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Cardiac GPCR-mediated EGFR transactivation: impact and therapeutic implications

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Abstract

G protein-coupled receptors (GPCR) remain primary therapeutic targets for numerous cardiovascular disorders, including heart failure (HF), due to their influence on cardiac remodeling in response to elevated neurohormone signaling. GPCR blockers have proven to be beneficial in the treatment of HF by reducing chronic G protein activation and cardiac remodeling, thereby extending the lifespan of HF patients. Unfortunately this effect does not persist indefinitely, thus next generation therapeutics aim to selectively block harmful GPCR-mediated pathways while simultaneously promoting beneficial signaling. Transactivation of epidermal growth factor receptor (EGFR) has been shown to be mediated by an expanding repertoire of GPCRs in the heart, and promotes cardiomyocyte survival, thus may offer a new avenue of HF therapeutics. However, GPCR-dependent EGFR transactivation has also been shown to regulate cardiac hypertrophy and fibrosis by different GPCRs, and via distinct molecular mechanisms. Here, we discuss the mechanisms and impact of GPCR-mediated EGFR transactivation in the heart, focusing on angiotensin II, urotensin II and β -adrenergic receptor systems, and highlight areas of research that will help us to determine whether this pathway can be engaged as future therapeutic strategy.

Keywords

G protein-coupled receptor; epidermal growth factor receptor; cardiac remodeling

Introduction

G protein-coupled receptors (GPCRs) are a large family of 7 transmembrane-spanning proteins that mediate responses to a diverse array of external stimuli including hormones, neurotransmitters, odorants and photons. GPCRs are known to play a role in the development of cardiovascular disorders such as hypertension and heart failure, and therapeutics that target GPCRs constitute ~40% of currently used drugs, demonstrating their continued importance as pharmacological targets. Stimulation of GPCRs initiates a number of signaling pathways to regulate extensive cellular responses, which in the heart includes

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contractility, hypertrophy, proliferation, survival and fibrosis (Figure 1A)¹⁻⁴. While the proximal mechanisms relaying GPCR signals are now commonly recognized to include engagement of G protein- and/or GPCR kinase (GRK)/ β -arrestin (β arr)-dependent pathways⁴, the distal mechanisms that regulate cardiac cell behavior are divergent and vary by cell type and pathophysiologic status. Understanding how these mechanisms regulate cardiac remodeling during disease progression remains a fundamental goal of cardiac GPCR research and development of therapeutics. One such mechanism that has been shown to modulate various aspects of cardiac remodeling downstream of GPCRs is via transactivation of epidermal growth factor receptor (EGFR).

EGFR is a receptor tyrosine kinase, of which there are four closely related members: EGFR (ErbB-1, HER1), HER2 (ErbB2/neu), HER3 (ErbB3) and HER4 (ErbB4). While ErbB2-4 are beginning to be studied more frequently for their potential roles in the regulation of cardiomyocyte proliferation and cardiotoxicities related to cancer therapeutics⁵⁻⁸, EGFR is the most well-characterized family member in the heart. Several endogenous ligands, including epidermal growth factor (EGF), heparin binding-EGF (HB-EGF) and transforming growth factor α (TGF α)^{9, 10}, bind EGFR to induce dimerization or stabilization of an existing dimer and allow trans-phosphorylation of C-terminal tyrosine residues by the kinase domain, thereby engaging various signal transduction pathways¹¹.

EGFR was first shown to be activated by GPCRs 20 years ago when Ullrich's group reported that EGFR phosphorylation increased following treatment with several GPCR agonists including endothelin and thrombin, which could be blocked using an EGFR inhibitor or dominant-negative mutant EGFR¹². Since the initial discovery of GPCR-dependent EGFR transactivation, an increasing number of GPCRs have been identified as being capable of transactivating EGFR, including adenosine^{13, 14}, adrenergic¹⁵⁻²⁰, aldosterone²¹, angiotensin II²²⁻²⁵, bradykinin²⁶, δ -opioid²⁷, endothelin-1^{28, 29}, muscarinic³⁰, prostaglandin E2³¹, protease-activated^{32, 33}, sphingosine-1-phosphate³⁴ and urotensin II³⁵⁻³⁷ receptors. A growing body of evidence demonstrates that GPCR-mediated EGFR transactivation occurs in the heart, playing potentially important roles in both normal physiology as well as pathophysiology.

