The role of the paracrine/autocrine mediator endothelin-1 in regulation of cardiac contractility and growth

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Endothelin-1 (ET-1) is a critical autocrine and paracrine regulator of cardiac physiology and pathology. Produced locally within the myocardium in response to diverse mechanical and neurohormonal stimuli, ET-1 acutely modulates cardiac contractility. During pathological cardiovascular conditions such as ischaemia, left ventricular hypertrophy and heart failure, myocyte expression and activity of the entire ET-1 system is enhanced, allowing the peptide to both initiate and maintain maladaptive cellular responses. Both the acute and chronic effects of ET-1 are dependent on the activation of intracellular signalling pathways, regulated by the inositol-trisphosphate and diacylglycerol produced upon activation of the ETA receptor. Subsequent stimulation of protein kinases C and D, calmodulin-dependent kinase II, calcineurin and MAPKs modifies the systolic calcium transient, myofibril function and the activity of transcription factors that coordinate cellular remodelling. The precise nature of the cellular response to ET-1 is governed by the timing, localization and context of such signals, allowing the peptide to regulate both cardiomyocyte physiology and instigate disease.

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Abbreviations
AngII, angiotensin II; CICR, calcium-induced-calcium release; Cn, calcineurin; ECC, excitation-contraction-coupling; IIICR, IP₃-induced calcium release; IP₃R, inositol triphosphate receptor; MyBP-C, myosin binding protein C; RyR, ryanodine receptor; TnI, troponin I

Introduction
Endothelin (ET)-1, ET-2 and ET-3 are a family of cyclic 21 amino acid peptides. The peptides are encoded within three separate yet highly conserved genes located on chromosomes 6, 1 and 20 in humans respectively (Inoue et al., 1989). ET-1 is the most well characterized member of this family. The peptide was initially isolated from the culture supernatant of porcine aortic endothelial cells and identified as a protease-sensitive vasoconstrictor. It remains the most potent vasoactive substance identified to date (Yanagisawa et al., 1988), performing an important function in the regulation of vascular smooth muscle tone. ET-1 also mediates effects on other cell types. Here, we discuss the role of ET-1 in the heart. We will focus on the actions of ET-1 on cardiac muscle and describe how the ET system contributes to cardiac regulation and autoregulation during health and disease.

ET-1 in the heart
Throughout life, ET-1 plays key roles in many aspects of cardiac physiology and pathology. It is involved in controlling aortic arch formation during development (Kurihara et al., 1995), is required for cardiomyocyte survival and prevents myocyte loss during ageing (Zhao et al., 2006). In the adult, ET-1 modulates coronary blood flow by regulation of vascular tone. Furthermore, by acting on its receptors expressed by atrial and ventricular myocytes, the peptide modulates cardiac muscle function directly (Hirata et al.,...
The effects of ET-1 on the heart are observed over distinct and wide timescales. Acutely (within minutes), ET-1 modulates muscle contractility and induces arrhythmias (Baltogiannis et al., 2005), whereas over longer time scales (days to weeks), the peptide induces cardiomyocyte growth. The long-term effects of ET-1 are associated with maladaptive hypertrophic remodelling of the heart (Yorikane et al., 1993) and its progression to failure (Francis et al., 1990). Hence, ET-1 is considered to contribute to both the aetiology and pathology of these conditions. Indeed, the circulating plasma level of ET-1 is positively correlated with severity of cardiac disease and thus is a reliable prognostic indicator of future heart failure (Selvais et al., 2000; Zolk et al., 2002).

Paracrine/autocrine ET-1 in regulation of cardiac function

Further contributing to its significant role in modulating cardiomyocyte function, ET-1 is synthesized and secreted by the myocytes (Nunez et al., 1990; Firth and Ratcliffe, 1992; Suzuki et al., 1993), fibroblasts (Fujisaki et al., 1995) and endothelial cells (Kedzierski and Yanagisawa, 2001) of the heart. The presence of the machinery for both the production and detection of ET-1 within the myocardium generates a local autocrine and paracrine ET-1 system, providing a tightly coupled and regulated means of control (Pikkarainen et al., 2006; Higazi et al., 2009). Supporting this notion, ET-1 production/secretion is signal-responsive and contributes to both normal adaptive physiological responses and to the induction and progression of pathology. During physiology, myocyte-secreted ET-1 is reported to contribute to stretch-induced enhancement of contractility, which can occur during periods of increased workload (Pikkarainen et al., 2006). In this context, ET-1 secretion/production is either directly stimulated or induced in response to ANGII, which is also secreted by the stretched myocyte (Sadoshima et al., 1993; Yamazaki et al., 1996; Pikkarainen et al., 2006; Cingolani et al., 2011). As a result, contractility is enhanced to accommodate for the change in cardiac demand. This mechanism may explain the slow force response or ANREP effect observed in stretched atrial preparations (Kockskämper et al., 2008a). Indeed, many of the cardiac effects of ANGII are abrogated by ET receptor inhibition (Ito et al., 1993). Should the initiating stimulus and thus ET-1 secretion persist, hypertrophy may result (Yamazaki et al., 1996). The acute effect of ET-1 is therefore a prequel to its longer term effect on cardiac remodelling (Cingolani et al., 2011). In aged individuals, the plasma concentration of ET-1 is elevated (Maeda et al., 2003), hence the importance of ET-1-induced cardiovascular effects increases with time. Furthermore, cardiomyocyte ET-1 generation is increased following ischaemia (Serneri et al., 2000), during the cardiac hypertrophic response (Yorikane et al., 1993; Zolk et al., 2002; Mayyas et al., 2010) and as a result of heart failure (Francis et al., 1990). This increased ET-1 contributes to pathologies such as arrhythmia and the initiation and development of cardiac remodelling, the incidence of which increases with age (Lakatta and Levy, 2003b). Increased expression of ET-1 has been observed at both the mRNA level of pre-pro-ET-1 and the protein level of the mature peptide itself (Arai et al., 1995; Kobayashi et al., 1999; Zhao et al., 2006), suggesting that increased transcription contributes to enhanced ET-1 production. This is accompanied by increased activity of the processing mechanisms that produce the mature peptide. Expression and activity of endothelin converting enzyme 1a (EC1E1a) is up-regulated in human failing hearts and animal models of congestive heart failure (Ergul et al., 2000a; 2000b), expediting the processing of big-ET-1 into mature ET-1. In conjunction with the increased level of ET-1 peptide, expression of the ETA receptor by ventricular myocytes is enhanced during disease (Arai et al., 1995; Pieske et al., 1999; Baltogiannis et al., 2005; Harzheim et al., 2010). Thus, under disease-associated conditions, mechanisms of ET-1 production and detection are up-regulated in a myocyte-centric manner, providing an entirely local mechanism for the pathological effects of ET-1.

ET receptors

ET-1 elicits its effects by association with one or both of its receptor isoforms, the ETA and ETB receptors (Arai et al., 1990; Sakurai et al., 1990; Webb, 1991; Yorikane et al., 1993; Alexander et al., 2011). Both receptor isoforms are classical GPCRs comprising 7 α-helical transmembrane spanning domains, an extracellular N-terminal domain and intracellular C-terminal domain. The intracellular C-terminal domain and loops between the transmembrane segments interact with heterotrimeric G-proteins composed of Gα, β and γ subunits. Activation of these receptors stimulates multiple intracellular signalling pathways and mediates diverse cellular effects (Ishikawa et al., 1988a; 1988b; Moravec et al., 1989). The relative abundance of ET receptors varies across the chambers and regions of the normal healthy heart, with the number of ET-1 binding sites in atrial cells far exceeding those found in ventricular cells (Molenaat et al., 1993). Functionally, the ETA and ETB receptors exhibit several key differences. Although the affinity of both ET receptors for ET is subnanomolar, the ETA receptor exhibits a higher affinity for ET-1 over ET-2 or ET-3 (Hosoda et al., 1991), whilst the ETB receptor binds non-selectively to each of the three ET isoforms (Sakurai et al., 1990). The downstream signals activated following engagement of the ETA receptor and ETB receptor also differ. Both receptors can couple to multiple G-proteins but the particular G-protein repertoire that each isoform engages may vary, governing the pattern of downstream pathways activated. The ETA receptor interacts with Gαq/11 (Aramori and Nakashii, 1992), Gα (Eguchi et al., 1993b; Yamazaki et al., 1996) and Gαi, activating polyphosphoinositide hydrolysis and production of inositol-(1,4,5)-triphosphate (IP3), diacylglycerol (DAG) and cAMP (Griendling et al., 1989; Takuwa et al., 1990; Serieri et al., 2000). The ETB receptor also couples to Gαq/11. However, in contrast to the ETA receptor, it preferentially interacts with Gαi and Gαi (Eguchi et al., 1993b; Gohla et al., 1999). Consequently, activation of the ETB receptor is associated with inhibition of cAMP accumulation as well as stimulation of polyphosphoinositide hydrolysis (Eguchi et al., 1993a). Although the heart expresses both receptor isoforms, in cardiomyocytes the ETA receptor predominates accounting for greater than 80% of ET binding sites (Allen et al., 2003).
The most well established mechanisms of ET₄ receptor-dependent signalling in cardiomyocytes concern those regulated downstream of Ga₁₁q. Inhibition of Ga₁₁q prevents activation of ET-1 dependent signalling pathways in rat adult ventricular myocytes (AVM), whilst reducing the activity of either Ga₁₂/₁₃ or Gaᵢ/o does not produce comparable effects (Snabaitis et al., 2005). Furthermore, ET-1-dependent signalling is enhanced by knockdown of the GTPase activating protein regulator of G-protein signalling 2 (RGS2), which negatively regulates GTP-bound Ga₁₁q by enhancing its GTP hydrolysing properties (Zhang et al., 2006). The downstream effects of Ga₁₁q activation are mediated through activation of PLC enzymes, which catalyse the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PI-4,5-P₂) to produce membrane-associated DAG and soluble IP₃ (Wu et al., 1992). Cardiomyocytes express multiple PLC isoforms, with PLCβ₁ and B₃ showing Ga₁₁q-dependent activity. ET-1 stimulation of cultured neonatal rat ventricular myocytes (NRVM) leads to phosphoinositide hydrolysis within seconds (Clerk and Sugden, 1997). The rapid increase in IP₃ and DAG activates multifarious downstream signalling pathways, including those regulated by calcium, small G-proteins and kinase cascades (Figure 1). In addition, ET-1 stimulates nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) (De Giusti et al., 2008) and production of reactive oxygen species (ROS) (Cheng et al., 1999). These pathways perform important roles in regulation of cardiomyocyte contractility, pacemaking and remodelling.

