A central role of EGF receptor transactivation in angiotensin II -induced cardiac hypertrophy

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In addition to their physiological roles in the cardiovascular system (CVS), G-protein-coupled receptor (GPCR) agonists such as noradrenaline, endothelin-1 and angiotensin II (Ang II) are known to be involved in the development of cardiac hypertrophy. Recent studies using targeted overexpression of the angiotensin AT_1 receptor in cardiomyocytes suggest that Ang II can directly promote the growth of cardiomyocytes via transactivation of the epidermal growth factor (EGF) receptor and subsequent activation of mitogen-activated protein kinases (MAPKs). This process is mediated by the production of heparin-binding EGF (HB–EGF) by metalloproteases. Blockade of the generation of HB–EGF by metalloprotease inhibitors, or abrogation of EGF receptor kinase activity by selective pharmacological inhibitors or antisense oligonucleotides, protects against Ang II-mediated cardiac hypertrophy. These approaches offer a potential therapeutic strategy to prevent cardiac remodeling and hypertrophy, and possibly prevent progression to heart failure.

Cardiac hypertrophy is an adaptive physiological process that occurs in response to a variety of extracellular stimuli, such as mechanical stress, G-protein-coupled receptor (GPCR) agonists, cytokines and growth factors. Increased hemodynamic stress, from both pressure and volume overload, is fundamental to the development of left ventricular hypertrophy (LVH), which is characterized by alterations in the structure and muscle content of the heart and blood vessels. These changes lead to significant morbidity and mortality. The cellular derangement in LVH is the consequence of remodeling processes involving the two major cell types of ventricular tissue: cardiomyocytes and fibroblasts [1]. Cardiomyocytes, which comprise 70–80% of the mass of the adult heart, are terminally differentiated cells that are responsible for the precise regulation of the contractile response of the heart through activation of their GPCRs. Adult ventricular myocytes (AVMs) have been used to delineate the acute signaling pathways by which GPCRs regulate cell excitability, Ca^{2+} and excitation–contraction coupling. However, these cells are less useful for examining the control of cell growth and most studies on cardiomyocyte hypertrophy have used primary cultures of neonatal rat ventricular myocytes (NRVMs). During cardiac remodeling, hypertrophy of cardiomyocytes causes an increase in cardiac mass accompanied by an enlargement of individual cell size, an increase in the content of contractile proteins such as myosin heavy chain, and expression of embryonic genes such as those encoding atrial natriuretic factor (ANF). However, cardiac fibroblasts undergo proliferation and produce extracellular matrix components such as fibronectin, laminin and collagen in the interstitium [1,2].

Renin–angiotensin system and cardiac hypertrophy

Sustained stimulation of GPCRs such as α-adrenoceptors and those for endothelin-1 (ET-1) and Ang II can culminate in cardiac hypertrophy, which is initially an adaptive response of the heart and involves cardiomyocyte growth and accumulation of extracellular matrix. However, LVH is also an independent risk factor, and if left untreated can lead to several adverse morphological and functional changes in the heart, including dilated cardiomyopathy, fibrosis and heart failure [3]. Such alterations have been associated, in part, with increased activity of the renin–angiotensin system (RAS) [4,5]. Angiotensins are peptide hormones derived from the protein precursor angiotensinogen by the sequential actions of proteolytic enzymes (Fig. 1). The classical pathway of Ang II synthesis includes a reaction catalyzed by renin, followed by another reaction catalyzed by angiotensin-converting enzyme (ACE), which occurs not only in plasma but also in the kidneys, brain, adrenal glands, ovaries and possibly other tissues [6]. Components of the RAS have been detected in multiple tissues including the heart. Sadoshima *et al.* [7] demonstrated that mechanical stretch caused the release of Ang II from cardiac myocytes, providing direct evidence for an autocrine mechanism in the load-induced growth of cardiomyocytes. Furthermore, in the hypertrophied human heart, angiotensinogen is increased in the subendocardial myocardium. However, Ang II concentrations vary widely in individual tissues, possibly as a result of differential uptake of RAS components and/or variations in their local synthesis [8].

Ang II activates diverse signaling molecules through the AT_1 receptor

Ang II is the dominant effector of the RAS and has an important role in cardiovascular and renal pathophysiology.

