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Cardioprotective Signaling by Endothelin

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Abstract

The endothelin axis promotes survival signaling pathways in the heart, inviting the idea to use antagonists of endothelin signaling for the treatment of heart failure. Promising results from animal trials, however, failed to show beneficial effects in heart failure patients. Here we review the role of major signaling pathways in the heart that are involved in cell survival initiated by ET-1. These pathways include MAPK, PI3K/AKT, NF- κ B and calcineurin signaling. A better understanding of endothelin mediated signaling in cardiac cell survival may allow a re-evaluation of endothelin receptor antagonists in the treatment of heart failure.

The Endothelin Axis in the Cardiovascular System

Endothelin was first identified as a potent vasoconstrictor peptide secreted by the endothelium (Yanagisawa et al. 1988). This action is mediated by endothelin synthesized in vascular endothelial cells that acts on the underlying smooth muscle of the media in a paracrine fashion through one of two G-protein coupled receptors, ET_A or ET_B. Endothelin has three isoforms that are synthesized in many organs and cell types and have a variety of physiologic roles. Endothelin-1 (ET-1) is expressed in the heart and has direct influence on cardiac myocyte function, including hypertrophic and inotropic effects (Ishikawa et al. 1988, Ito et al. 1991). In cardiomyocytes, the ET_A receptor is more abundant (90%) and has been considered more important for the cardiac effects of ET-1, although the ET_B receptor may be more responsive to physiological stress (Kedzierski et al. 2001). While endothelin receptors expressed on vascular smooth muscle cells promote vasoconstriction, ET_B receptors expressed on endothelial cells mediate vasodilation (Brunner et al. 2006).

A role of endothelin in heart failure was recognized early. The amount of ET-1 increases in the plasma of animals or patients with heart failure, and in the failing hearts of animals. The

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specific cells responsible for this synthesis have not been identified (Margulies et al. 1990, Sakai et al. 1996b, Wei et al. 1994) as endothelial cells, fibroblasts and cardiac myocytes can synthesize ET-1. Multiple physiological stimuli in heart failure can lead to enhanced endothelin expression, including both hypertrophy and cardiac damage. In animal models, treatment with endothelin receptor antagonists (ETRAs) produced promising results for the treatment of heart failure, as they improved ventricular remodeling and prolonged survival after infarction (Sakai et al. 1996a). This prompted efforts to examine the effect of these agents in patients. In humans, endothelin receptor (ETR) blockade leads to a promising hemodynamic profile (Schalcher et al. 2001, Torre-Amione et al. 2001), including reduced peripheral resistance and increased cardiac output with little effect on heart rate. Nonetheless, a series of clinical studies, each with hundreds of patients with different degrees of heart failure, testing both selective ET_A and non-selective ETR antagonists, was uniformly unimpressive (Abrahams 2001, Kalra et al. 2002, Kelland 2006, Mylona 1999). Currently, these drugs have been approved only for the treatment of pulmonary hypertension (Sastry 2006).

Recent information on the role of the endothelin axis in cardiomyocyte survival may provide at least a partial explanation for these disappointing clinical results, and point towards strategies that would be more successful. In this review we will discuss survival pathways in the heart mediated by ET-1. These include MAPK, PI3K/AKT, NF[.kappa]B and calcineurin signaling. Each of these pathways can provide a survival benefit by distinct mechanisms.

Endothelin-1 and Cardiomyocyte Survival Signaling

The ET-1 axis has pleotropic effects in the heart, modulating function by activating signaling pathways that impinge upon hypertrophic, proliferative and cell survival responses. These effects seem to be tissue-dependent, and the specific signaling pathways involved are not yet always well defined. Here we summarize research investigating the role of endothelin in apoptosis and cell survival.