Mechanisms of GPCR-mediated EGFR transactivation and association

With regard to the EGFR transactivation process, there are several molecular mechanisms that comprise the means by which GPCR stimulation may lead to EGFR-dependent signaling, often occurring in both GPCR- and cell-specific manners, via either intracellular or extracellular pathways, as previously reviewed³⁸. Briefly, GPCR stimulation rapidly leads to both G protein-dependent and GRK/ β arr-dependent signaling responses, either of which may lead to EGFR transactivation. Several GPCRs induce EGFR activation via matrix metalloprotease (MMP)- or a disintegrin and metalloprotease (ADAM)-mediated cleavage of extracellular EGFR ligand, often HB-EGF, which can be induced in either a G protein- or GRK/ β arr-dependent manner. While this process typically involves intermediate signaling adapters or kinases such as Src, it was recently shown that G $\beta\gamma$ subunits can directly activate MMP14 via to induce HB-EGF cleavage and EGFR transactivation in cardiac

myocytes and fibroblasts²⁵. Thus, in a GPCR- and cell type-specific manner, EGFR transactivation may be engaged via multiple mechanisms (Figure 1B).

In addition to intermediate signaling components, some GPCRs structurally interact with EGFR in absence of receptor ligands which may enhance the efficiency of EGFR transactivation or focus the downstream signaling response to particular subcellular locales^{39–41}. Such multireceptor complexes may form on membrane microdomains, since blockage of lipid rafts has been demonstrated to disrupt a specific interaction between EGFR and the bile acid receptor TGR5⁴². This may be an especially important concept with respect to fully differentiated adult cardiomyocytes that are known to have sarcolemmal microdomains containing various GPCRs⁴³, some of which, like β 2AR, have been well-established to both interact with and transactivate EGFR^{39, 44}. Aside from membrane localization, modulation of GPCR activity may influence the stability of their interaction with EGFR, as stimulation of β 2AR with the full agonist isoproterenol (ISO) increased, while treatment with the inverse agonist ICI 188,551 reciprocally decreased, its association with EGFR³⁹. Interestingly, not all GPCR-EGFR complexes respond to their ligands in the same manner since β 1AR-EGFR interaction did not increase following stimulation with ISO, but dissociated in response to EGF⁴⁰, while stimulation of an AT1R-EGFR complex with either AngII or EGF increased its association in a temporal manner⁴¹.

The impact of receptor ligands on modulation of GPCR-EGFR interaction appears to be dependent on the GPCR-EGFR complex in particular and may suggest different mechanisms of both basal and post-stimulation association with EGFR. While β arr-dependent signaling has been implicated in maintaining or increasing β AR association with EGFR following catecholamine stimulation^{39, 40}, less is known about the actual structural requirements at the receptor level that allow for EGFR association. With regard to β 1AR, it was shown that mutation of all the putative C-terminal GRK phosphorylation sites almost completely abolished its ability to interact with EGFR, even in under non-stimulated conditions⁴⁰. Whether this was due to a GRK-specific effect or occurred as a by-product of a structural change in the C-terminus of β 1AR is not known, although another study showed that the third intracellular loop and a portion of the C-terminus of AT2R was required for interaction with ErbB3⁴⁵. Similarly, current knowledge regarding the specific region of EGFR that is responsible for GPCR interaction is lacking. However, it has been reported that the juxtamembrane domain of EGFR binds Gs protein and that an increase in Gs activity disrupts this interaction⁴⁶, thus the juxtamembrane domain of EGFR could serve as a point of association with Gs protein-coupled receptors.

Following ligand binding and EGFR dimerization, C-terminal trans-phosphorylation leads to adaptor protein recruitment and formation of signaling networks⁴⁷. EGFR has numerous tyrosine phosphorylation sites in its C-terminus and distinct phosphorylation patterns may occur in response to differential activation by various GPCRs, which could result in the recruitment of unique sets of adaptor proteins⁴⁸. This concept is similar to the “Barcode Hypothesis” for GPCRs where distinct phosphorylation patterns, or barcodes, on the C-terminal tail of GPCRs have been shown to produce differences in adaptor protein recruitment^{49–52}. While differential EGFR phosphorylation patterns have been observed in response to EGF stimulation in distinct cell lines⁴⁷, the impact of transactivation by various

GPCRs on EGFR phosphorylation patterns has not been extensively investigated. Indeed, a majority of studies have reported the impact of GPCR-mediated transactivation on phosphorylation of a limited number of tyrosine residues in the C-terminus of EGFR. However, unique GPCR-mediated EGFR phosphorylation patterns could contribute to divergent adapter protein recruitment, network formation and downstream signaling responses produced in different cardiac cell types in response to different ligands, processes that await discovery.

