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Transactivation of the Epidermal Growth Factor Receptor in Responses to Myocardial Stress and Cardioprotection

Running Title: EGFR and Cardioprotection

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HIGHLIGHTS

- ErbB1/EGFR can be activated by multiple GPCRs through a process termed ‘transactivation’.
- EGFR acts as a nexus for GPCR-mediated protective signalling.
- EGFR localisation to caveolae microdomains is critical to signal transduction.
- EGFRs appear to be a critical determinant of myocardial tolerance to ischaemia-reperfusion.
- Signalling mediators downstream to EGFR include arrestins, PI3K, PKB/Akt, ERK and PKC.
- EGFR signalling is perturbed in cardiovascular pathologies, including ischaemic heart disease, hypertrophy/heart failure, diabetic cardiomyopathy and obesity/dyslipidaemia.
- Despite a predominance of evidence for a protective role for the EGFR, EGFR over-activity may be detrimental specifically in the context of vascular injury.

ABSTRACT

The epidermal growth factor receptor (EGFR) family comprises the ErbB1 (EGFR) and ErbB4 receptors as well as the 'co-receptors' ErbB2 (which does not bind EGF ligands) and ErbB3 (which lack tyrosine kinase activity). This family of receptors is essential for cardiac development, myocardial, renal and vascular function, and cardiac responses to physiological and pathological perturbations. The EGFR appears critical in protecting cardiac cells from injury, while considerable attention has focussed on neuregulin/ErbB4 signalling in potentially ameliorating cardiomyopathy/ heart failure. Indeed, the EGFRs provide a signalling nexus, upon which multiple cardioprotective stimuli appear to converge, including ischaemic preconditioning and various G protein-coupled receptors (opioid, muscarinic, adenosine, adrenergic, bradykinin, sphingosine 1-phosphate). These stimuli engage the EGFR axis (in a process referred to as transactivation) in differing ways, involving both G protein-dependent and -independent mechanisms, to promote myocardial cell survival during and following ischaemia/infarction. Elucidating the molecular processes that underpin EGFR transactivation and mediate cardiac protection will advance our understanding of the intrinsic capacity of the heart to withstand pathological insult. It should also reveal new approaches to facilitate cardioprotective therapy to limit damage during and following myocardial ischaemia/infarction, which despite intense investigation remains an unrealised, yet highly desirable, clinical goal. This review focuses on the cardiovascular functions of the EGFR, its role in cardioprotection, and the potential influences of common disease states on this signalling.

Key Words: EGFR; ErbB; cardioprotection; G protein-coupled receptors; ischaemia-reperfusion; preconditioning; transactivation

1. The EGFR Family

The epidermal growth factor receptor (EGFR) family of RTKs is comprised of 4 members: EGFR (ErbB1, HER1), EGFR2 (ErbB2, Neu, HER2), EGFR3 (ErbB3, HER3) and EGFR4 (ErbB4, HER4) (Jorissen et al., 2003; Roskoski, 2014). All 4 are expressed in the heart, with ErbB1, ErB2 and ErbB4 declining with postnatal development (Camprecios et al., 2011). Although earlier studies documented expression of ErbB1, ErbB2 and ErbB4 only in adult heart (Sundaresan et al., 1998; Zhao et al., 1998; Rohrbach et al., 1999), more recent work also confirms adult expression of ErbB3 (Lorita et al., 2009; Camprecios et al., 2011).

The EGFR is a 175-kDa glycoprotein activated by multiple ligands, including epidermal growth factor (EGF), heparin-binding EGF (HB-EGF), and transforming growth factor α (TGFα), amphiregulin, betacellulin, epigen and epiregulin. Receptor binding induces ErbB homo- or hetero-dimerisation (Figure 1). The ErbB2 protein lacks a ligand-binding domain and relies on heterodimerisation for signalling functionality (though may independently exert cardiac effects when abnormally expressed). ErbB3 lacks the ability to phosphorylate exogenous peptides, heterodimerising with ErbB receptors, in particular ErbB2 (Shi et al., 2010; Steinkamp et al., 2014) to confer a unique pro-survival properties, especially relating to tumor progression (Ma, J. et al., 2014).

The ErbB1 monomer may be bound by EGF, HB-EGF or TGFα, while HB-EGF also binds to ErbB3, neuregulin-1 and -2 to ErbB3 and ErbB4, and neuregulin-3 and -4 to ErbB4 (Fuller et al., 2008; Roskoski, 2014; Schilling and Patel, 2015; Forrester et al., 2016). When considering the protective functions of EGFR signalling in heart, both ErbB1 and the ErbB2 dimer partner are thus relevant. However, this is

complicated somewhat by the important role of ErbB2 in ErbB4 (neuregulin) receptor responses.

The molecular basis of ErbB dimerisation has been extensively reviewed (Jorissen et al., 2003; Lemmon, 2009). Distinct from other receptor kinases, phosphorylation of the kinase activation segment of EGFR is not required for activation (Gotoh et al., 1992; Tice et al., 1999). Instead, ligand-activated kinase domains form an asymmetric dimer: ligand (EGF, TGFα, HB-EGF) independently binds with low affinity to extracellular domains I and III (LR1, LR2) of ErbB1, inducing a conformational change that facilitates high affinity binding to both domains (Figure 1). The molecular tether between domains II and IV is also broken, and a 'dimerisation arm' hidden within domain IV (where it stabilises a conformation that inhibits ligand binding/dimerisation) is exposed for binding via interaction with domain II, leading to ErbB dimerisation. Within this dimer, one kinase domain acts as activator/donor, the other as receiver/acceptor: activated receiver kinase phosphorylates activator kinase tyrosine residues which function as docking sites for downstream signalling (Figure 1). This results in phospho-regulation of effector molecules including Ras, Raf, extracellular signal-regulated kinase 1/2 (ERK1/2), p38 mitogen activated protein (MAP) kinase, and the phosphatidylinositol 3 kinase (PI3K)/protein kinase B (Akt) pathway (Figure 2). The preferred ErbB2 dimer partner increases ligand-binding affinity (Karunagaran et al., 1996) and reduces EGF dissociation to prolong signal activation. Heterodimers containing ErbB2 are also more stable and endocytosed at lower rates than other ErbB dimers (Lenferink et al., 1998; Wang et al., 1999; Hommelgaard et al., 2004). Thus, signalling via ErbB1/2 heterodimers may be more powerful and sustained than that arising via ErbB1 homodimers.

The EGFRs play essential roles in cardiac development, regulate myocardial function and participate in adaptation/remodelling responses to physiological or pathological loads (Iwamoto et al., 2003; Fuller et al., 2008; Forrester et al., 2016). A recently identified role includes promotion of myocardial survival and adaptation to injurious stress. This stress signalling appears an important determinant of myocardial responses to disease/insult (albeit detrimentally influenced by diverse co-morbid states), and may represent a novel avenue for therapeutic cardioprotection. Herein we specifically review evidence for EGFR/ErbB1 involvement in governing myocardial responses to ischaemic and other injurious insult, and transduction of diverse cardioprotective stimuli.

1.1. Mechanisms of EGFR Transactivation

Additional to direct agonism, the EGFR is transactivated via diverse G protein-coupled receptors (GPCRs). In cardiac cells, EGFR transactivation has been linked to angiotensin (Rakesh et al., 2010), muscarinic (Krieg et al., 2002; Krieg et al., 2004; Miao et al., 2015), endothelin (Kodama et al., 2002; Chen et al., 2006), opioid (Cao et al., 2005; Cohen et al., 2007; Forster et al., 2007; Zhang et al., 2015), bradykinin (Methner et al., 2009), adrenergic (Noma et al., 2007; Grisanti et al., 2014), adenosine (Williams-Pritchard et al., 2011), and sphoingosine-1 phosphate (S1P) (Hofmann et al., 2009) GPCRs, with transactivation via angiotensin II (Ang II) perhaps the most well studied. Several G_q-linked receptors (Ang II, ET-1, α-ARs) may promote cardiac hypertrophy/remodelling, whereas transactivation by adenosine, opioid, bradykinin or muscarinic receptors may be protective, enhancing cell survival. The transactivation of EGFR signalling is conventionally attributed to a process termed the 'triple-membrane-passing-signalling' model (Figure 2), which involves

GPCR activation and initial heterotrimeric G protein dissociation, activation of ligand-specific intermediates (including non-receptor tyrosine kinase, Ca^{2+} and reactive oxygen species), followed by metalloprotease activation and shedding of mature extracellular EGFR ligand.