Figure 1
Signalling pathways activated downstream of the ET₄ receptor in cardiomyocytes. ET-1 binds to the Ga₁₁q-coupled ET₄ receptor inducing production of IP₃ and DAG. These second messengers activate protein kinase cascades, ROS production and IP₃-induced calcium release. Following ET-1 binding, the ET-1-ET₄ receptor complex is internalized in a GRK3-β-arrestin dependent manner. β-arrestin has been postulated to induce activation of MAPK cascades.

Terminating ET-1 signalling
In order to limit the effects of ET-1 on the heart, signalling from ET receptors must be tightly regulated. The ET₄ receptor forms a very high affinity interaction with ET-1 and the rate of disassociation of ligand/receptor complex is slow; hence, this process is not straightforward (Hilal-Dandan et al., 1997). Indeed, the resistance of the ET-1/ET₄ receptor complex to acid or exposure to inhibitor has led to the proposal that ET-1/ET₄ receptor binding is quasi-irreversible, possibly as a result of covalent binding of ET-1 to the receptor. Thus, it is likely that the receptor is internalized in a ligand-bound state.
and that internalization of this complex, rather than ET-1 dissociation, determines the duration of the ET-1 response. Indeed, an association of ET-1 with the ETα receptor within the endosome for up to 2 h has been reported (Chun et al., 1994; 1995). It is unclear whether ET-1 continues to signal via the ETα receptor when internalized in this manner but this hypothesis could explain certain of the observed effects of ET-1 stimulation. Co-localization of internalized ET-1-ETα receptor with caveolar domains may aid interaction with G-proteins, enabling ET-1-ETα receptor signalling to persist (Chun et al., 1994). Furthermore, ET-1-ETα receptor complex internalization is GRK (G-protein receptor kinase)-β-arrestin dependent (Freedman et al., 1997). GRKs 2, 3 and 5 are expressed in cardiac myocytes (Vinge et al., 2001) with GRK2 activity and expression being up-regulated in heart failure – an adaptation that limits β-adrenergic signalling (Ping et al., 1997). In AVMs, GRK3 targets the ETα receptor and inhibition of GRK3 in AVM enhances ET-1 induced signalling, suggesting that this kinase is important for attenuation of the ET-1 response (Vinge et al., 2007). Following GRK-dependent phosphorylation, recruitment of β-arrestins to activated receptors targets them for internalization. However, β-arrestin is also implicated in the activation of the MAPK cascade (Lefkowitz and Shenoy, 2005). Given these potential mechanisms of alternative and persistent ET-1-induced signalling, it appears that a simple model of ET-1-GPCR binding mechanisms of alternative and persistent ET-1-induced signalling cannot fully account for the cellular effects of ET-1.

Interestingly, internalized ETα receptor and ETβ receptor are targeted to different intracellular compartments after stimulation with ET-1. The ETα receptor co-localizes with the pericentrliolar recycling compartment and can be recycled to the plasmalemma so that cellular ET-1 sensitivity is maintained. In contrast, ETβ receptor are targeted to lysosomes and degraded after agonist-induced internalization. The lysosomal fate of the internalized ET-1-ETβ receptor complex provides a mechanism for clearance of the peptide (Attinà et al., 2005). Exemplifying this, deletion of the ETβ receptor in mice impairs ET-1 clearance and selective pharmacological blockade of this isoform increases the circulating concentration of ET-1 (Gariepy et al., 2000; Kelland et al., 2010). The vasculature of the lung provides the major site of clearance of ET-1 from plasma (Dupuis et al., 1996), with approximately 80% of the peptide being removed from the bloodstream by the ETβ receptors expressed by pulmonary endothelial cells. Efficient removal of the peptide via this mechanism generates a half-life for ET-1 in the circulation of less than 5 min and maintains the plasma concentration of the peptide in healthy individuals at approximately 1 pM (Kedzierski and Yanagisawa, 2001), significantly below its activity threshold. However, local ET-1 concentration at sites of ET-1 production and release is expected to be much higher, raising the level of the peptide to that which can modulate cardiac function.

ET-1 in cardiac pacemaking and contractility

Cardiac contraction during systole is initiated by an action potential generated following automatic depolarization of the pacemaking SA node. From the SA node, the action potential propagates across the atria and induces contraction. Following a pause at the atrio-ventricular node, the action potential is rapidly conducted to the apex of the heart by specialized Purkinje fibres. Subsequent spreading of the action potential throughout the ventricles induces contraction and thus expels blood into the aorta. At the molecular level, the mechanism that links membrane excitation and myocyte contraction is termed excitation-contraction coupling (ECC) (Figure 2) (Bers, 2002; Fearnley et al., 2011). This process is initiated by the depolarization-induced opening of L-type voltage-gated calcium channels (also known as dihydropyridine receptors), generating the I_{Ca,L} current. Calcium entry through these channels generates a much larger release of calcium from the sarcoplasmic reticulum (SR) via the ryanodine receptor (RyR2), in a process termed ‘calcium-induced-calcium-release’ (CICR) (Fabiato, 1983; Roderick et al., 2003). This calcium binds to the contractile apparatus and induces cellular shortening. To terminate systole and allow relaxation, calcium is extruded from the cell or re-sequestered in the SR. Synchronous release of calcium throughout the ventricular myocyte is achieved as a consequence of the highly specialized subcellular structure of the cell. The sarcolemma is extensively invaginated to form the t-tubular network; hence, depolarizing action potentials can spread into the depths of the cell and induce coordinated cellular contraction. Furthermore, the L-type calcium channel and RyR2 are co-localized within specialized structures known as the cardiac dyads, facilitating CICR.

ET-1 has an established role in the regulation of cardiac contractility, in particular as a positive inotrope (Ishikawa et al., 1988b; Moravec et al., 1989; Shah et al., 1989). Other effects upon the heart include negative inotrope, arrhythmogenesis, decreased relaxation (lusitropy) and chronotropy (Ishikawa et al., 1988a; Li et al., 2005). Despite these well-described aspects of ET-1 in cardiac biology, controversy remains regarding the effects of ET on cardiac function, its mechanism(s) of action and the specific molecular targets involved. A number of factors contribute to the apparent diversity of responses reported, although variable expression levels of ET receptors account for many of the discrepancies. For example, responses are different between species and vary with age. ET-1 does not exert an inotropic effect in adult mice and dogs, which have low levels of ET receptor expression (Takanashi and Endoh, 1991; Nishimaru et al., 2011). This is not surprising given that the distribution and abundance of ET receptor isoforms varies across the chambers of the heart (Molenaar et al., 1993; Russell and Davenport, 1996). Further diversity is provided by the experimental preparation – intact heart, chamber muscle strips or isolated cells.