Impact and implications of GPCR-dependent EGFR transactivation

An expanding cohort of GPCRs (as listed above) has been shown to influence cardiac remodeling, including hypertrophy, fibrosis and survival, through the transactivation of EGFR. Here, we focus our discussion on three GPCR systems (AT1R, UTIIR and β AR) with distinct G protein-coupling characteristics and how they may engage EGFR to elicit changes in cardiac remodeling processes.

Angiotensin Type 1A Receptor (AT1R)

Angiotensin II (AngII), acting through AT1R, has been demonstrated to relay both pro-hypertrophic and pro-fibrotic effects via EGFR transactivation in the heart. For instance, AngII infusion-induced pathological cardiac remodeling in vivo was shown by several groups to be largely abrogated by EGFR inhibition, either via siRNA targeting EGFR⁵³, co-infusion with the EGFR inhibitor erlotinib⁵⁴ or overexpression of inhibitors of EGFR signaling, including receptor-associated late transducer (RALT)⁵⁵ and a dominant-negative EGFR mutation⁵⁶. At a cellular level, AT1R enhances both cardiomyocyte hypertrophy and cardiac fibroblast proliferation. AT1R-mediated EGFR transactivation-sensitive neonatal cardiomyocyte hypertrophy was initially demonstrated to require G α q protein activation, MMP activation and downstream ERK signaling²⁴, but was insensitive to PKC inhibition. Similarly, AngII treatment of cardiac fibroblasts was demonstrated to enhance EGFR phosphorylation and downstream ERK activation, leading to increased *cfos* expression and DNA synthesis, in a PKC-independent manner²². Instead, these responses were sensitive to treatment with either the EGFR inhibitor AG 1478, a dominant-negative EGFR mutant, a Ca²⁺ chelator or Ca²⁺/calmodulin kinase inhibitors. Further, DNA synthesis was unaffected by the addition of an anti-EGF antibody to the culture media and conditioned media taken from AngII-treated cells, suggesting an intracellular activation mechanism.

While the distal responses to AT1R-mediated EGFR transactivation appear to commonly involve ERK activation, the proximal mechanism(s) linking AT1R stimulation to EGFR transactivation-mediated effects on these processes are varied and somewhat controversial. While AT1R couples to both Gq/11 and G12/13 protein families⁴, Gq protein-mediated AT1R signaling was initially associated with EGFR transactivation-dependent effects on cardiac remodeling, although Gq protein-independent signaling via phosphorylation of AT1R at Tyr319 was later shown to contribute to remodeling processes. It was postulated that Tyr319 allowed binding with SHP2 to mediate interaction with and transactivation of EGFR and an AT1R-Y319F mutant no longer induced cardiac fibroblast proliferation⁵⁷. In fact, transgenic mice overexpressing AT1R-Y319F did not develop cardiac hypertrophy, in

contrast to mice overexpressing WT-AT1R, and were resistant to AngII infusion-induced hypertrophy, cardiomyocyte apoptosis and fibrosis, similar to mice overexpressing a dominant negative EGFR²³. Additionally, PKC, STAT3, ERK, Akt and Gαq activation were unaltered in Tg-Y319F mice compared to Tg-WT, suggesting that these pathways are not involved in the adverse effects of AngII treatment. However, more recent work has again highlighted the importance of AT1R-Gq protein-dependent EGFR transactivation in mediating cardiomyocyte hypertrophy⁵⁸, independent of Tyr319, and an elegant screening study identified numerous kinases that are likely to be involved in transmitting AT1R stimulation to EGFR transactivation, confirming roles for triple functional domain/PTPRF interacting (TRIO), bone marrow kinase in chromosome X (BMX) and choline kinase α (CHKA) in the process⁵⁹. BMX, a non-receptor tyrosine kinase, was subsequently shown to be involved in AngII-mediated cardiac hypertrophy, though the impact of BMX on EGFR transactivation was not specifically investigated in this study⁶⁰. Thus, the role these newer targets play in AT1R-dependent EGFR transactivation-induced cardiac hypertrophy or fibrosis remains to be directly tested.