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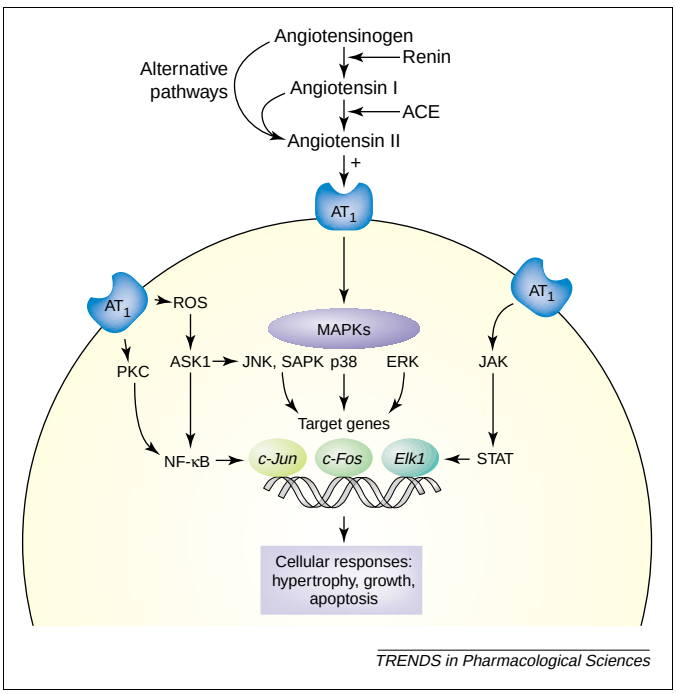

Fig. 1. Signaling pathways activated by angiotensin II (Ang II). The peptide hormone Ang II is derived from the protein precursor angiotensinogen by the sequential actions of proteolytic enzymes. The classical pathway of Ang II synthesis includes the conversion of angiotensinogen to Ang I, catalyzed by renin, followed by the conversion of Ang I to Ang II by the action of angiotensin-converting enzyme (ACE), However, Ang II can also be formed via other pathways. Stimulation of the angiotensin AT_1 receptor leads to the generation of the second messengers protein kinase C (PKC) and Ca^{2+}, through $G_{q/11}$ or via $G\beta\gamma$-subunits following G_i activation, and results in the activation of multiple signaling proteins including: (1) mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinases 1 and 2 (ERK1,2), p38 MAPK, c-JUN N-terminal kinase (JNK) and stress-activated protein kinase (SAPK); and (2) janus-activating kinase (JAK) and signal transducers and activators of transcription (STATs). Activation of AT_1 receptors also causes activation and nuclear translocation of nuclear factor κB (NF-κB) following stimulation by PKC or following stimulation by apoptosis signal-regulating kinase 1 (ASK1; also known as MAPKKK), which in turn is stimulated by reactive oxygen species (ROS). These signals activate target genes such as *c-Fos*, *c-Jun* and *Elk1* in the nucleus, leading to cell growth, hypertrophy and apoptosis.

Although the vasopressor action of Ang II can be life-saving under physiological conditions, over-stimulation of the RAS can result in hypertension, atherosclerosis, tissue remodeling and cardiac hypertrophy. Indeed, elevated Ang II levels have been implicated in several hypertension-associated pathologies. LVH is a well-recognized manifestation of end-organ injury in hypertension. The pathogenesis of LVH is linked to activation of the RAS because ACE inhibitors and AT_1 receptor antagonists such as losartan, valsartan and candesartan can reverse LVH in hypertensive patients [5,9].

The effects of Ang II are mediated primarily through the activation of the angiotensin AT_1 receptor (Fig. 1), which, via the G protein $G_{q/11}$, stimulates phospholipase C (PLC) to generate inositol (1,4,5)-trisphosphate and diacylglycerol, thereby increasing the intracellular concentration of Ca^{2+} and activating protein kinase C (PKC) [6]. In cardiac fibroblasts, the AT_1 receptor is coupled to G_i in addition to G_q [6,10]. Several signaling molecules have been implicated in the Ang II-induced hypertrophic response, including: (1) mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinases 1 and 2 (ERK1,2), p38 MAPK and c-JUN N-terminal kinase (JNK) [11,12]; (2) tyrosine kinases such as Src and Pyk2 [13,14]; (3) phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB; Akt) [15,16]; (4) janus-activating kinase (JAK) and signal transducers and activators of transcription (STATs) [17,18]; (5) receptor tyrosine kinases (RTKs) such as the epidermal growth factor (EGF) receptor or platelet-derived growth factor (PDGF) receptor [19,20]; (6) immediate early genes (IEGs) such as *c-Fos* and *c-Jun* [21]; and (7) transcription factors such as nuclear factor κB (NF-κB) [22]. The interplay of these signaling molecules during the activation of AT_1 receptors by Ang II is shown in Fig. 1.