Endothelin-1 binding to endothelin receptors on cardiac myocytes stimulates signaling cascades that include activation of protein kinase C (PKC) and phosphatidylinositol-1,4,5-triphosphate kinase (PI3K), with subsequent effects on intracellular calcium that can stimulate calmodulin-dependent pathways. PKC and/or Ca²⁺/calmodulin (CaM)-dependent protein kinase (CaMK) activate receptor and non-receptor tyrosine kinases such as Src and proline-rich tyrosine kinase 2 (Pyk2). These and other stimuli activate MAP kinase pathways and direct nuclear translocation of nuclear factor of activated T-cells 1 (NFAT-1). All of these potential survival pathways are initiated by G_q transduction. G_i-dependent pathways, when stimulated by ligand-binding to endothelin receptors, are not required for the anti-apoptotic effect of ET-1 in cardiomyocytes (Araki et al. 2000, James et al. 1994).

Endothelin-1 Activates the Mitogen-activated Protein Kinase (MAPK) Signaling Pathway

Mechanism of MAPK Cascade Activation

Binding of ET-1 to its ET_A receptor induces a conformational change in the receptor that allows GTP binding to the α -subunit of the trimeric receptor associated G_q protein. Activation of G_q- α results in dissociation from the $\beta\gamma$ -complex and the initiation of downstream G-protein signaling. In this way, ET-1 initiates the MAPK pathway in various cell types, including cardiomyocytes, vascular smooth muscle cells and fibroblasts. G_q- α activates the small GTPase Ras in cardiomyocytes (Chiloeches et al. 1999). There is evidence that G_q- α through activation of PKC, activates Ras, which undergoes conformational changes that in turn allow the exchange of Ras-bound GDP for GTP with the help of guanine exchange factors (GEFs) such as the son of seven (SOS). GTP-bound Ras has a high affinity for Raf, which is recruited to the membrane, where it is phosphorylated (Lezoualc'h et al. 2008).

Activated Raf phosphorylates MEK1/2 or MAPKK at specific serine/threonine residues, which subsequently phosphorylates MAPKs such as ERK1/2 on threonine and tyrosine residues (Chiloeches et al. 1999). The primary MAPKs are extracellular signal regulated protein kinase (ERK), c-Jun NH₂-terminal protein kinase (JNK) and p38 MAP kinase. Activation of ERK1/2 through phosphorylation by MEK1/2 plays an important role in cardiac hypertrophy as well as in cardioprotection. MEK 4/7 and MEK 3/6 phosphorylate JNK and p38 MAPK, respectively. These pathways have distinct, occasionally countervailing, roles in cardiac signaling with many of the details still undefined (Wang 2007).

Activation of ERK

It appears that ET_A-mediated activation of ERK is at least one of the routes by which ET-1 promotes cardiomyocyte survival (Figure 1). In neonatal cardiomyocytes, ET-1 inhibits isoproterenol-induced apoptosis, whereas the ET_A receptor antagonist FR139317 blocks this anti-apoptotic effect (Araki et al. 2000). Moreover, an ET_B receptor antagonist (BQ788) does not alter isoproterenol-induced apoptosis. A MAPK kinase inhibitor (MEK1-specific-PD098059) blocks the ET-1 mediated anti-apoptotic effect in cardiomyocytes, further indicating that the ERK1/2-MAPK pathway plays a role in endothelin-1 mediated cell survival.

Activation of JNK via Pyk2

The focal adhesion-dependent pathway is also stimulated by ET-1 signaling (Kodama et al. 2003). The non-tyrosine receptor kinase Pyk2 can be activated by phosphorylation on one or more tyrosine residues in response to integrin activation (Luttrell et al. 2004) or activation of G-protein-coupled receptors that elevate intracellular calcium levels or activate PKC in various cell types (Lev et al. 1995, Sabri et al. 1998).

In cardiac myocytes, Pyk2 can be activated upon ET-1 stimulation. Once phosphorylated, Pyk2 recruits other signal-transducing molecules with SH2 domains such as the tyrosine

kinase c-Src and the adapter molecule Grb2 (Lev et al. 1995, Luttrell et al. 2004), which can ultimately lead to MAP kinase activation. Activated c-Src bound to Pyk2 can directly phosphorylate adjacent cellular proteins, such as the 130 kDa Crk-associated substrate (p130Cas) (Dikic et al. 1996) which in turn phosphorylates Crk and activates JNK in a Rac-dependent manner (Dolfi et al. 1998). In ET-1 stimulated cardiac myocytes, p130Cas and Pyk2 mediate activation of JNK signaling by this pathway (Kodama et al. 2003) (Figure 1).