Due to greater receptor abundance in the atria, the effects of ET-1 are most well established in this chamber (Ishikawa et al., 1988b; Vigne et al., 1989; Molenaar et al., 1993). The atrial response to ET-1 (e.g. in rat, rabbit and guinea pig) is characterized by an initial negative inotropic phase (first 1–2 min), followed by a slowly developing and persistent positive
inotropic phase (Takanashi and Endoh, 1991; Bootman et al., 2007). Although cAMP independent, the positive inotropic effect of ET-1 in the atria can reach levels comparable to that induced by the catecholamines (Vigne et al., 1989). The cardiac ventricle also exhibits a positive inotropic response to ET-1, albeit at a substantially reduced magnitude (Takanashi and Endoh, 1991; Proven et al., 2006) and unlike the situation in the atria, a negative inotropic response is rarely observed (Proven et al., 2006). Notably, ET-1 also affects heart rate by modulating the physiology of the SA node and Purkinje fibres (Ishikawa et al., 1988a). The combined effect of ET-1-induced positive inotropy and chronotropy increases cardiac output under conditions of stress, contributing to the adaptive response of the heart. However, these actions of ET-1 also have deleterious consequences, promoting arrhythmogenic, action potential-independent calcium transients (Baltogiannis et al., 2005; Li et al., 2005; Mayyas et al., 2010).

Similarly to other cardiotonic agonists, ET-1 changes cardiac contractility by modulating components of the ECC machinery (Figure 3). These include alterations in the dynamics and amplitude of systolic calcium transients (Bootman et al., 2007; Kockskamper et al., 2008), changes in myofilament sensitivity to calcium (Yang et al., 1999; Westfall et al., 2005; Cuello et al., 2007a) or altered frequency of stimulation (Ishikawa et al., 1988a).

ET-1 regulation of NCX activity

ET-1 effects systolic calcium predominantly via modulation of the Na+/Ca2+ exchanger (NCX) (Ballard and Schaffer, 1996; Yang et al., 1999) and L-type channel activity (Ono et al., 1994). The primary function of NCX is calcium clearance from the cytosol during diastole, accounting for up to 20% of the calcium extruded, a contribution that increases further under disease conditions (Bers, 2002; 2006). NCX uses the electrochemical gradient for Na+ between the extracellular space and the cytosol, created by the Na+K+-ATPase, to exchange 3 Na+ for every calcium ion extruded from the cell (Philipson and Nicoll, 2000). Where intracellular calcium is increased independently of an action potential, NCX-mediated Na+ entry may cause sufficient depolarization to cross the threshold for action potential generation (Gilbert et al., 1991). As well as this forward mode of activity, NCX can also operate in reverse, inducing calcium influx (Leblanc and Hume, 1990). This may be precipitated by raised intracellular Na+ concentration under conditions of membrane depolarization, for example during the initial phase of the action potential (Shattock and Bers, 1989). As a result of calcium influx at this point, calcium transients are increased in amplitude and contractility enhanced (Meyer et al., 1996; Yang et al., 1999). ET-1 regulates NCX either by engaging signalling mediators activated directly downstream of the ETA receptor such as PKC (Ballard and Schaffer, 1996; Zhang and Hancox, 2009), or by increasing activity of the sodium-hydrogen exchanger (NHE) (Cingolani et al., 2011). The NHE is the major mechanism by which intracellular pH is regulated in cardiac myocytes. It extrudes one H+ ion from the cell in exchange for 1 Na+ (Cingolani and Ennis, 2007), exploiting the Na+ gradient created by the Na+K+-ATPase. The resulting increase in intracellular Na+ activates NCX in reverse mode, increasing [Ca2+], and producing an inotropic effect (Alvarez et al., 1999). H+ extrusion by NHE would also be expected to modulate contractility via cytosolic alkalanization, which
would influence myofilament calcium sensitivity. Despite support for this hypothesis in earlier studies, it is now clear that the small changes in intracellular \([H]_i\) produced by NHE are compensated for by the bicarbonate anion transporter; providing bicarbonate is present in the extracellular medium (Alvarez et al., 1999). Therefore, this mechanism is unlikely to contribute to the ET-1 response. ET-1 increases NHE activity via kinases such as PKC, ERK1/2 and p90 RSK (Bianchini et al., 1997; Fliegel and Karmazyn, 2004; Maekawa et al., 2006; Cuello et al., 2007b; Snabaitis et al., 2008). Although ERK1/2 and therefore p90 RSK may both be activated via canonical GPCR signalling, NADPH oxidase activation and ROS formation are necessary for these kinases to phosphorylate NHE in response to ET-1 (Cingolani et al., 2011).

**Figure 3**

ET-1 regulates ventricular myocyte contractility. Downstream of the ET\(_A\) receptor in the ventricular myocyte, IICR, PKC, PKD, p90 RSK and ERK are activated. Protein-kinase-dependent phosphorylation of NHE1 increases intracellular Na\(^+\) concentration and depolarizes the cell. Subsequent activation of NCX in reverse mode allows calcium entry to be enhanced. When combined with PKC-dependent amplification of calcium entry through the L-type calcium channel, the stimulus for CICR is increased. IP\(_3\)-dependent calcium release from the SR sensitizes the closely opposed RyR2, hence facilitating CICR. The resulting elevated cytosolic calcium concentration and PKC/PKD-dependent phosphorylation of myofilament proteins allows the myocyte to produce a larger force of contraction.

**ET-1 regulation of L-type calcium current**

L-type calcium channel activity is subject to extensive regulation by GPCRs, which elicit their effects through PKC and PKA dependent phosphorylation (Puri et al., 1997; Kamp and Hell, 2000). Phosphorylation of the pore-forming \(\alpha_{1C}\) and accessory \(\alpha_{1I}\) and \(\beta_{2A}\) subunits of the channel has been detected biochemically (Puri et al., 1997). Although intensively studied, there is a lack of consensus regarding modulation of L-type calcium current (\(I_{Ca,L}\)) by ET-1, with evidence for both a positive (Lauer et al., 1992; He et al., 2000) and negative effect (Ono et al., 1994; 2001; Cheng et al., 1995). A biphasic effect of ET-1 has been reported in rabbit ventricular myocytes, consisting of a reduction followed by an enhancement of \(I_{Ca,L}\) (Watanabe and Endoh, 1999). In these cells the ET-1 stimulated augmentation of \(I_{Ca,L}\) approached a level similar to that induced by \(\beta\)-adrenoceptor stimulation. However, the mechanisms by which ET-1 and sympathetic stimuli affect \(I_{Ca,L}\) are not shared. When ET-1 and sympathetic stimuli occur concurrently, ET-1 exerts an anti-adrenergic effect through a pertussis toxin-sensitive pathway (i.e. \(G_{\beta\gamma}\) mediated). This suppresses the enhancement of \(I_{Ca,L}\) by \(\beta\)-agonists and prevents their associated inotropic (Boixel et al., 2001; Munzel et al., 2005) and arrhythmic responses (Kolettis et al., 2008). In addition to the inter-species variation in the ET-1 response, the lack of a consistent effect of ET-1 has also been ascribed to the methods used for recording \(I_{Ca,L}\) (He et al., 2000). In order to mitigate this, the perforated patch technique has been used to minimize intracellular dialysis and loss of signalling mediators. Using this approach, a consistent PKC-dependent positive regulation of \(I_{Ca,L}\) by ET-1 has been reported (Kamp and Hell, 2000). In order for such a mechanism to operate efficiently, co-localization of the main players is required to facilitate interaction of the DAG generated by phosphoinositide hydrolysis with its targets (Robu et al., 2003). The importance of DAG localization is illustrated by the contrasting effects of bath-applied and intracellular-released DiC8, a DAG analogue (He et al.,
ET-1 stimulated IP$_3$-induced calcium release and cardiac contractility

As well as DAG, ET-1-dependent phosphoinositide hydrolysis in the myocytes and conduction system of the heart generates the important signalling effector IP$_3$. Chen et al., 2004; Remus et al., 2006; Hirose et al., 2008; Kockskämper et al., 2008b; Ju et al., 2011. Due to the relatively small increase in IP$_3$ and low expression level of IP$_3$ receptors (IP$_3$R) in heart preparations relative to other tissues, the role, if any for IP$_3$ in modulating intracellular [Ca$^{2+}$] and contractility was for a long time unclear (Wu et al., 2006; Higazi et al., 2009). Furthermore, since expression of IP$_3$R is 50–100 fold lower than that of the RyR (Moschella and Marks, 1993; Perez et al., 1997; Lipp et al., 2000), it was difficult to discern what additional function IP$_3$R could perform. This contrasts with the prominent role for IP$_3$Rs in the development and physiology of the embryonic heart. Indeed, mice in which the type 1 and type 2 IP$_3$R isoforms (IP$_3$R-I and IP$_3$R-II) are subject to germ-line deletion are not viable and exhibit defects in cardiac development (Uchida et al., 2010). Moreover, IP$_3$ stimulated calcium signals play a key role in pacemaker activity of the embryonic heart (Kapur and Banach, 2007). Evidence has recently accumulated which indicates that despite low expression of IP$_3$Rs, the IP$_3$ signalling pathway also plays a significant role in the physiology and pathophysiology of the adult heart, contributing to many of the effects of ET-1 and other G$_{q/o}$ coupled receptors on cardiac function (Wu et al., 2006; Kockskämper et al., 2008b; Higazi et al., 2009; Nakayama et al., 2010). As would be expected, the contribution of IP$_3$ signalling to the action of ET-1 across the regions of the heart varies according to the level of expression and subcellular localization of the IP$_3$R. Similarly to ET$_3$ receptors, IP$_3$Rs are most abundant in atrial myocytes and in nodal tissue (Lipp et al., 2000; Ju et al., 2011). The IP$_3$R-II predominates in SA node, atrial and ventricular myocytes (Perez et al., 1997; Lipp et al., 2000; Ju et al., 2011), whereas in Purkinje fibres, IP$_3$R is most abundant (Gorza et al., 1993).