Additional mechanisms may participate in transactivation in an agonist-specific manner, including G protein-independent activation, EGF-ligand independent mechanisms or direct GPCR/ErbB interaction (Figure 2). Transactivation may also occur in the absence of detectable ligand, involving protein tyrosine kinase (e.g., Src family kinase) phosphorylation of the cytoplasmic EGFR domain to provide docking sites for signalling proteins (Figure 1). Generation of ROS is important in both ligand-dependent and -independent transactivation, with membrane-associated NADPH oxidase considered the primary source of GPCR-dependent ROS, donating an electron from NADPH to O_2 to produce superoxide (Murdoch et al., 2006). Control of NADPH oxidase occurs pre- and post-translationally. Agonism of GPCRs (e.g., by Ang II) induces NADPH oxidase subunit expression and complex assembly at the cell membrane, increasing cardiomyocyte ROS production (Nakagami et al., 2003; Hingtgen et al., 2006). Activation of the small GTPase Rac also promotes membrane recruitment of NADPH oxidase complex components (Gregg et al., 2003), while the p47phox subunit (necessary for oxidase activation) is phospho-regulated by both PKC (Fontayne et al., 2002) and the PI3K/Akt pathways (Hoyal et al., 2003). Generation of ROS plays a key role in MMP activation (Wetzker and Bohmer, 2003), increases PTK activity and EGFR phosphorylation via inhibitory oxidation of tyrosine phosphatases targeting Src kinase and EGFR (Salmeen et al., 2003; Chen et al., 2006), and may enhance proteolysis of proteins that repress PTK activity (Liebmann, 2011; George et al., 2013a). These important functions may, in turn, render EGFR transactivation

sensitive to age- and disease-dependent perturbations in myocardial ROS and phosphatases.

In addition to conventional G protein-dependent induction of EGFR ligand shedding, MMPs can function as direct G protein effector molecules: Overland and Insel (2015) have identified a novel path of EGFR transactivation in which MMP14 acts as a direct heterotrimeric G protein effector, activated by Gβγ (potentially Gα) to trigger HB-EGF release. Other studies identify G protein independent/β-arrestin dependent mechanisms of EGFR activation (Tilley, 2011). Noma *et al.* (2007) report β1-adrenoceptor transactivation of cardiomyocyte EGFR independently of G protein activation, involving β-arrestin recruitment via G protein-coupled receptor kinase (and a subsequent canonical cascade of EGFR transactivation involving Src, a metalloprotease, and HB-EGF shedding). Conversely, Zhang *et al*. (2015) showed that the cardioprotective δ opioid receptor transactivates the EGFR via PKCδ dependent ligand shedding, with β-arrestin2 recruited to the distal EGFR (not the δ opioid receptor). Finally, a recent functional siRNA screen in human epithelial cells has identified novel mediators of EGFR transactivation, including TRIO, BMX and CHKA (George et al., 2013b). Their knockdown impairs EGFR phosphorylation in response to GPCR agonism, but not direct activation with EGF, locating the proteins between the GPCR and EGFR.

The specific identities of the metalloproteases involved in EGFR transactivation remain to be detailed, though ADAMs (a disintegrin and metalloprotease) are more commonly implicated. Importantly, shedding of EGFR ligands by ADAMs not only yields extracellular ligand but an intracellular carboxyl-terminal fragment that is functionally relevant, resulting in independent signalling. This remnant peptide may translocate to the inner nuclear membrane and regulate

gene expression (Higashiyama et al., 2008). The importance of carboxyl-terminal fragment signalling to cardioprotection has yet to be examined.

1.2. Cell Membrane Localisation - Membrane Rafts, Caveolae and Caveolins

The membrane domain localisation of EGFR (within plasma and endosomal membranes) appears critical to signal transduction, yet opposing models of microdomain dependence have emerged. Detailed below, earlier studies employing detergent-free methods of membrane fractionation and/or less select modes of cholesterol disruption provide support for caveolar localisation of EGFR, with disruption of this association (experimentally and during internalisation/degradation) inhibiting or terminating EGFR signalling (Smart et al., 1995; Mineo et al., 1996; Furuchi and Anderson, 1998). In contrast, studies utilising different membrane fractionation methodologies or more selective levels of cholesterol sequestration support localisation and activation of EGFR signalling within plasma and late-endosomal membrane rafts (Balbis and Posner, 2010).

Many signalling molecules involved in EGFR transactivation appear localised to caveolar domains and influenced by associated caveolins, with caveolae and caveolin-3 both essential to diverse cardioprotective responses (Schilling et al., 2015). Thus, the EGFR, relevant GPCRs (AT1, endothelin, β-adrenergic, muscarinic, opioid, adenosine), G proteins (α and βγ), Src family kinases, and ADAM17 have been reported to be, at least in part, localised to caveolae in non-cardiac cells (Smart et al., 1995; Mineo et al., 1996; Ushio-Fukai and Alexander, 2006; Takaguri et al., 2011).

Not only were initial signal transduction steps, including EGF triggered tyrosine kinase activation, and adaptor and kinase recruitment found to occur within caveolar fractions (Mineo et al., 1996; Furuchi and Anderson, 1998), activated EGFR

was shown to subsequently migrate from caveolae in association with attenuation of signalling (Mineo et al., 1996). Studies in cancer cells similarly supported sequestration of active EGFR within caveolae (Abulrob et al., 2004), while there is also evidence for signalling via both caveolar and non-caveolar EGFR (with prolonged ERK1/2 activation by extra-caveolar EGFR *vs*. transient ERK1/2 activation by caveolar EGFR) (Rimoldi et al., 2003). Interestingly, the protective A_1 adenosine receptor, implicated in preconditioning responses and shown to protect via EGFR transactivation (Williams-Pritchard et al., 2011), appears to translocate out of myocardial caveolae upon activation (Lasley et al., 2000), though there is evidence A_1 activation also translocates other signal elements, including PKC, into caveolae (Yang et al., 2009).

In contrast to these findings, more recent studies support a role for non-caveolar raft domains in sequestration of active EGFR and intracellular recruitment/ activation of kinases in late endosomes (Balbis and Posner, 2010). Studies employing Triton X-100 extraction to isolate low-buoyancy detergent-resistant membranes support involvement of membrane rafts in promoting EGFR phosphorylation, adapter recruitment and kinase signal activation (Zhuang et al., 2002; Puri et al., 2005; Balbis et al., 2007; Wang et al., 2009). Immunoelectron microscopy, and select cholesterol and clathrin disruption, reveal no role for caveolae in concentrating the EGFR, or influencing EGFR binding and internalisation (Ringerike et al., 2002; Kazazic et al., 2006), while cholesterol itself may influence binding, dimerisation and phosphorylation (Ringerike et al., 2002). Initially viewed as a mechanism of signal termination (*ie*. dissociation of EGFR signal elements from caveolar domains), internalisation of raft-localised EGFR may thus be fundamental to signal transduction (Balbis and Posner, 2010). Data collectively support activity-dependent EGFR

internalisation, with low-level agonism triggering clathrin-dependent/cholesterol-independent recycling to the cell membrane (Sigismund et al., 2005; Sigismund et al., 2008), whereas higher level agonism results in clathrin-independent/cholesterol-dependent EGFR internalisation and trafficking to late endosomes (Lai et al., 1989). Caveolin independent rafts in these late endosomes may constitute a functional platform for the activation of local MAPK signalling (Teis et al., 2002; Balbis et al., 2007; Taub et al., 2007; Nada et al., 2009).

