasts, with effects on DNA synthesis that substantially exceed those elicited by other GPCR agonists. (2) PAR-1 signaling to ERK, p38-MAPK, and Akt displays a stringent requirement for Src family kinases and EGFR transactivation; EGFR transactivation contributes to the mitogenic actions of PAR-1. (3) PAR-1 displays highly contextual signaling to the ERK cascade and Akt. Strong ERK and Akt activation is detected in cardiac fibroblasts in which EGFR transactivation is prominent; PAR-1 activates ERK (but induces a much more modest level of Akt activation) through a mechanism that displays little to no requirement for EGFR kinase activity in cardiomyocytes.

Figure 8 provides a model for PAR-1 signaling in cardiac fibroblasts. PAR-1 coupling to PTX-sensitive and PTX-insensitive (presumably Gi/o and Gq) proteins leads to rapid activation of PLC. This results in the formation of IP3 and mobilization of intracellular calcium through both PTX-sensitive and PTX-insensitive pathways; diacylglycerol formation and PKC activation are predicted to occur in parallel. Thrombin also activates Src family kinases; both Src and Fyn
are activated, but only Fyn displays constitutive association with EGFRs. Recent literature identifies the preferential association of Src (and the p85 subunit of PI3K) with EGFRs that have been phosphorylated by Src (rather than with EGFRs autophosphorylated at distinct sites19). This supports a model in which the thrombin-dependent activation of Src and/or Fyn leads to an increase in EGFR phosphorylation and the formation of consensus-binding sites for SH2 domain proteins such as Src itself, the p85-p110 complex (leading to the activation of Akt), and the Ras guanine nucleotide exchange factor complex Grb2-Sos (which assembles the Ras activation complex and activates the Raf/MEK/ERK cascade). Of note, studies reported herein are the first to identify preferential complex formation between EGFRs and Fyn (rather than Src). These studies are also the first to place the p38-MAPK cascade and proliferation downstream from EGFR transactivation in the PAR-1 signaling pathway. Of note, the present study also identifies two distinct roles for Src kinases. Src kinases lie upstream in the pathway leading to EGFR transactivation; Src kinases also link EGFRs to the activation of p38-MAPK (but not ERK or Akt). Previous experiments in model systems have implicated βγ dimers from PTX-sensitive G proteins as a mechanism that initiates signaling via the Src-Ras-MAPK cascades.19 However, there is ample evidence that Gα-coupled GPCR pathways also converge on Src kinase–operated EGFR transactivation.21 Whereas PAR-1 activates Gα in cardiac fibroblasts, it is the PTX-insensitive pathway that leads to EGFR transactivation; this PTX-insensitive pathway is the major mechanism whereby PAR-1 stimulates ERK, p38-MAPK, and Akt (and promotes proliferation) in cardiac fibroblasts.

EGFR transactivation is increasingly identified as a paradigm for cross talk between GPCRs (including PAR-1) and receptor tyrosine kinases. However, several recent studies described failed attempts to implicate EGFR transactivation in PAR-1 signaling (including evidence that EGFR kinase activity is not required for thrombin-dependent activation of ERK or induction of DNA synthesis in Swiss 3T3 cells and airway smooth muscle cells).22,23 Mechanisms that facilitate cross talk between GPCRs and EGFRs in some cells but restrict it in others are incompletely understood; differences in signaling machinery intrinsic to the cell type in which the GPCR resides likely is contributory. In this context, it is noteworthy that GPCR signaling to ERK in cardiomyocytes typically follows mechanisms described in other cell types, but (with one notable recent exception24) a pathway involving EGFR transactivation generally is not reported in cardiomyocytes. Although the paucity of literature identifying EGFR transactivation by cardiomyocyte GPCRs could be construed as indirect evidence that cardiomyocytes are deficient in this pathway, the present study directly compares GPCR signaling to MAPK cascades in cardiomyocytes and cardiac fibroblasts in a manner that supports this negative conclusion. A mechanism that restricts PAR-1 transactivation of EGFRs in cardiomyocytes is not obvious. Cardiomyocytes express EGFRs, including ErbB2 (which reportedly can be transactivated by PAR-1) and ErbB4 (HER4); these receptors stimulate signaling pathways (ERK and p70S6k) that promote growth and cardiomyocyte survival.25 However, studies reported herein indicate that the role of cardiomyocyte EGFRs does not extend to mediating GPCR responses. In this context, cardiac fibroblasts and cardiomyocytes exemplify the two distinct modes for PAR-1 signaling (EGFR-dependent and EGFR-independent, respectively), providing a potentially informative model system to explore the distinct requirements for EGFR transactivation by PAR-1.

