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REVIEW

Pharmacological hypothesis: Nitric oxide-induced inhibition of ADAM-17 activity as well as vesicle release can in turn prevent the production of soluble endothelin-converting enzyme

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Abstract

Endothelin-1 (ET-1) and nitric oxide (NO) are two highly potent vasoactive molecules with opposing effects on the vasculature. Endothelin-converting enzyme (ECE) and nitric oxide synthase (NOS) catalyse the production of ET-1 and NO, respectively. It is well established that these molecules play a crucial role in the initiation and progression of cardiovascular diseases and have therefore become targets of therapy. Many studies have examined the mechanism(s) by which NO regulates ET-1 production. Expression and localization of ECE-1 is a key factor that determines the rate of ET-1 production. ECE-1 can either be membrane bound or be released from the cell surface to produce a soluble form. NO has been shown to reduce the expression of both membrane-bound and soluble ECE-1. Several studies have examined the mechanism(s) behind NO-mediated inhibition of ECE expression on the cell membrane. However, the precise mechanism(s) behind NO-mediated inhibition of soluble ECE production are unknown. We hypothesize that both exogenous and endogenous NO, inhibits the production of soluble ECE-1 by preventing its release via extracellular vesicles (e.g., exosomes), and/or by inhibiting the activity of A Disintegrin and Metalloprotease-17 (ADAM17). If this hypothesis is proven correct in future studies, these pathways represent targets for the therapeutic manipulation of soluble ECE-1 production.

Abbreviations

ADAM-17, a disintegrin and metalloprotease-17; BigET, big endothelin; CAD, coronary artery disease; cGMP, cyclic guanosine monophosphate; DCM, dilated cardiac myopathy; ECE, endothelin-converting enzyme; ET-1, endothelin; NO, nitric oxide; NOS, nitric oxide synthase; PKC, protein kinase C; PKG, protein kinase G; PMA, phorbol-12-myristate-13-acetate; SNP, sodium nitroprusside.

Introduction

Endothelin-1 (ET-1), first discovered in 1988, remains one of the most potent vasoconstrictors known (Yanagisawa et al. 1988). In addition to its role in vasoconstriction, ET-1 exerts mitogenic effects (Fukuda et al. 1996) and plays a role in growth and development.(Kurihara et al. 1995) ET-1 is produced by the cleavage of its precursor big endothelin (BigET) by ECE-1 (Fig. 1). Due to the vasoconstrictor effects of the ET system and its impact on cardiovascular diseases, both ET-1 and ECE are attractive therapeutic targets. At present, antagonists of ET-1 receptors such as bosentan are in clinical use (Maguire and Davenport 2015).

Nitric oxide (NO) is a gaseous metabolite that is a potent vasorelaxant (Fig. 1). It is synthesized by a family

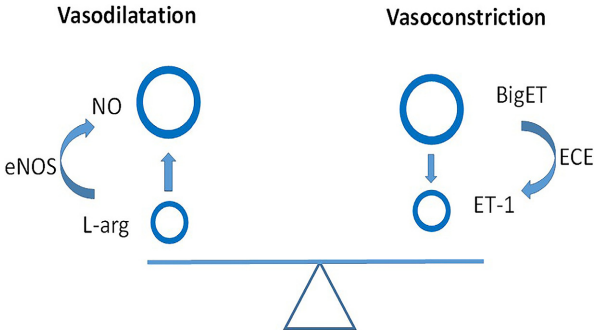

Figure 1. Opposing effects of NO and ET-1 on the vasculature. The action of ECE converts BigET into the ET-1, while the action of eNOS converts L-arg into NO. ET-1 is a vasoconstrictor, while NO is a vasodilator.

of enzymes known as the NO synthases (NOS) (Forstermann and Sessa 2012). NO donors are among the treatment options available for a range of cardiovascular diseases including essential hypertension, stroke, coronary artery disease, and atherosclerosis.(Katsumi et al. 2007) One of the major actions of NO is the activation of guanylyl cyclase leading to the production of cyclic guanosine monophosphate (cGMP).(Lee et al. 2004)

Both NO and ET-1 are produced by the vascular endothelium.(Raoch et al. 2011) Their opposing effects on the vasculature have led to numerous studies examining the mechanisms behind their reciprocal regulation (Kolb-Bachofen et al. 2006; Bourque et al. 2011). Inhibition of ET-1 by NO has been covered in a previous review (Bourque et al. 2011) and therefore will not be discussed further here.