During Ang II-induced cardiac hypertrophy, G_q-linked signaling predominantly operates in cardiomyocytes, and G_i-mediated activation of Src, Grb2, Ras and Raf occurs in cardiac fibroblasts [10,23,24]. The importance of G_q-linked signaling in the development of cardiac hypertrophy was demonstrated in transgenic mice overexpressing $G_{q\alpha}$ in the heart. Fourfold overexpression of $G_{q\alpha}$ resulted in increased cardiac weight and myocyte size, in addition to marked increases in the levels of ANF and skeletal actin [25]. Similarly, stimulation of NRVMs with the $G_{q\alpha}$ agonist *Pasteurell multocida* toxin enhanced cardiomyocyte enlargement, ANF expression and activation of MAPKs such as ERK1,2, JNK and p38 MAPK [26]. Interestingly, although transgenic mice lacking $G_{q/11\alpha}$ showed no detectable LVH in response to pressure overload induced by aortic constriction [27], mechanical stretch caused hypertrophic responses in cardiac myocytes of AT_{1A} receptor knockout mice [28].

Expression of AT_1 receptors in cardiomyocytes causes hypertrophy

The basis of Ang II-induced cardiomyocyte growth is controversial because there is compelling evidence that ET-1, cytokines and growth factors released from cardiac fibroblasts can act in a paracrine fashion to promote myocyte growth. Recently, transgenic studies in mice and rats have provided novel insights into the pathophysiological role of the RAS. Inactivation of individual RAS components by gene targeting in embryonic stem cells, and overexpression of genes in specific tissues *in vivo,* have begun to define the specific functions of the RAS [29]. To determine whether Ang II action in the heart is direct or a consequence of pressure overload, Paradis *et al.* used transgenic mice overexpressing human AT_1 receptors exclusively in cardiomyocytes. These mice, despite their normal blood pressure and heart rate, developed cardiac hypertrophy and remodeling that was accompanied by increased expression of ANF and interstitial collagen deposition, leading to congestive heart failure and premature death [30].

In a complementary study, Hoffman *et al.* [31] developed a transgenic rat model that overexpressed the human AT_1 receptor under the control of the α-myosin heavy-chain promoter specifically in the myocardium. These animals showed normal cardiac growth and function under baseline conditions but had pronounced hypertrophic growth and contractile responses to Ang II when challenged by volume and pressure overload. The reasons for the differential responses observed in this study and that of Paradis *et al.* [30] are not known but possibly include differences in: (1) the species used; (2) the onset and level of

transgene expression; and (3) the length of AT_1 receptor cDNA used.

More recently, Thomas *et al.* [32] used recombinant adenovirus to deliver modest levels of the AT_{1A} receptor into NRVMs to mimic the upregulation of AT_1 receptors that occurs during cardiac hypertrophy. In these cells, stimulation by Ang II caused robust hypertrophy that was independent of the release of cardiac fibroblast-derived factors. Moreover, activation of ERK1,2 by Ang II occurred through metalloprotease-dependent EGF receptor transactivation. These studies demonstrate that Ang II can directly promote cardiac myocyte growth via AT_{1A} receptor activation and indicate that cardiac hypertrophy in response to AT_1 receptor stimulation is not necessarily dependent on factors released from cardiac fibroblasts.

Role of EGF receptor transactivation in Ang II action

Much attention has focused on the molecular mechanisms that mediate the cardiomyocyte response to stimulation by Ang II. A major recent development in this area is the finding that many of the mitogenic effects of Ang II are mediated through transactivation of the EGF receptor. Substantial evidence indicates the existence of receptor tyrosine kinases, including the EGF receptor, in the heart [28,32–34]. Blocking EGF receptor kinase activity with the selective inhibitor AG1478 (see Chemical names) abolishes Ang II-mediated downstream signaling such as activation of ERK1,2 in several cell types, including vascular smooth muscle cells (VSMCs) and cardiac myocytes [13,20], and growth and migration of VSMCs [35]. Thus, transactivation of EGF receptors by GPCRs, in particular the AT_1 receptor, accounts for the majority of growth-promoting responses in the myocardium.