Akt activation generally is reported in the context of signaling by growth factors that activate tyrosine kinase receptors, but Akt activation by GPCRs (including by thrombin in human platelets and rodent cardiomyocytes) has also been reported.14,25 However, the magnitude of Akt phosphorylation/activation by GPCRs typically is modest, relative to the robust activation by tyrosine kinase growth factor receptors. To date, no coherent mechanism(s) linking GPCR stimulation to Akt activation has been identified. Two recent studies reported Akt activation by βγ dimers (presumably originating from the abundant PTX-sensitive G proteins). A role for G protein α subunits (particularly αq and its downstream effector PKC) is disputed.26–28 In this context, studies reported herein indicate that Akt activation by PAR-1 is robust in cardiac fibroblasts that engage the EGFR transactivation mechanism but not in cardiomyocytes. This suggests a novel paradigm for GPCR control of the Akt pathway, with physiologically relevant Akt activation by GPCRs only in tissues in which GPCRs effectively support EGFR transactivation. However, because EGFR transactivation may involve distinct EGFR family members (which differ in their mechanisms of activation and signaling phenotypes, including their ability to support signaling to PI3K/Akt), differences in EGFR expression or activation could allow for diversity in signaling (between cell types and for different GPCR species). Previous studies identified the effects of thrombin to promote tyrosine phosphorylation of ErbB1 or ErbB2,4,11,18,29 but (with one notable recent exception24) a pathway involving ErbB3 transactivation by thrombin has not been reported. However, ErbB3 is transactivated by other GPCRs30 with its six consensus YXXM p85-binding motifs in the C-terminus. ErbB3 represents the EGFR family member best suited to activate the PI3K-Akt pathway. Given the current enthusiasm for Akt as a therapeutic target to halt the progression of heart failure syndromes, efforts to identify mechanisms that enable GPCR networking to EGFR transactivation (and a potential role for ErbB3, as an ancillary mechanism to promote Akt...
activation by GPCRs) would carry clinically important implications.

Recent studies support the concept that thrombin and serine proteases generated during coagulation and inflammation play important roles in tissue remodeling after injury.31 Substantial inflammatory infiltration accompanies certain cardiac disorders. These include the border zone of a myocardial infarct, where there is prominent cardiomyocyte hypertrophy, apoptosis, and tissue remodeling. Previous studies have established that cardiomyocytes express PAR-1 and are therefore likely to be targets for the cellular actions of serine proteases elaborated during inflammation and coagulation. Studies reported herein are the first to identify PAR-1 as a potent agonist for cardiac fibroblasts. PAR-1 actions in cardiac fibroblasts might be predicted to influence the extent of tissue injury, the resultant remodeling, and, hence, the outcome during myocardial infarct. According to this formulation, drugs targeted to PAR-1 (currently under development for other indications) must be considered as possible modifiers of cardiac function.

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References

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Supplemental Figure 1: SFLLRN- and thrombin-dependent activation of ERK, p38-MAPK, and AKT is inhibited by the EGF receptor inhibitor AG1478, but not by the PDGF receptor inhibitor AG1295. Protocols were performed as described in Figure 3, except that pretreatment was with vehicle or AG1295, or AG1498 (each at 2 μM, starting 45 min prior to stimulation with SFLLRN, thrombin, or EGF). Similar results were obtained in two separate experiments.
Supplemental Figure 2: IP₂+IP₃ accumulation (at 2 min) and IP₁ accumulation (at 30 min) is similar in cardiac fibroblast cultures pretreated for 45 min with vehicle or AG1478 (2 μM, n=4, NS).
**Supplemental Figure 3:** Heparin does not inhibit thrombin- or SFLLRN-dependent activation of ERK, p38-MAPK, or AKT. Protocols were performed as described in Figure 3, except that pretreatment was with vehicle or heparin (100 μg/ml for 60 min). Similar results were obtained in two separate experiments.
Supplemental Figure 4: Immunocomplex kinase assay of Yes activity. Protocols were performed as described in Figure 6A. Stimulation was with 1 U/ml thrombin or 5 mM H$_2$O$_2$. Similar results were obtained in another experiment.
Table 1. PAR-1 increases intracellular calcium via PTX-sensitive and PTX-insensitive pathways in cardiac fibroblasts.

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Values are calcium concentration (nM) before (basal) or 10 sec following bolus administration of SFLLRN or thrombin (mean±SEM). SFLLRN and thrombin induced significant increases in intracellular calcium in both control and PTX-treated cardiac fibroblasts (P<.05 vs basal). Stimulation by both SFLLRN and thrombin is significantly attenuated in PTX-treated cells (P<0.5 vs -PTX).