Engagement of IP$_3$ signalling in the different cell types of the heart has varying consequences. In the SA node, ET-1 causes increases in firing rate, calcium transient amplitude, diastolic calcium levels and the frequency of elementary calcium release events (Ju et al., 2011). The recapitulation of these effects by exposure of nodal cells to cell-permeant IP$_3$ and the decreased firing rate and loss of ET-1 responses in nodal cells from IP$_3$R knockout mice demonstrates the importance of ET-1/IP$_3$ signalling in this region of the heart (Ju et al., 2011). Although ET-1 also elicits a positive chronotropic effect in guinea pig SAN (Ishikawa et al., 1988a), rabbit SAN exhibits a negative chronotropic response to ET-1 (Ono et al., 2001). The effect of ET-1 in these rabbit cells was attributed to modulation of calcium and potassium currents. The role of IP$_3$ signalling in eliciting the effects of ET-1 upon the SAN in species other than mouse remains to be fully resolved. ET-1 also elicits spontaneous events in Purkinje fibres. Despite evidence for expression of functional IP$_3$Rs that contribute to spontaneous calcium release events in these cells (Gorza et al., 1993; Wu and Tseng, 1993; Boyden et al., 2004), it is not determined whether these IP$_3$Rs are engaged by ET-1 (Wu and Tseng, 1993). The IP$_3$R is however stimulated in Purkinje cells by IP$_3$ generated following activation of $\alpha$-adrenergic GPCR$\beta$s, resulting in wide long lasting calcium release events (Hirose et al., 2008).

In atrial myocytes, IP$_3$ signalling contributes to the inotropic and pro-arrhythmic action of ET-1 (Li et al., 2005), and IP$_3$-mediated calcium signals are localized to the subsarcolemmal and perinuclear regions (Mackenzie et al., 2004; Zima et al., 2007). Whilst perinuclear IP$_3$-mediated calcium signals have been proposed to control transcription (see later) (Wu et al., 2006; Higazi et al., 2009), IP$_3$-mediated calcium signals at the plasma membrane play a role in modulating atrial myocyte contractility and may underlie the enhanced automaticity of ET-1 stimulated myocytes (Mackenzie et al., 2004). A similar pattern of IP$_3$R expression and activity is observed in cells of the SAN (Ju et al., 2011). As a result of their lack of t-tubules, ECC is restricted to the peripheral sarcolemma of atrial myocytes (Ju et al., 2011). Therefore, IP$_3$-mediated calcium signals exert a greater than anticipated effect on the physiology of an ET-1-stimulated atrial or SAN myocyte due to their co-localization with the ECC machinery in these cells. ET-1-stimulated IP$_3$-mediated calcium signals promote transition of the sub-sarcolemmal ring of calcium normally observed following depolarization of atrial myocytes to a global calcium elevation, enhancing ECC and recruitment of RyRs deeper in the myocyte cell volume (Mackenzie et al., 2004). As a result, contractility is increased and blood propelled with greater force into the ventricles. Since the extent of ventricular stretch determines the contractile force produced by the ventricle – the Frank Starling relationship – greater atrial contraction brings about increased cardiac output. In addition to enhancing electrically evoked calcium transients, ET-1 application also increases the incidence of elementary calcium release events in atrial myocytes (Mackenzie et al., 2001; Li et al., 2005). These events contribute to an increase in diastolic calcium levels beneath the sarcolemma, which if sufficiently elevated, activates NCX-dependent inward Na$^+$ flux and action potential generation. As a result, a potentially arrhythmogenic ectopic event is induced (Lipp et al., 2000; Mackenzie et al., 2002). Functional interactions between IP$_3$Rs and RyRs are also observed in ventricular myocytes exposed to ET-1 (Proven et al., 2006; Domeier et al., 2008; Harzheim et al., 2009). However due to the lower abundance of ET and IP$_3$ receptors in these cells (Lipp et al., 2000; Domeier et al., 2008), their interaction and influence upon myocyte physiology is not as
great. Most notably, IP₃ signalling does not make a prominent contribution to the inotropic action of ET-1 in the ventricular myocyte. This is not surprising given the limited effect of ET-1 upon calcium fluxes in these cells when compared with atrial myocytes (Proven et al., 2006; Domeier et al., 2008). Prolonged exposure of ventricular myocytes to ET-1 does however lead to the generation of ectopic calcium transients and IP₃-dependent extra-systolic contractions (Proven et al., 2006) and IP₃-dependent calcium signals also promote sodium–calcium exchange (Gilbert et al., 1991). Thus, similarly to the situation in atrial myocytes, ET-1 activated IP₃ signalling has the potential to precipitate ventricular arrhythmias.

As discussed earlier, both circulating and local cardiac ET-1 levels are increased with age (Maeda et al., 2003) and during cardiac pathologies such as arrhythmia, infarction and hypertrophy (Yorikane et al., 1993; Serner et al., 2000). Although this increase in ET-1 may initially support ECC, it also has deleterious consequences and is likely to exacerbate pathology and/or cause it to persist (Tuinenburg et al., 1998; Baltogiannis et al., 2005; Mayyas et al., 2010). Furthermore, IP₃Rs are increased in their abundance during cardiac pathology (Moschella and Marks, 1993; Harzheim et al., 2009; 2010). As a result, the effects of ET-1 on myocyte physiology are enhanced. Notably, the additional IP₃Rs in the hypertrophied heart are not homogenously distributed throughout the cell volume but localized to the sub-sarcolemmal dyadic region, proximal to the RyRs that participate in ECC (Harzheim et al., 2009). Thus, under conditions of increased IP₃s, calcium emanating from these IP₃Rs stimulates calcium release from their neighbouring RyRs. The effects of this activity are twofold. First, IICR may promote ECC, thereby enhancing contractility. However, second, IICR may promote potentially arrhythmogenic ectopic calcium release events (Harzheim et al., 2009). On balance, the enhancement of calcium release via RyRs by the additional IP₃Rs may outweigh the potential deleterious effects of ectopic calcium signals. During hypertrophy and the progression to failure, decreased coupling efficiency between L-type channels and RyRs arises as a result of increased width of the dyadic cleft (Song et al., 2006; Xu et al., 2007; Heinzel et al., 2011). In this situation, calcium release through additional IP₃Rs could provide a mechanism to overcome this defect in ECC by bringing RyRs closer to their threshold for activation. This effect is analogous to the priming of RyRs by PKA phosphorylation following β-stimulation, which sensitizes the receptors to calcium influx via LTCC (Zhou et al., 2009). In this way, increased IP₃R expression may be considered an adaptive response that sustains contractile function as the heart hypertrophies.

**Effects of ET-1 upon the contractile apparatus**

The relationship between the enhancement in [Ca²⁺], and contractility observed in cardiac preparations following exposure to ET is non-linear, indicating that the inotropic response does not solely result from enhancement of intracellular calcium levels. This non-linearity is particularly apparent in ventricular preparations where, despite a significant increase in contraction, [Ca²⁺] is not substantially augmented (Wang et al., 1991; Pieske et al., 1997). This contrasts with the response to the catecholamines, which cause a robust increase in calcium transient amplitude and contractility. The mechanism by which the alteration in contraction is brought about by catecholamines is well defined and occurs primarily through PKA-dependent phosphorylation of contractile filament regulatory proteins, most notably the inhibitory subunit of troponin (TnI) and myosin binding protein C (MyBP-C) (Chen et al., 2010). Phosphorylation of TnI and MyBP-C differentially contributes to regulation of contractility. Phosphorylation of TnI reduces calcium sensitivity of the myofilaments (Pi et al., 2003), and phosphorylation of MyBP-C is responsible for enhanced cross-bridge cycling, thereby enhancing relaxation (Chen et al., 2010). GPCRs that signal via Goα such as the ET₄ receptor elicit an opposing effect on myofilament action – generally increasing sensitivity to calcium and decreasing cross-bridge cycling (Pi et al., 2003; Wang et al., 2006). As such, their modulation of contractility is energetically less expensive. PKC, RSK and PKD have all been identified as ET-1-activated TnI and MyBP-C kinases (Cuello et al., 2007a; 2011; Chen et al., 2010).