Questions of caveolar *vs*. non-caveolar localisation notwithstanding, caveolins themselves appear to regulate EGFR signalling. Caveolins influence myocardial signalling and stress-tolerance via both canonical (e.g., caveolar localisation) and non-canonical mechanisms (Schilling and Patel, 2015). While studies identify caveolin influences on EGFR signalling, most focus on caveolin-1 with relatively little information regarding control of myocardial EGFR by the principle caveolin in striated muscle cells - caveolin-3.

Caveolin-1, the major structural protein in non-cardiac caveolae, suppresses EGFR function in other cell types. Infection of vascular smooth muscle with adenovirus encoding caveolin-1 inhibits Ang II-induced HB-EGF shedding, EGFR transactivation, ERK activation, hypertrophy and migration (Takaguri et al., 2011). Other studies support inhibitory effects of caveolin-1 on EGFR (Couet et al., 1997; Mineo et al., 1999; Wang et al., 2007). Nonetheless, caveolin-1 also appears essential in EGFR transactivation: silencing caveolin-1 expression inhibits Ang II-induced EGFR transactivation in vascular tissue (Takayanagi et al., 2014); caveolin-1 deletion inhibits EGFR transactivation by TGF-β in hepatocytes, in association with failed TACE/ADAM17 activation (Moreno-Caceres et al., 2014); and caveolin-1 deletion or caveolar disruption abolishes stretch-dependent EGFR transactivation and Akt

signalling in mesangial cells (Zhang et al., 2007). Caveolin-1 may thus act to limit EGFR activity in the basal state whilst promoting EGFR transactivation. This differential control may be relevant to stress-dependent EGFR signalling in cardiac fibroblasts, with fibroblast caveolin-1 depressed following infarction (Gao et al., 2014) and evidence such shifts influence other growth factor signalling (Miyasato et al., 2011).

Regarding the cardiomyocyte, there is some evidence ErbB1/ErbB2 associate with cardiac caveolae and caveolin-3 (Zhao et al., 1999; Liu et al., 2003; Ueda et al., 2005), potentially within T tubules (Ueda et al., 2005). Relevant GPCRs and effector molecules also associate with cardiac caveolae/caveolin-3 (Head et al., 2005). Receptor stimulation may differentially impact ErbB/caveolin-3 association, with ErbB4 but not ErbB2 dissociating from caveolin-3 upon neuregulin stimulation (Zhao et al., 1999). As for caveolin-1, caveolin-3 expression appears to suppress EGFR signalling (Brauers et al., 2010), consistent with early reports of EGFR inhibition by caveolin-3 (Couet et al., 1997). Dissociation from caveolin-3 upon receptor activation, as observed for ErbB4 (Zhao et al., 1999), could be important in initiating EGFR signalling (caveolin-3 suppressing basal activity, dissociation facilitating signalling activation). Further work is warranted in unravelling the roles of caveolae and caveolin-3 in control of cardiomyocyte EGFR signalling. Importantly, significant reductions in myocardial caveolin-3 expression/localisation with ageing (Kawabe et al., 2001; Ratajczak et al., 2003; Peart et al., 2007), infarction (Ratajczak et al., 2003), and disease (Piech et al., 2003; Penumathsa et al., 2008; Crossman et al., 2011; Sharma et al., 2011; Lei et al., 2013) have the potential to dysregulate myocardial EGFR signalling.

2. Myocardial and Coronary Control via the EGFR

The ErbB system (receptors and ligands) is essential for heart development and maintenance of structural integrity (Fuller et al., 2008). In adult heart, the most abundant ErbB receptors are ErbB2 and ErbB4 (Zhao et al., 1998), with HB-EGF and NRG-1 being important endogenous ErbB ligands. The influence of ErbB receptors on myocardial physiology was poignantly revealed in the cardiomyopathy observed in breast cancer patients receiving antibody inhibitors of ErbB2 (Ewer et al., 1999; De Keulenaer et al., 2010). Cardiac toxicity was noted in ~5% of these patients, increasing to >25% when co-administered with anthracyclines (Ewer et al., 1999; McKeage and Perry, 2002). It is now clear neuregulin-1β (NRG-1β) and its receptors (ErbB4 and the dimer ErbB2) are essential for cardiac development, and maintenance of adult cardiac function.

However, the heart also expresses ErbB1, and its endogenous ligands EGF and HB-EGF induce varied cardiac effects that include modulation of stretch-dependent ion channels (Browe and Baumgarten, 2006), Na^+ currents (Liu et al., 2007), prostacyclin formation (Braconi Quintaje et al., 1998), and cAMP accumulation (Yu et al., 1992). Rabkin *et al.* (Rabkin et al., 1987) observed novel positive chronotropy in response to EGF in chick embryo cardiomyocytes, with subsequent studies identifying positive inotropic and chronotropic effects of EGF in the heart (Nair et al., 1993; Lorita et al., 2002). These stimulatory effects appear to involve Gαs phosphorylation, adenylyl cyclase activation and cAMP accumulation (Nair et al., 1989; Nair et al., 1990; Yu et al., 1992; Nair et al., 1993).

Chronic EGFR/ErbB1 inhibition *in vivo* leads to myocardial dysfunction in mice (Barrick et al., 2008), as does cardiomyocyte expression of a dominant negative ErbB1 mutant (Rajagopalan et al., 2008). Other studies confirm cardiac dysfunction

following chronic inhibition of EGFR tyrosine kinase activity, with evidence for involvement of Mg^{2+} loss and substance P dependent inflammation (Weglicki et al., 2012; Mak et al., 2015). Conditional deletion of HB-EGF, which binds both ErbB1 and ErbB4, also leads to contractile dysfunction (Iwamoto et al., 2003). These findings contrast studies of *acute* EGFR inhibition, which generally reveal no significant changes in baseline myocardial or coronary function. However, Villa-Abrille *et al.* have shown that the cardiac Anrep effect (delayed inotropic response to stretch), and stretch-dependent phosphorylation of ERK1/2 and the Na^+/H^+ exchanger, are dependent upon acute EGFR signalling (Villa-Abrille et al., 2010). Other studies report involvement of EGFR in myocardial strain-dependent signalling (Anderson et al., 2004; Duquesnes et al., 2009), while hypertrophic effects of Ang II appear to involve EGFR activation (Kagiyama et al., 2002).

The EGFR also influences coronary vascular function: Muramatsu *et al.* (1985) identified cyclooxygenase dependent vasoconstrictor effects of EGF in different arteries, with subsequent studies revealing EGF-dependent constriction of coronary and other vessels via both prostaglandin dependent and independent mechanisms (Gan and Hollenberg, 1990; Hollenberg, 1994), a response potentially perturbed in and contributing to hypertension (Swaminathan and Sambhi, 1996; Florian and Watts, 1999). Hong *et al.* (2014) have also identified an essential role for EGFR activity in O_2-induced contraction of the ductus arteriosus, involving EGFR transactivation via mitochondrial ROS, and downstream p38 MAPK and JNK signalling.

The clinical importance of the myocardial EGFR is evidenced by association between EGFR polymorphisms and both acute coronary syndrome (Gao et al., 2008) and cardiomyopathy (Zhou et al., 2009). Shifts in EGFR functionality may contribute

to pathogenesis of ischaemic heart disease and cardiomyopathies. This is consistent with the cardiotoxicity of anti-cancer therapies targeting EGFR tyrosine kinase (Yeh and Bickford, 2009), and cardiac dysfunction with EGFR (ErbB1) mutation (Rajagopalan et al., 2008) or inhibition (Barrick et al., 2008).