Urotensin II receptor (UTIR)

Stimulation of UTIR, upregulated during congestive heart failure in human hearts⁶¹, also leads to EGFR transactivation in both in vitro and in vivo models. Urotensin II (UII) treatment has also been shown to engage EGFR transactivation in neonatal cardiomyocytes, independent of PKC, PI3K or Ca²⁺ mobilization, leading to increased ERK and p38 activity and resulting in hypertrophy³⁶. This effect on cardiomyocyte hypertrophy has also been shown to involve the generation of reactive oxygen species (ROS), which oxidize and inhibit the activity of the phosphotyrosine phosphatase SHP-2, thus increasing EGFR activation³⁷. UII-induced ROS generation was also shown to relay EGFR transactivation in cardiac fibroblasts, leading to enhanced ERK activation and proliferation⁶². Like AT1R, UTIR also couples to Gq protein to exert many of its effects⁶³, however it has also been demonstrated to signal via Gi protein, though an essential role for either G protein in relaying EGFR transactivation has not been defined. Indeed, G protein-independent signaling may be more crucial for UII-induced EGFR transactivation since UII treatment of HEK 293 cells initiated βarr-dependent EGFR transactivation and internalization, which was essential for relaying a decrease in cell death³⁵. Regardless of the proximal mechanisms involved, blocking UTIR using the UII antagonist urantide in a mouse model of pressure overload (transaortic constriction, TAC) was shown to worsen cardiac function, fibrosis and cell death outcomes, changes associated with decreased EGFR and ERK phosphorylation³⁵. Thus, the cardiac urotensin II-EGFR signaling system may represent an as-yet untapped therapeutic target for HF drug development.

β-Adrenergic Receptors (βAR)

Both β1AR and β2AR have been shown to interact with EGFR and mediate its transactivation using a similar molecular mechanism. Both βAR isoforms rely on G protein-independent, GRK/βarr-dependent signaling to promote Src- and MMP-mediated HB-EGF shedding and subsequent EGFR activation^{15, 16, 39, 64}. In addition, PI3K-dependent Src activation is required for β2AR-mediated EGFR transactivation and significantly increases their association in a multi-receptor complex⁴⁴. While the β1AR-EGFR complex does not

appear to be enhanced upon β 1AR stimulation, GRK- β arr-dependent signaling is required to maintain their association in HEK 293 cells⁴⁰. β 1AR-EGFR association also occurs in human heart⁴⁰ and adult feline ventricular cardiomyocytes (AFVM, Figure 2), however the kinetics of complex association following β 1AR stimulation appears to differ among different model systems. While β 1AR-EGFR association can be observed for prolonged timepoints after treatment with isoproterenol (ISO) in HEK 293 cells⁴⁰, activation of β 1AR in AFVM results in rapid co-localization with internalized EGFR and β arr2, but at longer timepoints is largely recycled back to the sarcolemmal membrane while EGFR undergoes prolonged internalization, including perinuclear localization (Figure 2).

As with other GPCRs, ERK activity is increased distal to β 1AR-dependent EGFR transactivation and, along with enhanced Akt activation, this mechanism promotes cardiomyocyte survival^{16, 18, 65}, while in cardiac fibroblasts, β 2AR-mediated transactivation leads to increased DNA synthesis and mitogenesis¹⁵. An initial investigation of the subcellular localization of signaling in response to β 1AR-mediated EGFR transactivation were performed in HEK293 cells and showed that activated ERK was restricted to the cytosolic compartment, with no nuclear accumulation observed even after 60 minutes of stimulation⁴⁰. In contrast, direct EGFR stimulation with either EGF or HB-EGF induced rapid nuclear accumulation of ERK, within 5 minutes, that persisted for 60 minutes and resulted in the activation of the transcription factor Elk-1, suggesting that β 1AR-mediated EGFR transactivation provides a mechanism to restrict EGFR signaling to cytosolic processes. However, subsequent studies in primary neonatal rat cardiomyocytes and in whole mouse hearts demonstrated that β AR-mediated EGFR transactivation actually resulted in nuclear accumulation of activated ERK, as well as Akt¹⁸. Further, our group discovered a role for EGFR transactivation-dependent repression of TRAIL, a pro-apoptotic factor whose decreased expression promoted cardiomyocyte survival¹⁸. Subsequently, we detailed both the acute and chronic transcriptome responses to β AR-mediated EGFR transactivation in the mouse heart, with foci on apoptotic genes and cytokines, including thrombospondin 1, Ccl2 and TNF α ^{66, 67}. Coupled with perinuclear localization of EGFR following β 1AR stimulation observed in AFVM (Figure 2), our cumulative studies suggest that in relevant primary cardiomyocyte and whole heart systems, β AR-mediated EGFR transactivation influences survival, hypertrophy and fibrosis via dynamic regulation of the transcriptome^{18, 66, 67}.