Endothelin-converting enzyme-1

The rate of ET-1 production is dependent on the transcription of its precursor BigET as well as the localization and expression of ECE-1. (Mitsutomi et al. 1999; Kuruppu and Smith 2012). ECE-1 cleaves BigET between Trp^{21}/Val^{22} producing the 21 amino acid peptide known as ET-1. (Opgenorth et al. 1992; Turner and Tanzawa 1997) ECE-1 exists as a homodimer on the cell surface, with each monomer being composed of a short N-terminal region, a single transmembrane region, and a large extracellular catalytic site containing a Zn^{2+} coordinating motif. The extracellular region of ECE-1 can be shed from the cell surface to produce a soluble form that retains catalytic activity (Kuruppu et al. 2007). ECE-1 has four different isoforms, all encoded by a single gene but under the control of different promoters (Valdenaire et al. 1995, 1999). The difference among the isoforms lie in the intracellular N-terminal domain, which contain phosphorylation sites for several kinases (Schweizer et al. 1997; Jafri and Ergul 2003). Phosphorylation is thought to play a key role in the subcellular localization and trafficking of ECE-1, and has been covered extensively in a previous review (Kuruppu and Smith 2012). ECE-1 is predominantly expressed in the endothelial cells, while low to moderate expression is found in the surrounding vascular smooth muscle cells (Davenport et al. 1998). Physiological relevance of ECE-1 expressed on smooth muscle cells is indicated by the production of ETs by these cells (Yu and Davenport 1995).

The closest homolog of ECE-1 is Neprilysin (NEP) sharing 40% sequence identity, and both enzymes are members of the M13 family of metalloproteases (Hoang and Turner 1997). NEP is primarily a membrane-bound metalloprotease, while intracellular pools of ECE-1 have been identified (Schweizer et al. 1997). Both NEP and ECE metabolize peptide hormones that play a role in cardiovascular and neurodegenerative disease. These include amyloid beta, substance P, encephalin (Nalivaeva et al. 2012), bradykinin (Connelly et al. 1985; Hoang and Turner 1997), and BigET (Takahashi et al. 1995; Barnes et al. 1998). However, the two enzymes differ significantly in their relative substrate specificities with BigET and amyloid beta being the preferred substrates for ECE and NEP, respectively (Nalivaeva et al. 2012). Furthermore, crucial role of ECE-1 in growth and development is evidenced by known difficulties in producing a viable ECE-1 knockout model (Yanagisawa et al. 2000).

Role of ECE-1 in disease processes

Given the vasoconstrictor effects of ET-1 and hence ECE-1, expression of ECE-1 is thought to play a key role in the initiation and progression of a number of cardiovascular diseases. Elevated ECE-1 expression has been reported in atherosclerotic plaque (Grantham et al. 1998). ECE-1 activity in endothelium denuded coronary arteries obtained from patients undergoing surgery for coronary artery disease (CAD) was compared with that of patients with dilated cardiac myopathy (DCM). Response of these tissues to BigET was taken as a measure of ECE-1 activity. EC50 of exogenous BigET was higher in arteries from DCM (274 nmol/L) patients compared to CAD (97 nmol/L), indicating elevated ECE-1 activity/expression in the latter group (Maguire and Davenport 1998).

Although ECE-1 expression is known to be elevated in coronary artery disease, its precise contribution to the disease process is unknown. Interestingly a previous study found a negative correlation between increasing vascular ECE-1 activity and systolic and diastolic BP, as well as LDL levels, while a positive correlation was found with fibrinogen (Ruschitzka et al. 2000). In this study, vascular ECE-1 activity was measured in the internal mammary

arteries of CAD patients. The rate of BigET to ET-1 conversion was taken as a measure of ECE-1 activity. The authors concluded that ECE-1 expression in the vasculature may modulate cardiovascular risk in patients with coronary artery disease (Ruschitzka et al. 2000). However, further studies are required to determine if the change in cardiovascular risk factors mentioned above such as blood pressure, LDL, and fibrinogen levels are the results of a feedback loop.

Increase in ECE-1 expression is also reported in idiopathic pulmonary fibrosis (Saleh et al. 1997). Elevated levels of ECE-1 was found in airway epithelium, as well as endothelial and inflammatory cells. Cell culture-based studies indicated that inflammatory cytokines such as TNFα increased the expression of ECE-1 mRNA and protein in normal bronchial epithelial cells (Saleh et al. 1997).

In addition to vasoconstriction, ET-1 is a known mitogen and thus has been implicated in the pathogenesis of human cancers including cancers of the colon, cervix, breast, and prostate. The role of the ET system in cancer progression, and as a therapeutic target in cancer has been the subject of other reviews and therefore will not be discussed here in detail (Smollich and Wulfing 2007). Elevated expression of ECE-1 in particular has been reported in prostate and breast cancer (Smollich et al. 2007; Rayhman et al. 2008). Overexpression of ECE-1 in cancer cells is known to occur through the alternative polyadenylation of the 3′untranstrated region of ECE-1 (Whyteside et al. 2014).