The EGF receptor, an RTK, is endogenously expressed in numerous cell types and is an important factor in the control of many fundamental cellular processes, including the cell cycle, cell migration, cell metabolism and survival, in addition to cell proliferation and differentiation [36]. RTKs possess an extracellular ligand-binding domain connected to the cytoplasmic domain by a single transmembrane helix. The cytoplasmic domain contains a conserved protein kinase core and additional regulatory sequences that are subjected to autophosphorylation and phosphorylation by other protein kinases [37]. Overexpression of the EGF receptor often results in uncontrolled growth and tissue remodeling. Not surprisingly, the EGF receptor and its ligands are frequently upregulated in human cancers. Consequently, blocking the proliferative effects of EGF receptor activation has potential therapeutic applications in cancer and aberrant tissue growth.

Mechanism of Ang II-induced EGF receptor activation

Ang II-mediated transactivation of the EGF receptor is mediated by several intermediary signaling molecules including Ca^{2+}, PKC, reactive oxygen species (ROS), tyrosine kinases (Src and Pyk2) and metalloproteases that generate EGF-like ligands (Fig. 2) [13,19,20,38]. In addition, stimulation by Ang II promotes the rapid movement of AT_1 receptors to caveolae and lipid rafts that are organized by the cholesterol-binding protein caveolin and are associated with EGF receptors in VSMCs. Depletion of membrane cholesterol by β-cyclodextrin, filipin or nystatin disrupts the structure of caveolae and concomitantly inhibits tyrosine phosphorylation of EGF receptor and the subsequent activation of PKB induced by Ang II [16]. Analysis of the specific AT_1 receptor motif(s) responsible for EGF receptor transactivation revealed that mutation of the receptor at Y319F prevented EGF receptor phosphorylation and proliferation of cardiac fibroblasts. Moreover, stimulation by Ang II increases AT_1 receptor association with EGF receptors through SHP-2 [39]. The paradigm emerging from recent studies by several laboratories implicates transactivation of the EGF receptor as a central point in mediating the growth-promoting effects of GPCRs, in particular those of Ang II in cardiovascular disorders including hypertension and cardiac hypertrophy [19,32,33,40]. EGF treatment of NRVMs stimulates protein synthesis, expression of the IEGs *c-Fos* and *c-Jun*, and STAT5 [41]. The central role of EGF receptors in Ang II-induced hypertrophic effects in cardiovascular tissues was well demonstrated by Kagiyama *et al.* [40], who showed that Ang II-induced elevation of blood pressure and cardiac hypertrophic responses were abolished by an antisense

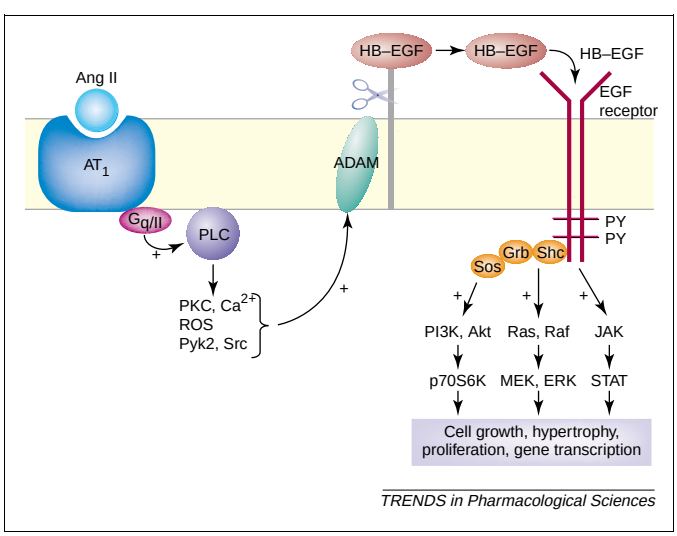

Fig. 2. The mechanism of angiotensin II (Ang II)-induced transactivation of the epidermal growth factor (EGF) receptor and downstream signaling pathways. Activation of the angiotensin AT_1 receptor by Ang II leads to the activation of phospholipase C (PLC) and the subsequent production of several intermediary signaling molecules including Ca^{2+}, protein kinase C (PKC), reactive oxygen species (ROS) and tyrosine kinases (e.g. Src and Pyk2). Activation of the metalloprotease ADAM (a disintegrin and metalloprotease) by these signaling molecules leads to the enzymatic conversion of pro-heparin-binding EGF (proHB–EGF) to HB–EGF, which binds to, phosphorylates and thus activates the EGF receptor. Activation of EGF receptors initiates a mitogen-activated protein kinase (MAPK) cascade through the recruitment of adaptor proteins, such as the Shc–Grb–Sos complex, that activates the small G protein Ras, and subsequent activation of Raf, MAPK kinase (MEK) and extracellular signal-regulated kinases 1 and 2 (ERK1,2). EGF receptor stimulation also leads to the activation of the janus-activating kinase (JAK) and signal transducers and activators of transcription (STATs) pathway, and the phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt) pathway, culminating in cell growth and hypertrophy.