3. The EGFR in Myocardial Stress Responses

Accumulating evidence indicates the EGFR is an important determinant of myocardial tolerance to ischaemia-reperfusion (I-R) injury, contributes to adaptive responses to ischaemic/hypoxic stress (exemplified in ischaemic preconditioning), and mediates protection via multiple GPCRs. Indeed, EGFR may serve as a signalling nexus in cardioprotection, raising the possibility of adverse impacts of ErbB-targeted anti-cancer therapies on myocardial responses to ischaemia/infarction and cardioprotective interventions. The advent of cell-type specific modulation of receptor activity opens a new avenue for potential therapeutic intervention, however a more nuanced understanding of EGFR activity in cardiac cell subtypes is required.

3.1. Role of ErbB1 (and ErbB2) in Intrinsic Stress-Tolerance

Several studies support roles for EGFR ligands and ErbB1/ErbB2 in governing cardiomyocyte stress-tolerance. However, in many of these studies the specific importance of EGFR *vs.* other ErbB2 partners remains to be established. The cardioprotective or 'anti-ischaemic' effects of EGFR detailed below are mirrored in other tissues. For example, Zhou *et al.* (Zhou et al., 2015) report EGFR phosphorylation and transactivation during cerebral ischaemia-reperfusion, with PI3K/Akt activation reduced by either EGFR or MMP inhibitors (AG1478, GM6001).

Inhibition of endogenous ErbB2 causes dysfunction and exaggerates apoptosis in rat cardiomyocytes (Grazette et al., 2004; Gordon et al., 2009), These effects

appear to occur via a pathway involving Akt and PKC-α (Gordon et al., 2009), and involve increased ROS production and expression of pro-apoptotic Bcl-sL (*vs.* decreased anti-apoptotic Bcl-xL), promoting apoptosis (Grazette et al., 2004). Mitochondria are a key target, with cyclosporine A and diazoxide (regulating pro- and anti-apoptotic mitochondrial channels, respectively) countering these deleterious impacts of ErbB2 blockade (Gordon et al., 2009). Moreover, mouse embryonic fibroblasts lacking Bax and Bak (mediators of the mitochondrial cell death pathway) are resistant to the detrimental effects of ErbB2 antibody. An insufficiency of HB-EGF similarly promotes apoptosis in normoxic and hypoxic H9c2 myoblasts, enhancing ROS formation, caspase-3 and JNK activities (Uetani et al., 2009). Chronic treatment with inhibitors of EGFR or ErbB2 significantly worsens myocardial ischaemic tolerance in hearts from healthy rats, and also diabetic rats exhibiting reduced expression and phosphorylation of EGFR and ErbB2 (Akhtar et al., 2012). Increased Gαq expression also promotes EGFR-dependent Akt phosphorylation in cardiomyocytes and limits apoptosis during metabolic stress (Howes et al., 2006).

These studies collectively support important roles for ErbB1 and/or the dimer partner ErbB2 in promoting myocyte survival under baseline and stressful conditions. However, the extent to which effects on stress-resistance involve shifts in intrinsic EGFR *vs.* ErbB4/NRG signalling remains to be detailed. Importantly, alterations in this signalling with disease and during stress may both impair intrinsic stress-resistance and render cells refractory to protective stimuli.

3.2. Role of the EGFR in GPCR-Triggered Cardioprotection

Transactivation of the EGFR has been implicated in cardiac protection via muscarinic, opioid, bradykinin, adrenergic, S1P, and adenosine receptors. Krieg *et al.* (2002) initially found that acetylcholine triggered myocardial EGFR and Akt

phosphorylation in an AG1478 sensitive manner, although cardiac protection appeared insensitive to the inhibitor. However, their subsequent work showed anti-infarct effects of acetylcholine and the δ-opioid agonist DADLE were associated with EGFR phosphorylation, and blocked by metalloproteinase inhibition (Krieg et al., 2004). The authors also reported acetylcholine-dependent ROS formation in cardiomyocytes was sensitive to metalloproteinase and HB-EGF inhibitors, and was similar to the effects of EGF and HB-EGF. More recent work supports an important role for EGFR transactivation in cardioprotection via muscarinic receptor agonism, activating PI3K/Akt signalling and inhibiting TNF-α mediated ER stress and apoptosis in H9c2 myoblasts (Miao et al., 2015)

Cao *et al.* (2005) confirmed EGFR involvement in opioid (met-enkephalin) mediated protection of cardiomyocytes, in association with activation of downstream PI3K/Akt signalling. Similarly, Forster *et al.* (2007) showed that DADLE activates myocardial Akt and ERK1/2 and protects against I-R injury in an AG1478 and MMP inhibitor dependent manner. Subsequent work by Cohen *et al.* (2007) indicated that while the δ-opioid agonist DADLE and bradykinin both increase myocyte ROS production and protect against infarction, only the effects of DADLE were sensitive to MMP and HB-EGF inhibitors. Effects of DADLE on ROS formation and infarction were also blocked by a Src kinase inhibitor, with phosphorylation of ventricular Akt and ERK1/2 blocked by AG1478. In contrast to these findings, Methner and colleagues (2009) report that cardiac and mitochondrial protection via bradykinin is blocked by AG1478 or GM6001. The basis of these differing responses to bradykinin is unclear.

The β_1-adrenoceptor has also been shown to induce EGFR transactivation/ internalisation and ERK1/2 activation in cardiomyocytes via a G protein independent

process of β-arrestin recruitment by G protein-coupled receptor kinases 5 and 6 (Noma et al., 2007). Inhibition of this signalling path promoted myocyte apoptosis. A G protein-independent process of EGFR transactivation may also occur with some β-blockers (e.g., alprenolol, carvedilol) in non-cardiac models (Kim et al., 2008), with these agents also known to protect against acute or long-term effects of myocardial I-R. Grisanti *et al.* (2014) more recently identified EGFR involvement in compartment-specific signalling responses to the β-agonist isoproterenol. Phosphorylation of ventricular ERK1/2 was EGFR-sensitive in all sub-cellular fractions studied, whereas Akt phosphorylation was EGFR-sensitive only within plasma membrane and nuclear fractions (results confirmed in neonatal cardiomyocytes). This β□adrenoceptor mediated EGFR transactivation was shown to be protective, reducing apoptosis in serum-depleted myocytes.

Other cardioprotective GPCRs engage cardiac EGFR signalling. For example, the sphingolipid S1P triggers GPCR-dependent protection, is implicated in ischaemic conditioning responses, and transactivates EGFR in heart (Hofmann et al., 2009) and vascular myocytes (Tanimoto et al., 2004). This transactivation appears essential to cardioprotection triggered by S1P receptor agonists. Cardiac protection and survival kinase activation in response to adenosine receptor agonism (and ischaemic preconditioning) is also associated with EGFR phosphorylation and blocked by EGFR, MMP or HB-EGF inhibitors (Williams-Pritchard et al., 2011). Finally, mechanical stress has been shown to trigger β-arrestin-dependent AT1 receptor activation, leading to EGFR transactivation and protection of cardiac myocytes from apoptosis (Rakesh et al., 2010).

These studies collectively reveal an important role for EGFR transactivation in cardioprotection via opioid, adenosine and S1P GPCRs, and also support protective

effects of AT1 and β–adrenergic receptor dependent EGFR transactivation. Involvement of EGFR in cardiac protection via acetylcholine and bradykinin remains to be more clearly established. This relatively broad-spectrum engagement of EGFR in different responses highlights a critical point of signalling convergence in cardioprotection – EGFR appears to be a key determinant of myocardial stress-responses, and a potentially potent target for therapeutic cardioprotection.