At present, there are no published studies on the effect of NO on ECE-1 expression in any disease setting. However, cell culture-based studies conducted by us and others have demonstrated the effect of NO on both membrane-bound (Raoch et al. 2011) and soluble forms of ECE-1 (Kuruppu et al. 2014a,b).

NO-mediated inhibition of cell surface ECE-1 expression

The effect of NO on cell surface ECE-1 expression has been examined using bovine aortic endothelial cells (Raoch et al. 2011). Treatment of these cells with the exogenous NO donors sodium nitroprusside (SNP) and diethylamine/nitric oxide (DEA-NO) reduced ECE-1 protein content and mRNA expression (Raoch et al. 2011). This effect appeared to be mediated via protein kinase G (PKG)-induced activation of cGMP, as evidenced by the use of KT5823 (a nonspecific PKG inhibitor), as well as transfection of cells with dominant negative PKG isoform (Raoch et al. 2011). The authors of this study report that activation of the PKG/cGMP pathway by exogenous NO, destabilizes the 3′-untransltaed region of the ECE-1, thus leading to a decrease in ECE-1 levels (Raoch et al. 2011).

Treatment with sodium nitroprusside (SNP) reduced ECE-1 protein content in lung tissue as well as circulating ET-1 levels in rats (Raoch et al. 2011). ECE-1 expression in lungs and aorta increased in eNOS-deficient mice compared to wild-type controls, and also in L-NAME-treated wild-type mice compared to respective control (Raoch et al. 2011). Together, these findings provide evidence that NO regulates ECE-1 expression via the soluble guanylyl cyclase/cGMP/PKG pathway.

We have shown that stimulation of Protein Kinase C (PKC) by phorbol esters such as phorbol-12-myristate-13-acetate (PMA) can induce the phosphorylation followed by subsequent trafficking of ECE-1 to the cell surface (Smith et al. 2006). Numerous studies have shown that NO can also stimulate PKC (Ping et al. 1999; Balafanova et al. 2002). This could be a potential mechanism offsetting the inhibitory effect of NO on cell surface ECE-1 expression, thus setting up a negative feedback loop.

NO-mediated inhibition of soluble ECE-1

We first reported on the presence of a soluble form of ECE-1 with catalytic activity in the media of the endothelial cell line Ea.hy926 (Kuruppu et al. 2007). This soluble form consists of the C-terminal extracellular domain and is a truncated version of the native membrane-bound form (Kuruppu et al. 2010). We later confirmed the presence of this soluble form in the cerebrospinal fluid of patients who have suffered subarachnoid hemorrhage (Kuruppu et al. 2014a,b). A circulating form of ECE-1 with catalytic activity can result in the production of ET-1 throughout the vasculature, thus having significant implications on vascular tone.

In subsequent studies, we examined the effect of exogenous NO on soluble ECE-1 production in Ea.hy926 cells (Kuruppu et al. 2014a,b). NO donor SNP inhibited the release of soluble ECE-1. This effect was mimicked by incubation of cells with L-arginine, the substrate for NOS (Kuruppu et al. 2014a,b). Furthermore, the presence of amino acids such as L-lysine, which compete with L-arginine for entry into cells, as well as the NOS inhibitor L-NAME, prevented the L-arginine-induced inhibition of soluble ECE-1 production (Kuruppu et al. 2014a,b). Our results indicated that endogenous NO produced by these cells prevented the secretion of ECE-1. In contrast, the level of ECE-1 expression in the cell membrane did not change in response to L-arginine or SNP (Kuruppu et al. 2014a,b). Therefore, according to studies conducted in our laboratory, both exogenous and endogenous NO can inhibit the release of soluble ECE-1. Furthermore, the inhibition of ECE-1 expression by exogenous NO donors has been confirmed both in vitro and in vivo (Raoch et al. 2011).

Possible mechanisms by which NO inhibits soluble ECE-1 production

At present, the precise mechanisms by which NO inhibits soluble ECE-1 is unknown. However, two mechanisms can be hypothesized taking into account the possible pathways for the production of soluble ECE-1, and the likely implications of NO on these pathways.