**Remodelling of the heart by ET-1**

In addition to its diverse actions in the regulation of myocyte contractility, ET-1 has been consistently demonstrated to perform a major role in disease-associated remodelling of the heart (Russell and Molenaar, 2000; Sugden, 2003). Heart disease encompasses a spectrum of interrelated and progressive disorders, which include hypertension, coronary artery disease, arrhythmia and, most severely, heart failure (Diwan and Dorn, 2007). Pathological enlargement of the heart, termed maladaptive cardiac hypertrophy, is observed clinically in many of these conditions and can be induced as a result of increased arterial blood pressure, genetic mutations or following myocardial injury. Hypertrophic remodelling can take place within the left and/or right ventricles, depending on the nature of the initiating stimulus. It develops as a consequence of the increased size of individual ventricular myocytes, without an increase in myocyte number. When hypertrophy occurs as a consequence of persistent cardiovascular stress, an increase in heart size is a pathological and maladaptive response. As the condition progresses to heart failure, myocyte apoptosis/necrosis and myocardial fibrosis are observed, impairing cardiac contractility and reducing cardiac output. At the cellular level, hypertrophic myocytes show remodelling of gene expression profiles, altered intracellular calcium handling and reorganization of the contractile machinery. Reprogramming of cardiomyocyte gene expression profiles is responsible for many of the phenotypic changes observed in hypertrophic myocytes and is perceived to be critical for disease-associated impairment of myocyte function. Left ventricular hypertrophy produced by reprogramming under pathological conditions is one of the major risk factors for subsequent progression of patients to congestive heart failure and sudden death (Jessup and Brozena, 2003), prompting much investigation into the mechanism of action of pro-hypertrophic stimuli. Exposure of cultured car-
diomyocytes to ET-1 has been shown to recapitulate many of the cellular features of hypertrophic myocytes (Ito \textit{et al}., 1991). Furthermore, ET-1 stimulation of NRVM alters expression of hundreds of genes, in a manner mirroring the remodelling of gene expression observed during hypertrophy (Cullingford \textit{et al}., 2008). Consequently, these observations have promoted much investigation into the role of the ET-1 system in pathological remodelling.

As indicated earlier, cardiomyocyte ET-1 biosynthesis and secretion and ET receptor expression are increased in heart failure patients and animal models of cardiovascular conditions (Hasegawa \textit{et al}., 1996; Motte \textit{et al}., 2003; McMullen and Jennings, 2007). This remodelling of the ET system thereby provides a local mechanism for the pathological effects of ET-1. The importance of a local role for ET-1 in the induction of myocardial remodelling has been demonstrated by the effects observed upon infection of an organism with the intracellular protozoan 	extit{Trypanosoma cruzi}. Parasite infection leads to the development of Chagas disease (Coura and Borges-Pereira, 2010), which in its chronic form induces a cardiomyopathy phenotype, consisting of cardiomyocyte hypertrophy, myocardial fibrosis and, eventually, myocyte death. Together, these changes promote the development of heart failure (Coura and Borges-Pereira, 2010). Similar to other forms of hypertrophic remodelling, plasma ET-1 concentration is high in mice and humans infected with 	extit{T. cruzi} and mRNA levels of both ET-1 itself and the enzymes involved in its processing are increased under these conditions (Petkova \textit{et al}., 2000; Salomone \textit{et al}., 2001). When the ET-1 gene is specifically deleted in cardiomyocytes, the maladaptive effects of 	extit{T. cruzi} infection are reduced. Such a protective effect is not observed upon deletion of ET-1 in endothelial cells, suggesting a myocyte-specific ET-1-dependent origin for the cardiomyopathic effect of the disease (Tanowitz \textit{et al}., 2005).

Manipulation of the ET-1 system experimentally in murine models of hypertrophy and heart failure has provided valuable further insight into the role of the peptide in left ventricular remodelling. Cardio-specific conditional overexpression of ET-1 in mice induces a pathological hypertrophic phenotype, which can be attenuated by combined blockade of ET\textsubscript{A} and ET\textsubscript{B} receptors (Yang \textit{et al}., 2004), whilst cardio-specific deletion of the ET-1 gene inhibits the hypertrophic response to treatment with the T3 thyroid hormone (Shohet \textit{et al}., 2004). Infusion of the ET\textsubscript{A} receptor antagonist, BQ123, inhibits the response to a pro-hypertrophic stimulus in adult rats (Ito \textit{et al}., 1994) and when administered to rats experiencing chronic heart failure, BQ123 impedes left ventricular remodelling and improves survival (Sakai \textit{et al}., 1996). These observations support the conclusion that ET-1 and the ET\textsubscript{A} receptor have an important role in pathological remodelling of the heart.

Based on these findings, the signalling pathways activated downstream of ET-1 and ET\textsubscript{A} receptor activation have been extensively studied within the context of the hypertrophic response. Overexpression of PLC\textsubscript{B2}, a primary proximal target of the ET\textsubscript{A} receptor, is sufficient to induce a hypertrophic response in NRVM (Filtz \textit{et al}., 2009). Moreover, by inducing calcium signals and activation of PKC, respectively, the IP\textsubscript{3} and DAG generated following ET-1 stimulated PLC-mediated phosphoinositide hydrolysis have pro-hypertrophic effects.

**Role of IP\textsubscript{3}-dependent calcium release in hypertrophy**

The low baseline expression of the IP\textsubscript{3}RII in ventricular myocytes (Perez \textit{et al}., 1997) has impeded investigation into its role in hypertrophic remodelling. Moreover, it has been unclear how IICR arising from the relatively few cardiomyocyte IP\textsubscript{3}RII could be discriminated from the cyclical global release and reuptake of calcium that mediates cellular contractility. However, it has been recognized that IP\textsubscript{3}RI expression increases during cardiovascular disease, with increased receptor expression being observed in human cardiomyopathic hearts and animal models of hypertrophic remodelling (Go \textit{et al}., 1995; Harzheim \textit{et al}., 2009; Nakayama \textit{et al}., 2010). As well as being expressed in the dyadic region, these receptors are also found in the perinuclear region. IP\textsubscript{3}-dependent nuclear calcium signals occur in response to ET-1 and are required to elicit a hypertrophic response (Wu \textit{et al}., 2006; Higazi \textit{et al}., 2009). Furthermore, a mouse-model with cardio-specific overexpression of the IP\textsubscript{3}RII demonstrated enhanced IP\textsubscript{3}-induced calcium release (IICR) and mild baseline hypertrophy (Nakayama \textit{et al}., 2010). Together, these data suggest that IICR has a pro-hypertrophic effect on the myocardium and that nuclear IICR plays a key role in the hypertrophic response to ET-1. The observation that ET-1 elicits release of calcium specifically in the nuclear domain is significant as it would allow pro-hypertrophic IP\textsubscript{3}-dependent calcium signals to be detected independently from the bulk calcium transients that elicit myocyte contractility. Furthermore, calcium elevation within the nucleus co-localizes the calcium signal with calcium-sensitive kinases and regulators of transcription, spatially coupling the stimulus with its effectors (Goonasekera and Molkentin, 2012). Such mechanisms are likely to be crucial for the remodelling of gene expression profiles observed in hypertrophy. Calcineurin-nuclear factor of activated T cells (Cn-NFAT) (Higazi \textit{et al}., 2009; Nakayama \textit{et al}., 2010; Rinne and Blatter, 2010) and CaMKII (Wu \textit{et al}., 2006), are examples of IICR-regulated proteins that can be localized within the nucleus. Significantly, both have important roles in the hypertrophic response.