3.3. Role in Adaptive Protection - Ischaemic Preconditioning

Evidence of EGFR involvement in responses to putative GPCR triggers of ischaemic preconditioning, including adenosine (Williams-Pritchard et al., 2011), opioid (Krieg et al., 2004; Cohen et al., 2007; Forster et al., 2007), bradykinin (Cohen et al., 2007; Methner et al., 2009), S1P (Hofmann et al., 2009) and β-adrenergic receptors (Noma et al., 2007; Grisanti et al., 2014), indirectly supports EGFR involvement in this protective response. More specific analyses confirms an essential role for EGFR in preconditioning: chronic inhibition of EGFR with AG1478 attenuates ischaemic preconditioning by 50% or more (Benter et al., 2005a); and acute inhibition of EGFR, MMP or HB-EGF negates protection via preconditioning, though no effects were observed on intrinsic I-R tolerance (Williams-Pritchard et al., 2011). Sensitivity to both GM6001 and CRM197 supports involvement of MMP-dependent HB-EGF shedding. On the other hand, Ichikawa *et al.* (Ichikawa et al., 2004) report a role for ADAM activity in tyrosine kinase dependent and PKC independent protection via ischaemic preconditioning, although AG1478 did not inhibit cardiac protection.

3.4. Cardioprotection via Exogenous EGF

Effects of exogenous EGFR ligands confirm the cardioprotective functions of this receptor tyrosine kinase. Studies to date support protection via EGF, while TGFα fails to protect against acute myocardial I-R injury (Manukyan et al., 2011), and exogenous HB-EGF has yet to be assessed. Injecting EGF (0.25 mg/kg) 20 min before exposing mice to the stress of restraint-and-cold exposure also reduces myocardial injury markers (Pareja et al., 2003), an effect abolished by AG1478. In isolated mouse hearts exogenous EGF protects against detrimental functional effects of continuous epinephrine stimulation (Lorita et al., 2002), with subsequent work showing EGF induces Tyr-phosphorylation of ErbB1 but not ErbB2, and protects against combined epinephrine/I-R injury (Lorita et al., 2009). These investigators also showed that EGF 5 min before and throughout myocardial ischaemia improves contractile function and prevented post-ischaemic elevations in anaerobic metabolism and leakage of intracellular LDH (Lorita et al., 2010). More recently, Akhtar *et al.* (2012) found that EGF treatment improved the functional recovery of both healthy and diabetic rat hearts from I-R, with effects more pronounced in diabetic hearts exhibiting suppressed EGFR and ErbB2 expression/phosphorylation. Thus, activation of ErbB1, with either endogenous or exogenous ligand, protects against multiple forms of injurious cardiac insult.

Whether direct activation of the EGFR *vs*. GPCR-dependent transactivation generate similar or distinct myocardial outcomes is unclear. Studies to date have focussed on contractile outcomes and release of cytosolic enzymes as indicators of injury (and protection), with less attention to specific pathways of cell death. Qualitatively, the protective effects of EGF vary, including augmented post-ischaemic pressure development without change in diastolic dysfunction (Akhtar et al., 2012), or

improved early but not late recovery of pressure development coupled with elimination of cell death, based on LDH release or myocyte viability (Lorita et al., 2010).

3.5. Influences of the ErbB2 Dimer Partner

Since ErbB2 is the preferred dimer partner for ErbB1, it is relevant to consider the potential protective actions of this protein together with those of ErbB1. However, this is complicated by the role of ErbB2 in neuregulin/ErbB4 signalling - altered expression or inhibition of ErbB2 is predicted to influence both EGFR and neuregulin responses.

Inhibition of cardiac ErbB2 activity (or endothelial NRG-1 expression) impairs functional recovery from cardiac ischaemia (Pentassuglia et al., 2009; Hedhli et al., 2011). Moreover, NRG-1 treatment is directly cardioprotective (Fang et al., 2010), and also neuroprotective (Xu et al., 2005). There is also evidence NRG-1/ErbB signalling is necessary for myocardial adaptation to the physiological stress of pregnancy (Lemmens et al., 2011). Pentassuglia *et al.* (2009) report that neither ErbB1 or ErbB2 inhibitors induce myocyte death, however ErbB2 (but not ErbB1) inhibition causes myofibrillar structural damage (additive to that induced with doxorubicin), in association with impaired excitation-contraction coupling and decreased ERK1/2 phosphorylation).

Sun *et al.* (2014) show that knockout of Gab1, which is essential to ErbB signal transduction, worsens myocyte tolerance to I-R and oxidative stress (exaggerating caspase-3 activation and apoptosis, impairing Akt and MAPK activation), and that ErbB (or Src) kinase inhibition reduces Gab1 phosphorylation, Akt/MAPK activation and cell survival during oxidative stress. The ErbB isoforms

involved in these survival effects remain to be determined. Sysa-Shah and colleagues (2012) have shown that ErbB2 overexpression induces concentric cardiac hypertrophy (without heart failure) and increases susceptibility to adrenergic receptor induced arrhythmias. An anti-apoptotic shift in the ratio of Bcl-xS *vs*. Bcl-xL was also evident, together with activation of translational machinery (involving S6, 4E-BP1 and eIF4E). Whether this shift in apoptotic signalling would benefit during I-R is untested, though others link similar ErbB-dependent shifts in Bcl proteins to improved survival in hypoxia/I-R (Grazette et al., 2004).

4. Molecular Basis of Cardioprotection Via EGFR Transactivation

Molecular mechanisms underlying cardiac protection distal to EGFR transactivation are not resolved. Studies of cardioprotective GPCR and preconditioning responses support transduction of protective signals via kinase (PI3K/Akt, ERK1/2, p70s6K, mTOR, PKC, PKG, PKA GSK3ß), NOS and ROS signalling, and modulation of distal effectors that include mitochondrial channels (mK_{ATP}, MPTP), gap junction, apoptotic and autophagic proteins (Hausenloy and Yellon, 2006; Heusch, 2015) (Figure 3). The JAK-STAT signalling path is also implicated. Studies focussed specifically on cardioprotective EGFR transactivation implicate Src tyrosine kinase (Cohen et al., 2007; Noma et al., 2007) PKCδ (Zhang et al., 2015), MMP and HB-EGF (Krieg et al., 2004; Cohen et al., 2007; Forster et al., 2007; Noma et al., 2007; Methner et al., 2009; Williams-Pritchard et al., 2011) upstream of the EGFR, together with downstream contributions from PI3K/Akt (Cao et al., 2005; Forster et al., 2007; Methner et al., 2009; Akhtar et al., 2012; Grisanti et al., 2014), MAPK/ERK1/2 (Cao et al., 2005; Forster et al., 2007; Akhtar et al., 2012; Grisanti et al., 2014; Zhang et al., 2015), PKC (Cao et al., 2005), and CaMK II-

dependent Ca^{2+} homeostasis (Benter et al., 2004). Studies examining the injurious effects of ErbB1 or ErbB2 inhibition/deletion also support involvement of ROS generation, mitochondrial channels and PKC (Gordon et al., 2009), and modulation of caspase-3 and pro- and anti-apoptotic Bcl proteins and JNK activity (Grazette et al., 2004; Uetani et al., 2009; Grisanti et al., 2014). The precise roles of these mediators in cardiac protection awaits further analysis. For example, while Akt and ERK1/2 are both phospho-activated upon GPCR transactivation of the EGFR, studies confirm negative effects of PI3K yet not ERK1/2 inhibition on cardioprotective outcomes.

4.1 G Proteins and Arrestins

As noted above, multiple G protein dependent and independent modes of EGFR transactivation have been identified (see ***1.1***). However, the precise roles of these distinct processes in protecting myocardial cells are yet to be defined. Expression of G proteins influences EGFR activation, with Howes *et al.* (2006) demonstrating that Gαq expression results in Src kinase and EGFR-dependent Akt phosphorylation in cardiomyocytes, limiting apoptosis during metabolic stress. Metalloproteases shedding mature EGFR ligands may also be a direct target of G proteins, as revealed in the recent study of Overland & Insel (2015).