In vivo studies to date do indeed demonstrate that chronic catecholamine stimulation leads to increased cardiac cell death, fibrosis and hypertrophy with decreased function, all of which are sensitive to EGFR inhibition. However, use of different EGFR inhibitors in vivo resulted in different outcomes, wherein erlotinib worsened cardiac dilation, decreased cardiac function and increased cell death in response to chronic ISO infusion¹⁶, while gefitinib reduced ISO-induced cardiac dysfunction, hypertrophy and cell death, but increased fibrosis on its own⁶⁷. These disparate results could arise from EGFR-independent, off-target effects since gefitinib and erlotinib each interact with a wide array of kinases⁷⁵. For instance, Fabian et al. demonstrated that gefitinib binds to at least 18 kinases and erlotinib to 23, with only 12 of these kinases shown to commonly bind to both⁷⁵. Such off-target effects could explain differences in β AR-mediated cardiac remodeling responses in the presence of an EGFR inhibitor and provide caution for in vivo pharmacologic studies.

Thus, more refined in vivo models, preferably genetic and cell type-specific, are essential to better understand the impact of β AR-mediated EGFR transactivation in the heart.

Although β AR-mediated EGFR transactivation shows promise as a cardioprotective pathway, it has been difficult to characterize. A major hurdle to defining its impact in cardiomyocytes has been the reliance upon loss-of-function analyses using EGFR inhibitors in the presence of an unbiased β AR agonist. While suggestive of the impact of this pathway on processes such as survival, EGFR inhibitors do not directly measure the contribution of EGFR transactivation in the absence of G protein-dependent β AR signaling. The orthosteric non-selective β -blocker carvedilol has been shown to induce β arr-biased β AR-mediated signaling, including EGFR transactivation^{68–70}; however, these studies were performed either in HEK 293 cell systems with exogenous receptor expression or in whole heart using a very high dose of carvedilol with no functional insight at a contractile level. Indeed, it has recently been demonstrated that carvedilol does not enhance cardiomyocyte contractility⁶⁴, likely due to its inverse agonist properties⁷¹. Further, a recent meta-analysis revealed a lack of clinical difference between carvedilol versus the unbiased β -blocker metoprolol succinate in HF patients⁷². Taken together, these data indicate that although carvedilol may have the capacity to induce β arr-biased β AR signaling and EGFR transactivation, it likely does not occur at therapeutically relevant doses, and if doses were increased to achieve such biased signaling, it may be at the expense of cardiac contractility. Future advancements in understanding both the molecular mechanisms and functional outcomes of β AR-mediated EGFR transactivation in cardiac cells and the whole heart are therefore likely to stem from next generation biased β AR ligands that can selectively activate the EGFR pathway.