Calcineurin (Cn) is a calcium-dependent heterotrimeric serine-threonine phosphatase (Clipstone and Crabtree, 1992; Fruman \textit{et al}., 1992). In response to increases in calcium concentration such as those elicited by ET-1-induced IP\textsubscript{3} generation, IP\textsubscript{3} the Cn autoinhibitory domain is displaced from the active site, stimulating Cn phosphatase activity. The major cellular target of activated Cn is a family of 4 Rel-homology domain containing transcription factors, NFATc1-4 (Jain \textit{et al}., 1993). Subcellular localization of NFATc1-4 is controlled by the phosphorylation status of the protein. Phosphorylated NFAT is predominantly localized in the cytosol. When Cn is active, NFAT is dephosphorylated, exposing two nuclear localization sequences (NLS) and inducing translocation to the nucleus, where it can activate transcription (Shaw \textit{et al}., 1995). Phosphorylation of NFAT by protein kinases such as GSK3\textbeta (Neal and Clipstone, 2001), DYRK1a (Kuhn \textit{et al}., 2009), p38-MAPK (Gomez del Arco \textit{et al}., 2000) and JNK (Chow \textit{et al}., 1997) leads to nuclear export of the protein (Hogan \textit{et al}., 2003). In response to ET-1 stimulation, multiple
NFAT isoforms have been shown to translocate to the nucleus of cultured cardiomyocytes (Kakita et al., 2001; Kawamura et al., 2004; Higazi et al., 2009), with identity of the ET-1-regulated isoforms species and cell-type specific (Rinne et al., 2010). Interestingly, nuclear import of Cn has also been observed in the cardiomyocyte, promoting sustained NFAT dephosphorylation and prolonging the NFAT-signal (Shibasaki et al., 1996). Indeed, nuclear translocation of Cn is required for the response to ET-1 in NRVM (Hallhuber et al., 2006; Higazi et al., 2009).

Cn-NFAT activity within the cardiomyocyte is undoubtedly important for the hypertrophic effects of ET-1, and there is a wealth of further evidence that convincingly demonstrates the pathological role of Cn-NFAT activity within the heart. Failing human myocardium exhibits increased expression and activity of Cn and translocation of NFATc4 to the nuclear compartment (Lim and Molkentin, 1999; Diedrichs et al., 2004). These observations have been further substantiated by in vitro and in vivo studies. Overexpression of a constitutively-active mutant of Cn in the mouse heart induces severe left ventricular hypertrophy, cardiomyocyte dysfunction and heart failure (Molkentin et al., 1998). To further confirm these conclusions, other studies have probed the role of Cn-NFAT downstream of hypertrophic stimuli such as ET-1 in vitro. ET-1 increases Cn enzymatic activity in NRVM, inhibition of which prevents the response to hypertrophic stimulation (Zhu et al., 2000). Moreover, multiple studies have verified that the deleterious effect of Cn on cardiac function is mediated through Cn-dependent regulation of one or more NFAT isoforms. Constitutively nuclear, constitutively active NFATc4 induces a phenotype of pathological left ventricular hypertrophy, confirming the damaging effect of increased NFAT-transcriptional activity in vivo (Molkentin et al., 1998). Moreover, cardiac-specific deletion of either NFATc3 (Wilkins et al., 2002) or NFATc2 (Bourrajaj et al., 2008) impairs the hypertrophic response to increased Cn activity. Although these mouse models do not directly interrogate the role of ET-1-induced Cn-NFAT activity during hypertrophy, when combined with the evidence for ET-1-dependent NFAT translocation in isolated myocytes and the requirement for Cn in the hypertrophic response to ET-1, this pathway can be considered to be an important mediator of ET-1 induced hypertrophic responses.

**Signalling to CaMKII, HDACs and MEF2 to induce hypertrophy**

Although Cn-NFAT are strongly implicated in the hypertrophic response to ET-1, other studies have shown that ET-1-induced nuclear IICR elicits its pro-hypertrophic effect by engaging co-localized CaMKII and eliciting gene expression dependent upon the pro-hypertrophic transcription factor MEF2 (Wu et al., 2006). Basally, MEF2 activity is restrained in the myocyte as a consequence of its association with class II histone deacetylases (HDACs 4, 5, 7 and 9), which negatively regulate transcription by deacetylating histone proteins, resulting in condensation of chromatin and impeding the access of transcriptional activators. In response to perinuclear IICR, CaMKII phosphorylates HDAC4 creating a docking site for 14-3-3 proteins. Association of HDAC4 with 14-3-3 induces conformational changes that mask NLS and expose a nuclear export sequence, facilitating HDAC4 nuclear export and depressing MEF2-dependent transcriptional responses (Lu et al., 2000; Zhang et al., 2002; Wu et al., 2006). ET-1 induces nuclear export of both HDAC4 and HDAC5 in rat ventricular myocytes (Wu et al., 2006; Peng et al., 2009), and ET-1 induced activation of MEF2-dependent transcription is likely to form a key component of the cellular response to the peptide. Deletion of MEF2D abrogates the hypertrophic response induced by hypertension-induced hypertrophy (Chang et al., 2004; Kim et al., 2008), whilst overexpression of MEF2A, C or D in vivo is sufficient to induce pathological cardiac remodelling (Kim et al., 2008).

Recent reports indicate that the spatial pattern of ET-1-induced signalling is important in the regulation of HDAC phosphorylation. ET-1 activates CaMKII kinase activity, and this activity is necessary for the hypertrophic response to the peptide in NRVM (Zhu et al., 2000). CaMKII is itself a key inducer of HDAC nuclear export, with cardiomyocytes predominantly expressing the CaMKIId isoform, localized to either the nucleus (CaMKIId) or cytosol (CaMKIIdc) (Anderson, 2005). CaMKIIdc is therefore present in the vicinity of its activating signal (ET-1-induced nuclear calcium transients) and its target (HDAC4). Although CaMKII directly targets HDAC4, it does not directly phosphorylate HDAC5. HDAC5 is rendered sensitive to CaMKII by forming oligomers with HDAC4 (Backs et al., 2008), indicating that ET-1-induced IICR could lead to nuclear export of both of the key MEF2 regulators. More recently, the novel calcium independent isoforms of PKC and the PKC target PKD have also been shown to phosphorylate HDAC5 and induce its nuclear export (Phan et al., 2011). ET-1 activates both PKC and PKD in cardiomyocytes (Phan et al., 2011); however, this is unlikely to form part of the mechanism whereby ET-1 depresses MEF2. ET-1-induced HDAC5 nuclear export is PKC-independent and although ET-1 activates subsarcolemmal PKD, it does not stimulate PKD translocation to the nucleus, preventing access of the kinase to its nuclear targets (Bossuyt et al., 2011). Therefore, perinuclear IICR and the CaMKII activity it elicits are considered to be the key signals for ET-1-induced MEF2 depression.

**ET-1 activation of PKC in hypertrophy**

ET-1 activates the novel isoforms of PKC in cardiomyocytes. PKC isoform expression is species, tissue and cell-type specific, with cardiomyocytes from murine species and humans expressing multiple differentially regulated PKC isoenzymes (Salazar et al., 2007; Palaniyandhi et al., 2009). NRVM express PKCs α, δ, ε and η (Clerk et al., 1995; Erdbruger et al., 1997; Markou et al., 2006), whilst human cardiac tissue also expresses PKC βI and βII (Bowling et al., 1999). In NRVM, ET-1 induces membrane association and activation of the DAG-sensitive, calcium-insensitive PKC δ and ε isoforms within 5 min of stimulation (Clerk et al., 1994; Clerk and Sugden, 1997), and PKC ε is the major ET-1-activated isoform in AVM (Bogoyevitch et al., 1993). The role of PKC isoenzymes in hypertrophy has been extensively investigated (Braz et al., 2002; Vega et al., 2004; Vijayan et al., 2004). This has
lead to the identification of many downstream targets of PKC relevant to hypertrophy in cardiomyocytes, such as the small G-protein Ras (Montessuit and Thorburn, 1999), mTOR, ribosomal S6 kinase (Moschella et al., 2007), GSK3β (Zhai et al., 2007), GATA4 (Wang et al., 2005) and PKD (Vega et al., 2004). Although PKC regulates multiple targets, activation of Ras and downstream MAPK cascades by the DAG-sensitive isoforms of PKC is considered to have a predominant role in cardiac remodelling (Braz et al., 2002).