Specific ligand-dependent conformational changes in GPCRs may promote signalling via arrestins independently of G proteins. Several studies highlight G protein independent/arrestin dependent signalling in cardioprotective EGFR transactivation (Noma et al., 2007). Mechanical stretch has also been shown to trigger an AT1 receptor-mediated conformational change in β-arrestin to selectively stimulate receptor signalling in the absence of G protein activation (Rakesh et al., 2010). In contrast, protective δ-opioid receptor activation of kinase signalling appears to involve PKCδ dependent control of ligand shedding, and β-arrestin2 recruitment

downstream of the EGFR (Zhang et al., 2015). Thus, β-arrestins can function both at the level of the triggering GPCR and downstream of the activated EGFR in transduction of cardioprotection.

4.2 ROS Signalling

Signalling via ROS is implicated in cardioprotective transactivation responses (Krieg et al., 2004; Cohen et al., 2007), and appears to be downstream of Src, MMP, HB-EGF, and PI3K/Akt (Cohen et al., 2007). In terms of cardiac protection via GPCR agonism/ischaemic preconditioning, ROS generation (via NADPH oxidase activity, mitochondrial electron transport chain) has been localised both up- and downstream of mitochondrial K_{ATP} channels and PKC (Hausenloy and Yellon, 2006; Murphy and Steenbergen, 2008; Heusch, 2015). However, ROS are also important in MMP activation and EGFR ligand shedding (Wetzker and Bohmer, 2003). In cardiac fibroblasts, for example, ROS are localised upstream of MMP-dependent HB-EGF shedding (in transactivation via endothelin-1), facilitating HB-EGF activation of EGFR via inhibitory oxidation of Src homology 2-containing tyrosine phosphatase (Chen et al., 2006). This is consistent with evidence of ROS-dependent control of protein tyrosine phosphatase 1B activity (Salmeen et al., 2003; van Montfort et al., 2003), known to dephosphorylate EGFR (together with insulin receptor kinase, JAK2 and TYK2 kinases). Thus, ROS are involved at multiple levels, modifying MMP activity and EGFR phosphorylation status whilst also transducing downstream cardioprotective signalling.

4.3 PI3K

The heart expresses three major Class 1 PI3K isoforms - PI3Kα, PI3Kβ and PI3Kγ (Crackower et al., 2002). Class IA and IB PI3Ks regulate metabolism, survival, and growth/differentiation in cells including cardiomyocytes (Cantley, 2002;

Engelman et al., 2006). These isoforms differ functionally and structurally: class IA catalytic subunits (p110α, β, δ) complex with regulatory p85 and are activated by tyrosine kinase signals; class IB catalytic subunits (p110γ) lack a C-terminus binding domain for p85, instead associating with p101, and are activated by GPCRs. In terms of cardiac protection, canonical activators of both PI3K IA (EGF, insulin, IGF) and IB (adenosine, opioid, bradykinin) can induce tissue protection, though specific roles in different modes of cardioprotection remain to be fully elucidated.

Interestingly, cardioprotective responses involving EGFR transactivation are negated with deletion of GPCR-sensitive PI3Kγ. Knockout of PI3Kγ worsens myocardial infarction and impairs Akt activation (Haubner et al., 2010), confirming its importance to I-R tolerance (albeit apparently independent of catalytic kinase activity). Moreover, knockout negates ischaemic and adenosinergic preconditioning (Ban et al., 2008), responses shown to require EGFR transactivation (Benter et al., 2004; Williams-Pritchard et al., 2011). On the other hand, PI3Kα appears key to protection via epoxyeicosatrienoic acid (Batchu et al., 2012) and ouabain (Duan et al., 2015). Unexpectedly, expression of dominant negative PI3Kα improves cardiac functional tolerance to I-R (Ban et al., 2008), though this potentially reflects augmented signalling via PI3Kγ, enhancing Akt and GSK3β phosphorylation. However, longer term outcomes from myocardial infarction (Lin et al., 2010) and pressure-overload (McMullen et al., 2003) are worsened. Collectively, data to date reveal different dependencies of cardioprotection on PI3Kα *vs.* PI3Kγ, and a paradoxical dependence of adenosine and ischaemic preconditioning responses on both EGFR transactivation and GPCR-sensitive PI3Kγ.

These data may reflect cross-talk between class IA and class IB isoforms during RTK transactivation via intracellular Src tyrosine kinase, MMP activation and

extracellular ligand release (Oudit et al., 2004). However, elimination of ischaemic preconditioning and adenosine protection via both PI3Kγ knockout (Ban et al., 2008) and EGFR inhibition (Williams-Pritchard et al., 2011) suggests a critical role for RTK-dependent p110γ rather than GPCR-coupled p110α (and/or compensatory changes in p110γ on p110α deletion) in these protective responses (Ban et al., 2008)

4.4 Protein Kinase B/Akt

Activation of PI3K leads to Akt phosphorylation. Both Akt1 and Akt2 isoforms are highly expressed in heart (Muslin and DeBosch, 2006), with Akt1 essential to normal cardiac growth (Cho et al., 2001b) and physiological hypertrophy (DeBosch et al., 2006b), and Akt2 involved in insulin-regulated glucose homeostasis and cardiomyocyte survival (Cho et al., 2001a; Garofalo et al., 2003; Etzion et al., 2010). Knockout of Akt2 results in severe hyperglycaemia (Cho et al., 2001a) and evidence of diabetic cardiomyopathy (Etzion et al., 2010). There is evidence cardioprotection involves specific Akt1 activation, with ischaemic preconditioning negated with Akt1 but not Akt2 deletion (Kunuthur et al., 2012). Other evidence supports Akt1-dependent inhibition of acute myocardial damage during I-R *vs.* a worsening of subsequent fibrosis and mortality (Ma, L. et al., 2014). Nonetheless, Akt2 has also been linked to apoptosis resistance in cardiomyocytes (DeBosch et al., 2006a).

There is little information regarding the Akt isoform specificity of cardiac EGFR (and PI3Kα *vs.* γ) signalling. Certainly, the EGFR selectively engages distinct Akt isoforms in other cell types, and at different stages of the cell cycle. Studies in cancer cells reveal cell-specific effects of EGF on different Akt isoforms (Okano et al., 2000; Grabinski et al., 2011; Khabele et al., 2014). Analysis of cell-cycle

dependent EGFR signalling reveals select activation of Akt2 and not Akt1 during mitosis (*vs*. both Akt1 and 2 activation in the interphase) (Wee et al., 2015).

4.5 ERK1/2

While activation of EGFR consistently enhances phosphorylation of cardiac ERK1/2 (Gotoh et al., 1992; Duquesnes et al., 2009; Tilley et al., 2009; Villa-Abrille et al., 2010; Akhtar et al., 2012), the importance of this MAPK to associated cardioprotection remains unclear. A number of studies provide support for ERK1/2 involvement in cardioprotection, whereas others report no role for the kinase in preconditioning and adenosine responses (Hausenloy and Yellon, 2006), both identified as EGFR-dependent stimuli. Few studies have tested the effects of ERK1/2 (or upstream MEK) inhibition on EGFR-dependent protection. In studying the EGFR dependence of protection with met(5)-enkephalin, Cao *et al.* (2004) confirmed inhibition of both protection and ERK1/2 activation with EGFR, MAPK and MEK1/2 (together with PI3K) inhibitors, whereas Grisanti *et al.* (2014) found the anti-apoptotic effects of ß1-adrenergic receptor mediated EGFR transactivation are inhibited by a MEK or PI3K inhibitor, supporting ERK1/2 and PI3K involvement. Further analysis of ERK1/2 involvement in EGFR mediated cardioprotection is needed.