Conclusion

An ongoing question in the field is how a single receptor, EGFR, can produce distinct effects and physiological outcomes depending on its mode of activation. Acting as a nodal input for numerous GPCRs, EGFR has the potential to fine-tune neurohormone signals both physiologically and pathologically. From in vitro cell model studies, various mechanisms of transactivation have been identified that can be engaged by distinct receptor systems, however how it is all put together in meaningful way in fully differentiated cardiomyocytes, or more generally in the whole heart in vivo is not well understood. Dynamic subcellular distribution of EGFR with various GPCRs and proximal regulators likely plays a role in the integration of such signals, but there have been major barriers to studying EGFR-sensitive cardiac GPCR signaling effects in vivo. EGFR KO mice are embryonic lethal, and another genetic model that employed constitutive vascular smooth muscle cell (VSMC)/cardiomyocyte EGFR loss was unable to dissociate the impact of EGFR deletion in VSMC from cardiomyocytes on remodeling in an adult HF model^{73, 74}. Further, pharmacologic studies using EGFR inhibitors are fraught with interpretability issues, especially regarding agent selectivity profiles⁷⁵, and have produced variable and conflicting reports regarding the impact of EGFR inhibition on cardiac function and survival^{13, 16, 67, 76, 77}. These issues have prevented succinct in vivo analysis of the impact of GPCR-mediated EGFR transactivation on cardiac function and survival during HF, or even EGFR signaling in more generalized contexts of HF. Future work on the impact of GPCR-mediated EGFR transactivation on

cardiac function and remodeling in relevant in vivo systems will thus benefit from the development of refined genetic models.

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Figure 1. The influence of GPCR-mediated EGFR transactivation on cardiac remodeling processes

A) Prolonged neurohormone stimulation of cardiac-expressed GPCRs leads to changes in cardiac remodeling, including myocyte survival and hypertrophy, fibroblast proliferation, fibrosis and inflammation. Blockers of these GPCR systems are commonly used to decrease cardiac remodeling during conditions such as heart failure. B) GPCR-mediated EGFR transactivation influences numerous cardiac remodeling processes and may occur via mechanisms including either G protein- or GRK/βarr-dependent signaling in a GPCR- and cell-type-specific manner. Research areas requiring further development include: 1) the proximal mechanism of EGFR transactivation by specific GPCRs, 2) the mechanism and contribution of GPCR-EGFR association in mediating transactivation-dependent outcomes and 3) the distal signaling pathways unique to specific GPCR-EGFR complexes that may influence cardiac remodeling.

Figure 2. EGFR co-localization with β 1AR and β arr2 following catecholamine stimulation in adult cardiomyocytes

Adult feline ventricular myocytes (AFVM) were infected with adenoviral constructs for EGFR-mCFP (MOI of 50), Flag- β 1AR (MOI 200) and β arr2-mYFP (MOI of 200) and plated on laminin (40 μ g/mL)-coated coverslips. Forty-eight hours following plating, cells were treated with isoproterenol (ISO, 10 μ M) for 0, 5 or 30 min, fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton-X 100. Cells were blocked in 0.1% BSA followed by incubation with an EGFR antibody (Millipore clone LA22; 1:100) with an AlexaFluor 568-conjugated secondary antibody (1:5000) and a Flag M2 antibody (Sigma Aldrich; 1:1000) with an AlexaFluor 350-conjugated secondary antibody (1:1000). ISO stimulation produced a rapid (5 min) co-localization of EGFR (red), β 1AR (blue) and β arr2 (green) primarily at the sarcolemma of adult feline ventricular myocytes (AFVM), with EGFR internalization and perinuclear localization also evident. At a later timepoint (30 min), β 1AR and β arr2 remain largely co-localized at the sarcolemma, whereas prolonged EGFR internalization and perinuclear localization is observed. Abbreviations: mCFP (monomeric cyan fluorescent protein), mYFP (monomeric yellow fluorescent protein).

Table 1

Evidence for the involvement of GPCR-mediated EGFR transactivation in cardiac remodeling processes (reference numbers indicated)

	In vitro			In vivo			Ex vivo	
GPCR	Hypertrophy	Survival	Proliferation	Hypertrophy	Survival	Fibrosis	Inflammation	Contractility*
Adenosine 1A Receptor (A1AR)								13
α1 Adrenergic Receptor (α1AR)	17, 19		19	17				
β-Adrenergic Receptor (βAR)		18	15	67	16, 67	67	67	
Angiotensin II Type 1A Receptor (AT1R)	24		22	23				26
Bradykinin Receptor (BR)								27
δ-Opioid Receptor (δOR)								
Endothelin Receptor	28							
Muscarinic Acetylcholine Receptor M2 (M2R)		30						
Prostaglandin E2 Receptor 4 (EP4)	31							
Protease-activated receptor-1 (PAR1)	33		32					
Urotensin II Receptor (UT-IIIR)	36, 37							

* I/R-induced contractile dysfunction