MAP kinases reduce doxorubicin cardiotoxicity

Doxorubicin induces apoptosis at least in part by activation of caspase-3 in cardiomyocytes (Ueno et al. 2006). This contributes to the dose-dependent cardiomyopathy that limits the usefulness of this potent chemotherapy. A transient cytoprotective effect of ET-1 in doxorubicin-induced cytotoxicity is mediated by ET_A. A role for phosphorylation-dependent signaling pathways, including MAPKs, was suggested as this cytoprotective effect of ET-1 was eliminated by the protein kinase inhibitor staurosporine. However, these experiments do not further specify a MAPK pathway. Also, ET-1 does not reduce cytotoxicity after longer (>48 h) doxorubicin treatment (Suzuki et al. 2001).

These findings suggest that MAPKs including ERK and JNK are activated by interacting with different scaffolds, receptor tyrosine kinases and focal adhesion proteins, which in turn may each respond to ET-1-mediated signaling. ET-1 can stimulate p38 phosphorylation in heart (Ueyama et al. 1999), but this appears to enhance rather than inhibit cell death (Adams et al. 1998, Wang et al. 1998).

Cross-talk of Integrin-mediated and ET-1 Signaling

Integrin binding to the underlying ECM promotes the formation of focal adhesions linking the ECM to the actin cytoskeleton. In focal adhesions, proteins such as focal adhesion kinase (FAK), Pyk2 (a close relative of FAK), p130Cas, and integrin-linked kinase (ILK) are activated and initiate downstream signaling pathways.

Integrin-linked kinase (ILK) appears to play a role in survival of cardiomyocytes (Hannigan et al. 2007). Activated by binding of the phosphoinositide phospholipid product of PI3K, PIP₃, to the PH-like domain of ILK (Dedhar 2000), ILK activates AKT and enhances cardiomyocyte survival (Bock-Marquette et al. 2004) (Figure 2). Whether ET-1 directly activates ILK in cardiomyocytes is unknown; however, it does activate ILK in ovarian carcinoma cells (Rosano et al. 2006) and protects these cells from apoptosis (Del Bufalo et al. 2002). Integrin binding to ECM also protects cardiomyocytes from oxidative stress through the PI3K/AKT survival pathway (Yoshida et al. 2007).

Endothelin-1 Activates the Phosphatidylinositol-3 Kinase/AKT Pathway

Phosphatidylinositol-3 kinases (PI3Ks) are activated by endothelin and influence endothelin-1 mediated cardiomyocyte survival (Araki et al. 2000, Proud 2004). The PI3K pathway generates phospholipids that act as second messengers that activate several protein kinases, including PI-3,4,5-triphosphate (PIP₃) dependent protein kinase (PDK), protein kinase B (PKB/AKT) and protein S6 kinase (S6K) (Rameh et al. 1999). ET-1 activates the

PI3K downstream effector PKB/AKT in cardiomyocytes (Pham et al. 2001), myofibroblasts (Shi-Wen 2004) and vascular smooth muscle cells (Dong 2005). Inhibition of PI3K-AKT activation with the PI3K specific inhibitor, wortmannin, blocks the protective effect of ET-1 against adrenergic stress in cardiomyocytes (Araki et al. 2000).

The anti-apoptotic effects of ET-1 are likely in part due to AKT activation that promotes the phosphorylation of the pro-apoptotic protein Bad, thereby sequestering it in the cytoplasm by binding to 14-3-3 proteins (Downward 1998) (Figure 2). Bad is a member of the Bcl-2 family and is a key regulator of the intrinsic pathway of apoptosis/cell survival. Phosphorylation of Bad, leading to retention in the cytoplasm, inhibits its ability to bind to Bcl-2 and Bcl-x_L at the mitochondrial outer membrane and subsequently promotes apoptosis. Similarly, AKT promotes phosphorylation of forkhead transcription factors (FOXOs) in the heart, which normally regulate the expression of the apoptosis-inducing FAS ligand (Brunet et al. 1999, Skurk et al. 2005). Phosphorylation of FOXOs also results in their inactivation via recruitment by 14-3-3 proteins. PKB/AKT also directly suppresses apoptosis via inactivation of caspase 9 (Cardone et al. 1998) (Figure 2).