Chemical names

AG1478: 4-(3-chloroanilino)-6,7-dimethoxyquinazoline
KBR7785: [4-(*N*-hydroxyamino)-*2R*-isobutyl-3S-methylsuccinyl]-L-phenylglycine-*N*-methylamide

oligodeoxynucleotide to the EGF receptor in cultured VSMCs and Ang II-infused hypertensive rats. However, the extent to which inhibition of EGF receptor kinase activity is of clinical significance in the prevention and treatment of cardiac hypertrophy remains to be determined.

Involvement of metalloproteases in EGF receptor transactivation

The mechanism of GPCR-mediated transactivation of the EGF receptor has only recently been identified. Several studies have revealed the crucial role of metalloproteases in the enzymatic conversion of pro-heparin-binding EGF (proHB–EGF) to HB–EGF, which binds to and activates the EGF receptor. The expression of HB–EGF and its receptor is enhanced in the hypertrophied left ventricle of spontaneously hypertensive rats [42] and after myocardial infarction [43]. Matrix metalloproteases (MMPs) are the major regulators of the extracellular matrix, and have been implicated in the genesis of myocardial infarction, left ventricular dilatation and heart failure. MMPs are a large family of structurally related endopeptidases that can degrade the components of the extracellular matrix. The myocardial extracellular matrix contains a fibrillar collagen network, a basement membrane, proteoglycans and glycoso-aminoglycans, and bioactive signaling molecules [44]. Pharmacological inhibition of MMPs blocks Ang II-induced transactivation of the EGF receptor and subsequent growth and migration of rat VSMCs [35]. Similarly, the MMP inhibitor KBR7785 blocks Ang II-induced EGF receptor transactivation and cardiac hypertrophy. Moreover, dominant-negative expression of a disintegrin and metalloprotease 12 (ADAM12) abrogated Ang II-induced cardiac hypertrophy by inhibition of HB–EGF shedding [33], which suggests that blockade of metalloproteases by specific inhibitors might prove to be a potent therapeutic strategy.

More than 20 MMPs have been identified and classified based on their specificity for substrates such as collagenase and gelatinase. Interstitial collagenases (e.g. MMP-1 and MMP-13), stromelysins (e.g. MMP-3) and gelatinases (e.g. MMP-2 and MMP-9) are present in the mammalian myocardium [45,46]. In animal models, a mechanistic relationship has been demonstrated between myocardial MMP expression and left ventricular remodeling. As a result of their crucial involvement in cardiovascular pathology, MMP inhibitors have been developed to prevent myocardial cell derangement. Studies using knockout mice and pharmacological MMP inhibitors have indicated the importance of these proteases in cardiac remodeling. Constitutive expression of human MMP-1 in transgenic mice lacking MMP-1 caused changes in myocardium collagen content over time, leading to increased mortality as a result of cardiac hypertrophy [12]. Thus, attenuation of MMP activation in cardiac tissue appears to have a specific inhibitory effect on hypertrophic responses.

The exact nature of the signals that contribute to the altered expression of MMPs during myocardial pathogenesis is currently a subject of great interest. MMP expression in different cell types is regulated by chemical agents, growth factors, neurohormones, cytokines and tumor promoters [47]. PKC has been shown to be involved in the activation of MMPs by GPCR agonists that are coupled to G_q [48]. The role of PKC as a growth signaling molecule in the myocyte has also been demonstrated by the use of constitutively active forms of PKC [49] and by overexpression of PKC-βII in NRVMs [50]. Stimulation of PKC by Ang II in NRVMs causes activation of MMP-9 through induction of nuclear transcription factor κB (NF-κB) [22], the transcription factor potentially involved in the hypertrophic growth of these cells [51].