MAPK signalling pathways in the hypertrophic response

Upon ET-1 binding to the ETA receptor, the classical small g protein Ras is converted into an active, GTP-bound form. Multiple mechanisms have been suggested to explain this effect, relying on the activity of PKC as described above and/or ET-1-induced production of intracellular reactive oxygen species (ROS) (Sugden and Clerk, 2006). Clinically, activating mutations in Ras and its target Raf result in Costello and Noonan syndromes respectively, both of which induce cardiomyopathy amongst other abnormalities (Lin et al., 2011; Wu et al., 2011). Induction of hypertrophy by activated Ras and Raf is ERK1/2 dependent, suggesting that this kinase is responsible for the hypertrophic response to Ras and Raf activation (Harris et al., 2004; Wu et al., 2011). However, although it has been intensively investigated, the precise role and function of activated ERK1/2 in the heart remains controversial (Bueno and Molkentin, 2002; Kehat and Molkentin, 2010). It has been appreciated for many years that ERK1/2 is activated in NRVM in response to hypertrophic agonists including ET-1 (Bogoyevitch et al., 1994). Moreover, inhibition of ERK1/2 activation using either pharmacological inhibitors of its activating kinases MEK1/2 or adenoviral-mediated overexpression of dominant-negative MEK prevents the hypertrophic response to ET-1 in NRVM (Ueyama et al., 2000; Yue et al., 2000). Indeed, microarray studies demonstrated the reliance of ET-1-induced hypertrophic gene expression on ERK1/2 activity (Marshall et al., 2010). However, these observations have been called into question following in vivo studies of transgenic mouse models. Cardiac-specific overexpression of constitutively-active MEK1 induces ERK1/2 phosphorylation and compensated concentric hypertrophy (Bueno et al., 2000). Therefore, activation of ERK1/2 above the baseline level, as occurs in vitro in response to ET-1, can induce cardiac remodelling. However, in vivo loss-of-function studies of ERK1/2 have demonstrated that under certain conditions the hypertrophic response is preserved, calling into question the necessity of these kinases during pathology. Echoing the current thinking regarding calcium-regulated signalling, interesting observations suggest that spatial and contextual aspects of ERK1/2 activation may govern whether it exhibits pro-hypertrophic activity. Interestingly, in vascular smooth muscle cells and fibroblasts, ET-1 induces nuclear translocation of ERK1/2 (Lenormand et al., 1993; El-Mowafy and White, 1999) where it can phosphorylate targets involved in regulation of gene transcription. These include transcription factors (e.g. GATA4 and Elk), nuclear kinases (e.g. MSK1) and chromatin modifying enzymes (e.g. the p300 histone acetylase). In cardiomyocytes, a novel phosphorylated form of ERK1/2 (p-Thr188-ERK1/2) accumulates within the nucleus over the duration of the hypertrophic response in vivo, an effect that could also be simulated in vitro by agonists of Gaαi-coupled receptors (Lorenz et al., 2009). Hence, it is conceivable that ERK1/2 activity becomes more important in the later stages of hypertrophy, once sufficient nuclear accumulation has occurred. Furthermore, the basal and stimulus-induced levels of ERK1/2 activity may have different functional effects within the myocyte (Kehat et al., 2011). In the context of cellular stimulation with agonists such as ET-1, mechanisms may be activated that permit ERK1/2 to perform functions other than those active under non-stimulated conditions, changing the cellular response to the kinase. Discriminating whether, when and how ERK1/2 regulates hypertrophy is an interesting topic of future research.

Although the requirement for ERK1/2 during remodelling remains controversial, many of the targets of the kinase have defined pro-hypertrophic roles. Genome-wide studies of myocyte gene expression profiles have shown that ET-1 induces distinct waves of gene expression, with the earlier phases of the response being largely ERK1/2 dependent (Cullingford et al., 2008; Marshall et al., 2010). Demonstrating the importance of these acute events, inhibiting activity of the activator protein-1 (AP1) complex of the immediate-early genes c-fos and c-jun prevents ET-1-induced hypertrophic remodelling (Oamura et al., 2002). Other transcription factors also exhibit ET-1-induced ERK1/2-dependent activity. For example, GATA4 shows ET-1-dependent DNA-binding activity in response to increased myocardial stretch (Hautala et al., 2001; 2002; Liang et al., 2001) and is regulated by ERK1/2-dependent phosphorylation (Liang et al., 2001). Expression of GATA4 harbouring phospho-mimetic activating mutations is pro-hypertrophic both in vitro and in vivo, whilst replacing endogenous GATA4 with a mutant that cannot be phosphorylated on its activating sites impedes hypertrophic responses (van Berlo et al., 2011). Interestingly, GATA4 can form higher order complexes with other pro-hypertrophic transcription factors such as NFAT and AP1, providing a mechanism whereby pro-hypertrophic signalling pathways can be integrated (Sanna et al., 2005).

Are JNK and p38-MAPK important for ET-1-induced remodelling?

Early studies in NRVM demonstrated that both cellular osmotic stress and ET-1 induce phosphorylation and activation of JNK1/2. However, the response to stress-associated stimuli was much greater than the response to ET, receptor activation. Subsequently, the role of JNK1/2 in hypertrophy has been extensively examined. Overexpression of dominant-negative MKK4, the kinase upstream of JNK1/2, attenuates the hypertrophic response to ET-1 stimulation of NRVM (Choukroun et al., 1998). These and other data suggest that JNK1/2 mediates a pro-hypertrophic effect in vitro. However, this observation has been questioned by other studies showing that activation of JNK1/2 in vitro inhibits the hypertrophic response to ET-1 (Nemoto et al., 1998). To attempt to
address the conflicting data sets, in vivo studies have been performed. Currently, the prevailing consensus suggests that although ectopic activation of JNK1/2 can induce cardiac remodelling and eventual heart failure (Choukroun et al., 1999), these changes occur in the absence of left ventricular hypertrophy. Instead, myocardial stiffening has been observed as a consequence of extracellular matrix remodelling (Petrich et al., 2004). Intriguingly JNK1−/− knock-out mice exhibited a hastened early deterioration in cardiac performance during hypertension-induced hypertrophy, suggesting that the basal level of activity of this isoform may be important for maintaining cardiac function (Tachibana et al., 2006). The cardio-protective actions of JNK have been attributed to JNK-dependent phosphorylation and subsequent nuclear export of the highly pro-hypertrophic transcription factor NFAT (Chow et al., 1997) and/or JNK1/2-dependent positive regulation of the anti-hypertrophic protein JunD. The importance of JNK activity within the hypertrophic response to ET-1 is likely to be governed by the balance of stimuli within the ET-1-regulated signalling network.

Similarly to JNK, the exact role of p38-MAPK within the myocardium has proven to be a matter for debate, with data generated in vitro and in vivo producing somewhat conflicting conclusions. Both ET-1 and cellular stress induce p38-MAPK phosphorylation and activation. However, the kinase has been demonstrated to either be necessary or dispensable for the ET-1-induced hypertrophic response. Furthermore, the different isoforms of p38-MAPK may have variable pathological roles, with p38-MAPKα implicated in the induction of apoptosis whilst p38-MAPKβ is involved in hypertrophy. The weight of evidence suggests that p38-MAPK activity is not required for the initial development of left ventricular hypertrophy (Liao et al., 2001; Braz et al., 2003; Zhang et al., 2003; Nishida et al., 2004). Indeed, akin to the action of JNK1/2, p38-MAPK isoforms may be anti-hypertrophic as a consequence of their NFAT-kinase activity during the early hypertrophic response (Gomez del Arco et al., 2000; Braz et al., 2003). In the latter stages of cardiac decompensation and heart failure, pathological functions for p38-MAPK have been described. The kinase promotes ventricular dilation, stiffening and fibrosis thereby impairing contractility (Liao et al., 2001; Zhang et al., 2003). Consequently, the effect of ET-1 stimulated p38-MAPK activity may not be important for the initial response to the peptide, but become more significant over time. A possible reason for this is that the remodelled physiology and signalling pathways of the hypertrophic heart creates a different profile of substrates or cellular context for the action of p38-MAPK.

**ET-1 signalling – a question of location and timing?**

When considered in their entirety, the importance of ET-1-induced signalling pathways within the hypertrophic response appears to be determined by two governing rules. First, as described above, as a consequence of the remodelling of gene expression that occurs during hypertrophy, the necessity of the various ET-1-regulated mediators for the induction of pathology may be altered over time. Secondly, subcellular localization of ET-1-dependent signals is a critical determinant of their pro-hypertrophic role. This is elegantly demonstrated by the specific pro-hypertrophic effect of peri-nuclear release of calcium in response to IP3R1 activation. Interestingly, the ET₃ receptor itself may also be subject to regulated localization, a mechanism that could be important for its cellular effects. The localization of GPCRs has been well studied and several GPCRs are localized at the nuclear membrane of cardiac myocytes and other cells types (Tadevosyan et al., 2010; Vaniotis et al., 2011a; 2011b; Wright et al., 2012). Although ET₃ receptors are largely found within the sarcomere, several studies have demonstrated functional ET₃ receptor and ET₉ receptor at the nuclear envelope of ventricular cardiomyocytes (Boivin et al., 2003; Bkaily et al., 2011). This may result from translocation of ET receptors from the cell surface or by de novo receptor synthesis. In order for nuclear receptors to be functional, a source of intracellular ligand must exist. It has been hypothesized that ET₁ produced intracellularly in the myocyte can signal in an intracellular manner and that ET-1 is taken into the cell from an extracellular source to act on nuclear ET receptors (Boivin et al., 2003; Tadevosyan et al., 2012). Activation of nuclear ET₁ receptor has been postulated to stimulate similar downstream pathways as sarcolemmal ET₁/R, such as PKC activation and IP3 generation, thus potentially activating hypertrophic gene expression and regulating cardiac homeostasis (Bkaily et al., 2011). As ET-1 production is increased in hypertrophic and failing myocytes, under hypertrophic conditions, there would be a larger stimulus to ET receptors located within the cell. Furthermore, if present in the nucleus specifically, the ET₃ receptor and the signalling pathways it initiates would be clustered in close proximity of their cellular effectors, facilitating ET-1-dependent cellular responses. Further studies to substantiate the localization of ET₃ receptors within the nucleus and their significance and relevance to the hypertrophic response are required to allow inclusion of subcellular ET-1 signalling within the accepted models of ET-1 action.