The anti-apoptotic effect of ET-1 during isoproterenol infusion can also be blocked by rapamycin. Rapamycin inhibits mammalian target of rapamycin (mTOR) signaling and subsequent activation of S6K (Araki et al. 2000). Activation of S6K promotes cell survival, at least in part, by phosphorylation of bad (Harada et al. 2001) which can inhibit apoptosis, as described above. These findings suggest that the anti-apoptotic effect of ET-1 in cardiomyocytes is mediated by more than one signaling pathway. Indeed, ET-1 also activates a S6K isoform in a MEK-dependent manner in primary adult cardiomyocytes (Wang et al. 2001). Taken together, these studies suggest that MAPK, PI3K and mTOR signaling may converge to activate the downstream effector S6K, which in turn contributes to the protective effect of ET-1 in cardiomyocytes by phosphorylation of downstream effectors of protein synthesis and apoptosis.

Endothelin-1 Activates a c-Src/Bcl-X_L Signaling Pathway

Anti-apoptotic proteins in the Bcl-2 family inhibit the leakage of cytochrome c from mitochondria that initiates the intrinsic apoptotic cascade. ET-1 ameliorates serum-deprivation-induced apoptosis in cultured cardiomyocytes via the ET_A receptor pathway. In this model, ET-1 mediated activation of Pyk2, as described above, promotes the binding of the autophosphorylation site of Pyk2 (Tyr-402) to the Src-homology 2 domain (SH2) of c-Src. This activates c-Src and subsequently upregulates the levels of the anti-apoptotic protein Bcl-X_L, leading to enhanced survival during serum deprivation (Ogata et al. 2003). Bcl-X_L is closely related to Bcl-2 (Zinkel et al. 2006), which also inhibits apoptosis in adult cardiomyocytes (Kakita et al. 2001).

Endothelin-1 Activates the Calcium-calcineurin Pathway

The calcium-calcineurin signaling cascade has been shown to play an important role in cardiac hypertrophy and is also activated by ET-1. In general, the GTP-bound α subunit of the G_q-protein coupled receptor family mediates the targeting of PLC- β to the membrane

which becomes activated as a result of interactions with the G_q - α subunit (Rhee 2001) and generates IP_3 .

ET-1 stimulation of cardiomyocytes increases intracellular calcium levels via IP_3 mediated Ca^{2+} release from intracellular calcium stores (Shubeita et al. 1990) that can activate calcineurin (Iwai-Kanai et al. 2004). Calcineurin is a calmodulin-regulated serine/threonine phosphatase that dephosphorylates residues within the regulatory domain of the nuclear factor of activated T cells (NFAT) and thereby promotes its translocation into the nucleus and subsequent activation of target genes.

Calcineurin activation is required for ET-1 inhibition of oxidant stress-induced apoptosis in cardiomyocytes (Kakita et al. 2001). Cyclosporine A and FK506, both of which antagonize calcineurin, block the protective effect of ET-1 in these cells. In this study, ET-1 also attenuated H_2O_2 -induced-apoptosis in cells constitutively overexpressing calcineurin. Translocation of cytoplasmic NFATc to the nucleus was stimulated by ET-1 in a calcineurin dependent manner. Further, cyclosporine A blocked ET-1 mediated induction of Bcl-2 gene expression, which is well placed to modulate proapoptotic oxidant stress on the mitochondria (Kakita et al. 2001). Together, these data demonstrate that activation of calcineurin and NFATc by ET-1 is sufficient to inhibit H_2O_2 -induced-apoptosis.

Endothelin-1 Activates Nuclear factor- κ B Signaling

Nuclear factor- κ B (NF κ B) signaling is a crucial integrator of immunological response and appears to impinge on the endothelin pathway. NF κ B dimers are sequestered in the cytosol by the inhibitor of κ B (I κ B) family. Activation of NF- κ B is initiated by a signal-induced degradation of the I κ B proteins, which occurs primarily via activation of I κ B kinase (IKK), exposing NF- κ B's nuclear localization signals. Defects in NF- κ B signaling results in increased susceptibility to apoptosis leading to increased cell death.