Role of reactive oxygen species (ROS) in Ang II action in the heart

Recent studies have shown that ROS have an important role in the normal physiology of the CVS. However, abnormal activation of the non-phagocytic nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase by Ang II or noradrenaline has been implicated in cardiac hypertrophy and heart failure [38,52–54]. All of the catalytic enzymes involved in the generation and degradation of oxygen radicals, such as peroxide, hydrogen peroxide (H_2O_2) and hydroxyl, are present in the three major cardiac cell types: myocytes, fibroblasts and endothelial cells [38]. Although the acute release of large amounts of ROS have been implicated in cell death associated with myocardial infarction or reperfusion injury, chronic overproduction of ROS contributes to the development of LVH. Earlier studies by Nakamura *et al.* [52] showed that Ang II-induced cardiac hypertrophy is attenuated by inhibition of ROS generation in NRVMs. Furthermore, N-acetylcysteine, an antioxidant known to alter the redox state of the cell, blocks Ang II-stimulated phosphorylation of Pyk2, EGF receptors and ERK in cardiac fibroblasts [55].

ROS are formed by several enzymatic electron transport systems, including mitochondrial complexes I and II, non-phagocytic NADPH oxidases, xanthine oxidases, cyclooxygenases, lipoxygenases, cytochrome P450 monooxygenases, nitric oxide synthases and heme oxygenases. Recently, Sano *et al.* [11] showed that Ang II-stimulated ROS production occurs in neonatal rat cardiac fibroblasts through activation of NADH/NADPH oxidase, with subsequent phosphorylation of MAPKs (ERK1,2, JNK and p38 MAPK) and interleukin 6 expression, which increases cardiac hypertrophy by acting in a paracrine fashion. Wenzel *et al.* [56] showed that Ang II-induced expression of transforming growth factor β (TGF-β), a potent mitogen, is mediated through ROS and p38 MAPK activation in AVMs. Recently, Frank *et al.* [57] showed that ROS (H_2O_2) transactivates EGF receptors through the release of HB–EGF by metalloproteases in VSMCs. Moreover, H_2O_2 activated Pyk2, JAK2 and STAT in a PKC-δ-dependent manner. Accordingly, the experimental use of antioxidants and ROS scavengers in various model systems has proved useful in blocking intracellular ROS release and cell death [54,58] in addition to cardiac hypertrophy [53,59].

Although Ang II is known to activate ROS generation and the transcription factor NF-κB, no clear relationship was evident until recently. However, Hirotani *et al.* [59] discovered that GPCR agonists such as Ang II,

noradrenaline and ET-1 cause rapid and transient activation of MAPKKK, also called apoptosis signal-regulating kinase 1 (ASK1), in NRVMs. Interestingly ASK1, which is well known as a mediator of tumor necrosis factor α (TNF-α) -induced apoptosis, is activated by ROS and NF-κB during Ang II-induced hypertrophy. Adenovirus-mediated overexpression of a dominant-negative ASK1 attenuated the agonist-induced NF-κB activation and cardiac hypertrophy. Conversely, overexpression of a constitutively active ASK1 led to NF-κB activation and development of LVH that was abolished by inhibition of NF-κB. The implications of this signaling pathway *in vivo* remain to be determined.

Concluding remarks

The concept that the mitogenic effects of Ang II in the CVS are mediated indirectly through the release of growth factors and cytokines from cardiac fibroblasts is not the only basis for agonist-induced cardiac hypertrophy. Ang II acts in endocrine, autocrine and paracrine manners in the myocardium and can promote cell growth via transactivation of the EGF receptor. Conversely, blockade of the EGF receptor attenuates Ang II-mediated cardiac hypertrophy. Because other GPCR agonists such as adrenaline, noradrenaline, endothelin, bradykinin and acetylcholine also regulate cardiac function [60], it will be of interest to determine the role of EGF receptor transactivation in the cardiac hypertrophy that results from stimulation of the GPCRs for these agonists. The clinical implications of this approach in the prevention and treatment of cardiac hypertrophy await further exploration. Despite the increasing knowledge of the role of various signaling proteins in Ang II-induced cardiac hypertrophy, we are only beginning to understand the complexities of tissue remodeling. The introduction of new technologies, including genomics and proteomics, should provide a broader view of the fundamental processes involved in Ang II signaling during remodeling of the myocardium. In conclusion, following AT_1 receptor activation, upstream mediators including PKC, Ca^{2+}, ROS, Pyk2 and Src cause activation of metalloproteases (ADAM) and ectodomain shedding of HB–EGF, which leads to phosphorylation of the EGF receptor. The activated EGF receptor recruits specific adaptor and signaling molecules (e.g. Shc, Grb2, Sos, Ras and Raf) that initiate ERK1,2 cascades that mediate the hypertrophic response. Activation of the EGF receptor also causes activation of PI3K and JNK pathways.

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