4.6 PKC

Protein kinase C appears to be located both upstream of EGFR activation, modulating MMP cleavage of EGFR ligands and receptor activation (Zhang et al., 2015), and downstream of the receptor in mediating cardiac protection (Cao et al., 2005). The enzyme is widely implicated in protective responses to preconditioning and GPCR stimuli (including PKC-α□□-δ, □ε, and□□η), though some controversy remains regarding the involvement and protective functions of PKC (Hausenloy and

Yellon, 2006). Within conventional protective signalling, PKC has been localised downstream of PI3K/Akt and NO/PKG and potentially both up- and downstream of mitochondrial K_{ATP} channels, with activation involving ROS generation (Hausenloy and Yellon, 2006; Murphy and Steenbergen, 2008; Heusch, 2015). Studies of preconditioning also localise PKC upstream of RTK activity (Baines et al., 1998), consistent with a role in regulating EGFR function and binding.

In non-cardiac cell models, the phorbol ester PMA stimulates PKC-dependent phosphorylation of EGFR at threonine 654, and blocks EGF induced high-affinity internalisation of the receptor (Lund et al., 1990). Kodama et al (2002) report that both ET-1 and PKC activation induce EGFR and ERK1/2 phosphorylation in cardiomyocytes: down-regulating PKC inhibits EGFR phosphorylation, and AG1478 partially inhibits PMA-dependent ERK1/2 phosphorylation while strongly inhibiting ET-1 dependent ERK1/2 phosphorylation. More recent work supports specific involvement of PKC-δ translocation in regulating metalloprotease activity (Zhang et al., 2015), and there is also evidence PKCα and PKCδ act in parallel to regulate ADAM activity and cell surface HB-EGF levels, respectively (Kveiborg et al., 2011). Studies of ErbB1 or ErbB2 inhibition/deletion in cardiac tissue also support PKCα involvement in protective signalling (Gordon et al., 2009). Additionlly, interaction of ouabain with cardiomyocyte Na^+/K^+ ATPase triggers EGFR transactivation and Ras/ERK1/2 activation (Haas et al., 2000), with PKC essential to this signalling (Mohammadi et al., 2001), though whether up- and/or downstream of EGFR is untested.

In contrast to these findings, several studies assessing EGF activation of myocyte signalling report no role for PKC in the EGFR response (despite ability of PKC activators to induce similar outcomes). Quintaje *et al.* (1998) showed both PMA

and EGF activate cardiomyocyte ERK1/2, however PKC effects were insensitive to RTK inhibitors and the EGF response appeared PKC-independent. Clerk *et al.* 2006 found that EGF increases cardiomyocyte Ras.GTP and activates c-Raf and ERK1/2 phosphorylation independently of PKC. Similarly, there is evidence that EGF activation of ERK1/2 in non-cardiac muscle is PKC-independent and Ras-dependent (Robin et al., 2004). These findings suggest that direct ligation of the EGFR removes the PKC dependence of signalling, implicating upstream functions of the kinase in regulating ligand levels and EGFR phosphorylation.

5. Impacts of Cardiac Disease/Co-Morbid States

Relatively little is known regarding impacts of disease states on cardiac ErbB1 expression and function, with studies to date focussed on ErbB2 and ErB4. While we briefly review effects of individual disease states on cardiac EGFR signalling, it is important to note that many of these may co-exist, for example within the so-called metabolic syndrome. The interactions between these conditions, and whether potentially synergistic impacts on EGFR signalling might arise, are unclear.

5.1. Ischaemia and Ischaemic Heart Disease

Ischaemic or hypoxic insult may impair myocardial ErbB expression and signalling. Proteosomal degradation of the dimer partner ErbB2 is limited by the ATP-sensitive chaperone function of Hsp90 (Xu et al., 2001), thus reductions in cellular ATP result in ErbB2 dissociation and degradation (Peng et al., 2005). Myocyte ErbB2 levels have been shown to decline with metabolic stress (glycolytic or mitochondrial inhibition) associated with ATP depletion (Peng et al., 2005) and hypoxia (Viswanath et al., 2011). Such findings support negative impacts of ischaemic/hypoxic stress on ErbB2 expression and thereby EGFR functionality.

Indeed, levels of ErbB2 are decreased in an animal model of ischaemic heart disease as well as in human ischaemic cardiomyopathy (Gordon et al., 2009). The latter investigators went on to demonstrate increased ROS generation and death in myocytes subjected to ErbB2 inhibition. Bodiga *et al.* (2015) report that ErbB1 and ErbB2 are the predominant EGFR members expressed in H9c2 myoblasts, as in intact heart (Zhao et al., 1998), and are differentially influenced by hypoxia-reoxygenation: ErbB1 mRNA was up-regulated and ErbB2 mRNA down-regulated with increasing hypoxic exposure. These shifts in ErbB expression are in line with changes observed in hypoxic myocardium of patients admitted for coronary artery bypass graft (Munk et al., 2012).

5.2. Hypertrophy/Heart Failure

Cardiac ErbB2 and ErbB4 mRNA and protein levels increase with ventricular pressure overload during compensatory cardiac hypertrophy in mouse models, yet decline on transition to heart failure (Rohrbach et al., 1999). Subsequent work from these investigators showed reduced ErbB2 and ErbB4 expression and activity in patients with advanced heart failure (Rohrbach et al., 2005). Interestingly, Uray *et al* (2002) found that ErbB2 mRNA was up-regulated following ventricular unloading in heart failure patients. Such changes may be relevant to myocardial outcomes and impaired stress-tolerance and cardioprotection in models of hypertension, hypertrophy and heart failure (Ferdinandy et al., 2014).

5.3. Diabetes

Limited and conflicting information exists regarding impacts of diabetes on myocardial EGFR signalling. Increased EGFR activity is reported in mice with streptozotocin induced type I diabetes, concomitant with ER stress, cardiac fibrosis and collagen deposition (Galan et al., 2012; Liang et al., 2015). Systemic inhibition of EGFR mitigated these effects of diabetes, though the high dose of AG1478 (10 mg/kg/day) also reduced the extent of hyperglycemia, complicating interpretation. In contrast, ErbB expression and EGFR phosphorylation is reportedly depressed in streptozotocin-dependent diabetic rats, in association with worsened myocardial I-R tolerance (Akhtar et al., 2012). In this study, systemic EGFR antagonism with AG1478 further aggravated I-R injury while EGFR agonism enhanced ischaemic tolerance. Others report reduced expression of ErbB2 and ErbB4 in rats with diabetic cardiomyopathy (Gui et al., 2012).

There is also evidence from studies of experimental diabetes that up-regulation of EGFR signalling (involving transcriptional induction and increased RTK activity) contributes to vascular dysfunction, which may be reversed with systemic receptor antagonism (Benter et al., 2005b; Benter et al., 2005c). Phosphorylation of vascular EGFR is increased in the murine *db/db* model of type II diabetes, in association with reduced eNOS expression and coronary vascular dysfunction (Belmadani et al., 2008), effects countered by inhibition of EGFR signalling. Inhibition of EGFR also counters abnormal vasoconstrictor/dilator responses in streptozotocin-dependent type I diabetic rats (Benter et al., 2005b; Yousif et al., 2005). Abnormal myogenic tone in diabetes may also involve EGFR (Matrougui, 2010), and vascular transcriptomic changes in type I diabetic rats are suppressed by EGFR inhibition (Benter et al., 2009).