We developed mice with a cardiomyocyte-specific deletion of ET-1 and found that local ET-1 expression is necessary to maintain normal cardiac function and survival in mice as a function of age or hemodynamic stress (Zhao et al. 2006). The cardioprotective effect of ET-1 is at least in part mediated by inhibition of the extrinsic FasL/TNF apoptotic signaling pathway which is associated with upregulation of NF κ B. Upregulation of NF κ B in the heart leads to expression of NF κ B initiated survival genes including TRAF1, cIAP1 and cIAP2, which inhibit TNF-induced apoptosis (Figure 2). While our findings point toward the extrinsic apoptotic pathway they do not exclude the involvement of the intrinsic mitochondrial apoptotic pathway, which, as we have discussed above, is also likely to play a role in cardiomyocyte apoptosis. NF κ B may also impinge on the Pyk2/AKT survival pathway described above (Shi et al. 2001).

Other Cytoprotective Mechanisms of Endothelin

Another interesting mechanism for the cytoprotective action of ET-1 during oxidative stress involves the disruption of sphingosine kinase-1 (SK1) and four-and-a-half-LIM domain 2 (FHL2 or SLIM3) interactions. These proteins are key elements in regulating relative levels of sphingosines and ceramides, which cause apoptosis and growth arrest, and sphingosine-1-

phosphates (S1P), which mediate proliferation and angiogenesis (Hannun et al. 2008). ET-1 decreases FHL2 interaction with SK1 in cardiomyocytes and increases SK1 activity, which leads to protection from oxidative stress-induced apoptosis (Sun et al. 2006). SK1 catalyzes the phosphorylation of sphingosine to sphingosine-1-phosphate (SPP or S1P) (Taha et al. 2006) that can then bind S1P-receptors, which are high-affinity G-protein coupled receptors (Hannun et al. 2008). S1P is cardioprotective (Peters et al. 2007), and it is possible that ET-1-mediated SK1 activation in the heart results in initiation of sphingolipid signaling that may activate similar survival pathways as described above.

Use of Endothelin Receptor Antagonists in Models of Heart Failure

Conditions that stimulate hypertrophy markedly increase expression of ET-1 in cardiac cells (Sakai et al. 1996b), and this activation commonly accompanies the increased wall stress and remodeling seen in heart failure. Initial *in vivo* studies with selective antagonists of the ET_A receptor were exciting because they reduced hypertrophic responses and the severity and mortality of post-infarction heart failure in rodents (Sakai et al. 1996a, Yamauchi-Kohno et al. 1999). The non-selective endothelin receptor inhibitor Bosentan improved ventricular remodeling, hemodynamics and function (Fraccarollo 1997, Mulder et al. 1997) and improved survival (Mulder et al. 1997) in rodent models of congestive heart failure. However, certain selective endothelin receptor antagonists had no effect on hypertrophic response (Mulder et al. 1998), or even worsened left ventricular dilation (Hu et al. 1998). The effects of endothelin receptor antagonists (ETRAs) in animal models of various diseases has been reviewed (Remuzzi 2002). We have also shown resistance to hyperthyroid-related cardiac hypertrophy in mice with cardiomyocyte specific ablation of ET-1 (Shohet et al. 2004). These mice showed increased apoptosis as well as increased cleavage of caspase-3 (Zhao et al. 2006) in the heart, implicating the ET-1 axis in both hypertrophy and cell survival during this specific stress.