**Non-myocyte effects of ET in the heart**

ET-1 signalling within the heart regulates a wide spectrum of cardiovascular processes, ranging from development through to life-threatening cardiovascular diseases. In this review, we have focused upon ET signalling in cardiac myocytes. By inducing the activity of a complex web of signalling pathways, this important peptide can directly influence myocyte survival, contractility and remodelling. However, the heart is composed of multiple cell types and despite providing the greater part of cardiac mass, myocytes account for only ~20% of the total number of cells in humans (Rubart and Field, 2006). The non-myocyte population is comprised of endothelial cells, smooth muscle cells and fibroblasts (Nag, 1980; Banerjee et al., 2007), all of which are responsive to ET-1 (Kedzierski and Yanagisawa, 2001). Cardiac endothelial and smooth muscle cells form the myocardial vasculature. ET-1 interacts with these cells to regulate vascular tone, influencing myocardial blood supply and metabolic activity (Wang et al., 1998). Cardiac fibroblasts are essential for secretion of extracellular matrix and for cardiac repair (Biernacka and
Frangogiannis, 2011). In response to elevated levels of ET-1, cardiac fibroblasts proliferate, differentiate into myofibroblasts and increase their secretion of matrix proteins (Shi-Wen et al., 2004a; 2004b). These fibroblast responses underlie scar formation following cardiac damage and the myocardium-wide fibrosis associated with cardiac hypertrophy, where the increased abundance of cardiac fibroblasts replaces the myocytes lost as a result of pathology (Swynghedauw, 1999). The reactive response of the fibroblast is essential for the maintenance of cardiac function during disease, without which the integrity of the heart is compromised (Shimazaki et al., 2008). However, fibroblast infiltration alters the electrophysiological properties of the myocardium, affecting both action potential propagation and allowing arrhythmic events at the cellular level to propagate (Mayyas et al., 2010; Karagueuzian, 2011). Cardiac fibrosis also decreases elasticity of the heart, stiffening the myocardial wall and impeding cardiac output (Lakatta and Levy, 2003a). Extensive fibrosis of the myocardium is not only a characteristic of pathology but is also a prominent feature of the aged, senescent heart and in common with pathophysiological situations, fibrosis of the aged heart arises due to hypertrophy and myocyte loss (Lakatta and Levy, 2003a; Biernacka and Frangogiannis, 2011). When combined with the decreased relaxation rate of the aged heart, these effects are manifest in diastolic dysfunction, impacting exercise tolerance, quality of life and contributing to future heart failure (Lakatta and Levy, 2003a). As ET-1 increases in concentration with age (Maeda et al., 2003) and induces fibrosis (Shi-Wen et al., 2004a), the importance of the ET-1 system for the decline in myocardial function associated with age should be considered paramount.

**Role of ET-1 in PAH and right ventricular hypertrophy**

The compelling evidence linking the ET-1 system to cardiovascular pathologies has prompted its targeting for therapeutic benefit. Currently, the combined ET$_A$-ET$_B$ receptor antagonist bosentan and the selective ET$_A$ receptor antagonist ambrisentan are licensed and successfully used for the treatment of pulmonary arterial hypertension (PAH) (Kirkby et al., 2008), a condition which induces right ventricular hypertrophy, right heart failure and eventually death (Gomberg-Maitland et al., 2011). PAH increases pulmonary arterial pressure and vascular resistance, stretching and increasing the load on the right ventricle. Under these conditions, the type 1 angiotensin receptor is activated in response to myocardial stretch and stimulates ROS formation. Subsequently, increased intracellular ROS indirectly triggers the release of ET-1. ET-1-induced increases in intracellular calcium stimulate NFAT and CaMKII, culminating in hypertrophic remodelling (Cingolani et al., 2010). Interestingly, during PAH, pro-hypertrophic GATA4 is also active within the right ventricle (Park et al., 2010), suggesting that multiple ET-1 regulated signalling pathways may be important in this condition. Significantly, ET$_A$ receptor blockade prevents right ventricular remodelling in an animal model (Jasmin et al., 2003), demonstrating the importance of the link between PAH, right ventricular hypertrophy and ET-1. These effects may be responsible for the therapeutic benefit of ET$_A$ receptor antagonists for the treatment of PAH.

**Why have ET receptor antagonists failed in the treatment of left ventricular hypertrophy and congestive heart failure and where may they provide benefit?**

Despite the myriad of lines of evidence from both animal models and humans for a role of ET-1 and ET-1-dependent signalling pathways in left ventricular hypertrophy and congestive heart failure (CHF), it is disappointing to note that ET-1 receptor antagonists have not yet proven efficacious for the treatment of CHF (Kirkby et al., 2008). Although initial studies were promising (Sutsch et al., 1998), clinical trials of the combined ET$_A$-ET$_B$ receptor antagonist bosentan for the treatment of CHF were halted owing to problems with liver toxicity. Furthermore, later studies were unable to demonstrate a reduction in the mortality or symptoms of patients suffering from CHF when a lower dose of bosentan was administered (Kalra et al., 2002; Kelland and Webb, 2006). A number of explanations for the failure of this approach have been proposed. Blockade of the ET$_B$ receptor impairs ET-1 clearance, increasing plasma ET-1 levels and allowing the effects of ET$_A$ receptor antagonism to be overcome (Cowburn et al., 2005). Furthermore, ET$_A$ receptor inhibition leads to systemic vasoconstriction, a deleterious response in patients with cardiovascular conditions (Cowburn et al., 2005). To avoid the complicating effects of ET$_B$ receptor inhibition, studies aiming to selectively block the ET$_A$ receptor have been carried out (Anand et al., 2004; Cowburn et al., 2005; Leslie et al., 2005). Although cardiac output was acutely improved, no long-term benefit of this treatment regimen was observed (Anand et al., 2004). However, these studies may have utilized a dose of drug at which antagonism of the ET$_B$ receptor cannot be completely excluded (Kelland and Webb, 2007). In view of such limitations, it would be informative if trials were performed using ET$_A$ receptor antagonists at a dose that more selectively targets the ET$_A$ receptor. In addition, the results of many of the clinical trials performed to date have not been fully published and analysed, meaning that a thorough interpretation of the data is lacking (Kelland and Webb, 2007). Further progress will only be made by a more detailed analysis of these trials. For example, there may be patient sub-populations where ET receptor inhibition is effective. It will also be important to gain a better understanding of the interaction of the ET-1 system with patient lifestyle and age. Indeed, ET-1 levels are elevated in aged and obese populations, which may suggest that ET receptor antagonists would provide greater benefit in these individuals. Interestingly, improved lifestyle benefits these populations, with weight loss and exercise reducing ET-1 levels in the obese and aged respectively (Maeda et al., 2003; 2006). Furthermore, our increased understanding of ET-1 biology and ET-1-induced cellular responses may henceforth guide more intelligent and targeted drug design. In view of the disparate effects of ET$_A$ and ET$_B$ receptor inhibition in humans, it will be
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important to consider the influence of dimers and heterodimers of the ET receptor subtypes upon downstream signalling and pharmacology (Evans and Walker, 2008a; 2008b). More knowledge regarding the persistence, localization and integration of these ET-1-induced responses is also required. Agents that allow selective control of the activation of ET\(_A\) and ET\(_B\) receptors and/or the individual limbs of the signalling pathways they activate downstream would be particularly informative. The development of biased agonists/antagonists akin to those for the angiotensin receptor would allow for the specific targeting of key signalling pathways (Watts, 2010) and perhaps provide a window of opportunity to manipulate ET-1-induced signalling for therapeutic benefit.

Cardiac failure is the dominant cause of mortality in the over 65 population. This population is likely to double in number in the next 25 years; hence, future therapies will need to be developed to tackle the increasing burden of cardiac disease. The prominent role that ET-1 performs in the pathological alterations in physiology and function of the multiple cell types of the heart, in particular during ageing, identify it as a key target for further development of therapeutic strategies.

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Conflict of interest

None.

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