5.4. Obesity/Dyslipidemia

There is limited evidence EGFR is perturbed and may be a useful target in obesity-related cardiovascular disease. Li *et al*. (2016) found that EGFR inhibitors reduce myocardial inflammation, fibrosis, apoptosis and dysfunction in two murine obesity models, together with inhibiting palmitate-dependent inflammation and apoptosis in H9c2 cells. This is consistent with saturated fatty acid inhibition of NRG-1b activation of myocyte PI3K/Akt signalling, an effect countered by a mono-unsaturated fatty acid (oleate) (Miller et al., 2009). Moreover, the endogenous mono-unsaturated fatty acid oleylethanolamide has been shown to activate ErbB2 and RAS-ERK1/2 signalling and improve cardiac function in doxorubicin-induced heart failure (Su et al., 2006). Thus, shifts in myocardial saturated *vs*. unsaturated fatty acids, as may occur with metabolic syndrome/hyperlipidaemia, may perturb normal myocardial EGFR signalling, though further work is warranted in terms of impacts on ErbB1/ErbB2 expression and function.

6. Understanding complexities in EGFR modulation

Despite a weight of evidence supporting cardioprotective outcomes with EGFR activity, there is also evidence for potentially detrimental impacts of this receptor in myocardial I-R. Feng *et al.* (2012) reported that the EGFR kinase inhibitor AG556, and EGFR siRNA significantly suppresses post-ischaemic arrhythmias. Inhibition of EGFR with cetuximab protects against cardiac rupture and improves survival post-infarction (Hammoud et al., 2011). Though in a model of endotoxemia, EGFR exerts injurious cardiac effects, promoting myocardial TNFα production and contractile dysfunction (Sun et al., 2015). Since TNFα worsens outcomes from myocardial I-R, such effects of EGFR might prove detrimental in the setting of myocardial I-R.

Reports of benefit via EGFR inhibition may reflect the importance of maintaining a normal range of receptor activity. Over-activity of EGFR in diabetes/metabolic disorders, for example, may exaggerate inflammation and oxidative stress in cardiac (Liang et al., 2015; Li et al., 2016) and other tissues (Fang et al., 2016). Inhibitors of EGFR have also been shown to reduce insulin-resistance (Prada et al., 2009), atherosclerosis (Gao et al., 2013) and vascular dysfunction in (Choi et al., 2012) in models of obesity, dyslipidemia and diabetes. Thus, dysregulated or excess EGFR signaling may promote some of the cardiac consequences of metabolic disorders. Indeed, excessive EGFR signaling may contribute to cardiac hypertrophy in chronic kidney disease and other scenarios, whereas under-activity may impair tissue stress-resistance. Distinct cardiac and vascular effects of this receptor system may also be relevant – cardiac hypertrophic outcomes may well reflect effects of EGFR on vascular control and blood pressure (Schreier et al., 2013; Schreier et al., 2016).

7. Summary and Conclusions

Despite the ongoing focus on ErbB signalling in cardiac development and hypertrophy, increasing evidence points to a key role for EGFR signalling in governing intrinsic myocardial stress-resistance and cardioprotective responses to diverse protective stimuli. Multiple GPCRs and ischaemic preconditioning stimuli appear to involve essential EGFR transactivation. The mechanistic details of this key signalling axis await further delineation. Questions include the identity of metalloproteases involved and the basis of their control, the roles of G protein *vs*. β-arrestin dependent mechanisms in cardioprotective EGFR signalling, the relative roles of PI3K and Akt isoforms in transducing protective signalling, the relative protective functionalities of ErbB1/1 *vs.* ErbB1/2 dimers, and the potential roles of altered neuregulin/ErbB4 *vs.* ErbB1 signalling in the detrimental impacts of ErbB2 inhibition.

Furthermore, how age, disease and infarction impact protective EGFR signalling requires specific study - shifts with age and disease may negatively impact myocardial stress-tolerance and impair our capacity to protect the diseased heart via conventional conditioning stimuli.

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Figure 1: ErbB1 structure and dimerisation. ErbB1 consist of: an extracellular domain containing 2 leucine-rich (LR) ligand binding domains (I and III) and 2 cysteine-rich (CR) regulatory domains (II and IV). ErbB2 lacks functional ligand binding domains. A transmembrane region connects to the intracellular domain, containing a short juxtamembrane sequence, dual protein tyrosine kinase sub-domains (smaller amino-terminal lobe – N; larger carboxyl-terminal lobe - C), and a non-catalytic C-terminal region containing regulatory Tyr-residues (Y) phosphorylated upon receptor activation. Ligands bind with low affinity to domains I and III on an ErbB1 monomer, inducing a conformational change that allows high affinity ligand binding to both sub-domains. The molecular tether between domain II and IV is broken, exposing a 'dimerisation arm' that can interact with another receptor arm to induce dimerisation.

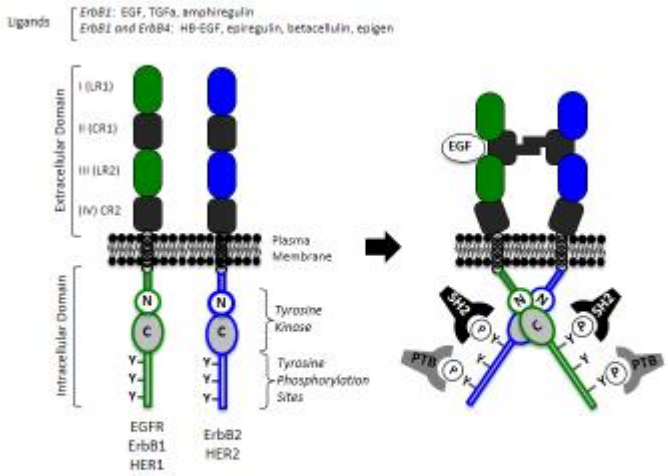

Figure 2: GPCR transactivation of EGFR signalling via G protein dependent and independent mechanisms. Differing G protein dependent and independent processes may trigger EGFR ligand shedding and EGFR activation. G protein dependent transactivation involves conventional (e.g., Ca^{2+}, PKC, ROS) and unconventional mediators (TRIO, BMX, CHKA) that modulate the activity of metalloproteases (and the EGFR). Activation of the EGFR results in PI3K/Akt and ERK1/2 phospho-activation.

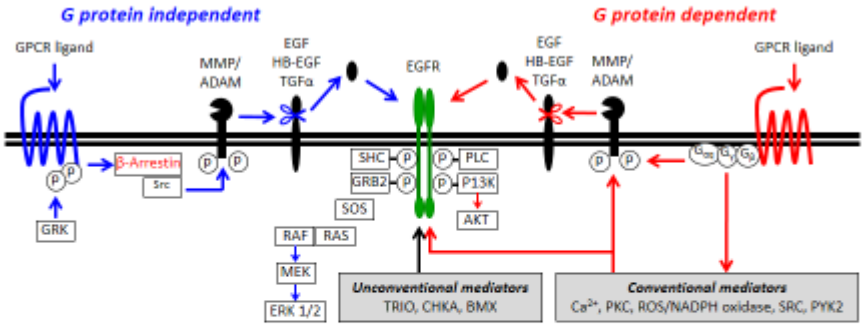

Figure 3: Cardioprotective signalling implicated in EGFR-dependent cardioprotective responses. A generalised scheme of kinase signalling and cardioprotective effectors implicated in GPCR and ischaemic preconditioning responses is shown. Red highlighted text refers to mediators directly implicated in studies of EGFR responses. Activation of PI3K/AKT, eNOS, PKG and PKC impacts on mitochondrial ATP-sensitive K^+ channel (mitoK_{ATP}) and mitochondrial permeability transition pore (mPTP) function, modifying mitochondrial ROS generation. ROS in turn influence a range of kinases mediating varied protective effects, including modulation of mPTP and mitoK_{ATP} function, gap junctional proteins (CX43) and function, oxidant stress, Ca^{2+} overload and apoptotic signalling. Evidence also implicates GPCR coupled PKA activity, and JAK-STAT signalling in conditioning/cardioprotective responses. Delayed protection may involve transcriptional induction of COX, iNOS and other mediators. Not shown are autophagy and mitochondrial fission/fusion paths, also implicated in cardioprotection.