Clinical Trials in CHF with Endothelin Receptor Antagonists

The discovery of the endothelin system, the recognition of its importance in heart failure and the success of ETRA treatments in animal models of heart failure promised a new treatment for heart failure. However, several large clinical trials testing ETRAs in the treatment of heart failure have failed to show benefit (reviewed in (Kelland 2006) and (Remuzzi 2002)). The Research on Endothelin Antagonism in Chronic Heart Failure (REACH-1) study, which assessed long-term effects of a relatively large dose of Bosentan in 368 patients with severe heart failure, was discontinued early owing to increased levels of liver transaminases and a worsening of heart failure in the treated group (Packer et al. 1998). Lower doses of Bosentan were tested in the Endothelin Antagonist Bosentan for Lowering Cardiac Events in Heart Failure (ENABLE) study of 1,613 patients with severe heart failure and again failed to demonstrate a reduction in morbidity and mortality by addition of Bosentan to standard treatment (Kalra et al. 2002). In the Enrasentan Cooperative Randomized Evaluation (ENCOR) study of 419 patients, treatment with Enrasentan resulted in higher heart failure rate than placebo and a higher mortality (Abrahams 2001). In the latest large study, the Endothelin A Receptor Antagonist Trial in Heart Failure (EARTH) trial, 642 heart failure patients were treated with Darusentan or placebo (Anand et al. 2004). No significant

differences were observed in mortality or progression of heart failure and ventricular dilation.

The reasons that these clinical studies failed to reproduce the success observed in animal models of heart failure might include that endothelin receptor blockers are applied in addition to standard treatment with ACE inhibitors, β -blockers, spiro lactone etc. These agents can obviously modify cardiac pathways and hence provide a different signaling milieu for endothelin receptor antagonists than that found in animal models that lack concomitant treatment with other CHF drugs. Another possible explanation derives from the theme of this review, namely that the beneficial hemodynamic effects of endothelin receptor antagonists are counter-balanced by a negative effect on cell survival. Thus an enhancement of cardiomyocyte cell death, which might be progressive with continued treatment, would gradually overtake the more immediate beneficial vascular effects. It is possible that endothelin antagonists that have reduced cardiac distribution, or enhanced vascular targeting, might provide the hemodynamic benefits seen in intravenous infusion of ETARs without the putative negative effects on cardiomyocyte survival. In general the focus of pharmacologic characterization of these receptor antagonists has focused on receptor-binding rather than tissue distribution. It also remains a possibility that endothelin receptor antagonism might work in isolation but is not beneficial in combination with established therapy. Other possible explanations for the failure of these clinical trials in comparison with the animal studies include the timing of treatment, receptor selectivity, and various limitations of animal studies (Kirkby et al. 2008)

Conclusion

Activation of the endothelin axis modifies several cell survival signaling pathways in the heart. Antagonism of ET-1 signaling was initially encouraging as a treatment for heart failure in animal models. Yet the clinical application of ETARs has not shown beneficial effects in patients with heart failure. Major pathways involved in the cytoprotective effects of endothelin signaling in the heart include MAPK, PI3K/AKT, NF κ B, and calcineurin signaling. An appreciation of the role of the endothelin axis in cell-survival pathways may usefully refocus and revive this approach to heart failure.

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Figure 1. MAPK related cardiac survival pathways initiated by endothelin-1

Definitions of abbreviations:

ET_A = Endothelin receptor A

Pyk2 = Proline-rich tyrosine kinase 2

PKC = Protein kinase C

Ras, Raf, Rac = Small GTPases

GTP = Guanosine triphosphate

MEK = Mitogen activated protein kinase kinase (also MAPKK)

P130Cas = Crk-associated substrate p130Cas

Scr = Tyrosine kinase c-Src

S6K = Protein S6 kinase

Erk = Extracellular signal regulated protein kinase

JNK = c-Jun NH₂-terminal protein kinase

p38 = p38 MAP kinase

-P = phosphorylated protein

Figure 2. AKT related cardiac survival pathways initiated by endothelin-1 Definitions of abbreviations

ET_A = Endothelin receptor A

Pyk2 = Proline-rich tyrosine kinase 2

S6K = Protein S6 kinase

PI3K = Phosphatidylinositol-3 kinase

ILK = Integrin-linked kinase

AKT/PKB = Protein kinase B

IKK = Inhibitor of κB Kinase

mTOR = Mammalian target of rapamycin

TRAF-1 = TNF receptor-associated factor 1

xIAPs = Cellular inhibitors of apoptosis

NF-κB = Nuclear factor-κB

FasL/TNF = Fas Ligand/Tumor necrosis factor

-P = phosphorylated protein