First, proteolytic cleavage at the cell surface can produce soluble ECE-1 (Fig. 2) (Kuruppu et al. 2007, 2010). A disintegrin and metalloprotease-17 (ADAM-17), has been implicated in the shedding of many cell surface proteins (Gooz 2010) including ECE-1. (Kuruppu et al. 2010). In addition to its role as an activator of PKC, PMA can also activate ADAM-17 (Althoff et al. 2000). Therefore, PMA-induced increase in soluble ECE-1 suggests a possible role for ADAM-17 in ECE-1 shedding (Kuruppu et al. 2010). ADAM-17 activity is known to be significantly reduced by exogenous NO donors. For example, exogenous NO donor S-nitroso-*N*-acetylpenicillamine significantly reduced ADAM-17 activity, as evidenced by a reduction in the levels of ADAM-17 substrates released into cell culture media (Bzowska et al. 2009). This is in agreement with our results which show that NO donor SNP can inhibit the production of soluble ECE-1 (Kuruppu et al. 2014a,b). Therefore, it appears likely that exogenous NO can reduce soluble ECE-1 production via the inhibition of ADAM-17 activity (Fig. 2). This should be confirmed in future studies by comparing the activity and expression of both ADAM-17 and ECE-1 in response to various NO donors.

Second, release from the cell surface via extracellular vesicles can also produce a soluble version of membrane-bound proteins (Stoeck et al. 2006). Our own studies indicate that soluble ECE-1 activity in cell culture media can be removed by ultracentrifugation, a process known to pellet extracellular vesicles including exosomes (Kuruppu et al. 2014a,b). Cleavage of ECE-1 within the vesicle itself (possibly by ADAM-17) can still produce a truncated version of the membrane-bound form. This has been reported in the case of CD23 which is sorted into exosomes in an ADAM10 dependant manner, and is cleaved within the exosome (Mathews et al. 2010). At present, the impact of NO on the production of extracellular vesicles (such as exosomes) is unknown.

ECE-1 is also known to be present in Weibel–Palade Bodies (WPB) which are granules specific to endothelial cells. Exocytosis of these granules is therefore likely to mediate the release of ECE-1 from endothelial cells. In line with this, studies conducted using nitric oxide synthease-2 knock outs have shown that NO can stabilize vessel wall, prevent endothelial activation by inhibiting the release of WPB (Qian et al. 2001). This further supports our data in cell culture models which show that

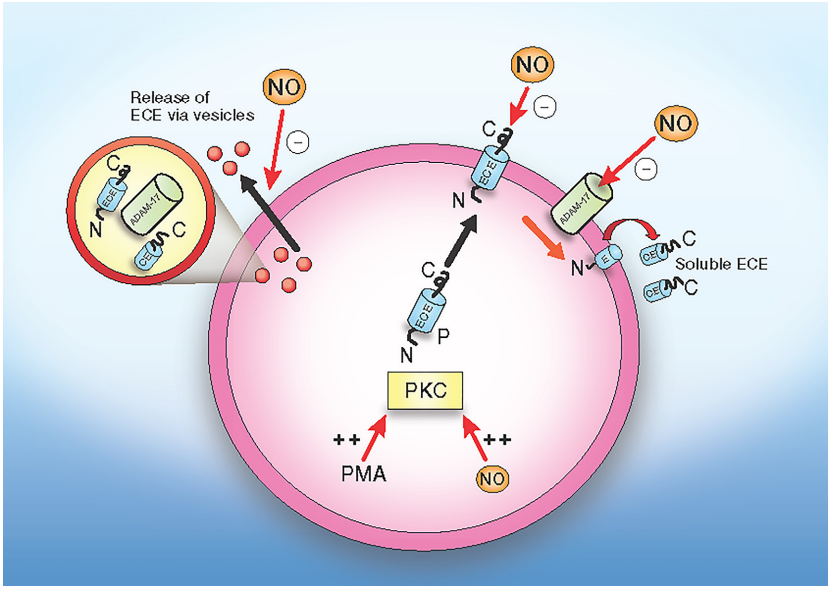

Figure 2. Possible mechanisms for the production of soluble ECE-1. PKC can be stimulated by both PMA and NO. This results in the phosphorylation and trafficking of ECE-1 to the cell surface. NO can inhibit the expression of ECE-1 on the cell surface thereby offsetting the effects of PKC stimulation. NO can inhibit the production of soluble ECE-1 via two possible mechanisms: (1) via the inhibition of ADAM-17-mediated cleavage of membrane-bound ECE-1 on the cell surface, or (2) by inhibiting the release of cellular vesicles that contain ECE-1. It is possible that the ADAM-17-mediated cleavage of ECE-1 occurs within these cells.

exogenous NO can inhibit the release of ECE (Kuruppu et al. 2014a,b).

Possible inhibition of vesicle release by NO could in turn reduce the production of soluble ECE-1 (Fig. 2). Future studies should aim to purify these vesicles which will facilitate the quantification ECE-1 levels as well as detailed studies on its structure. This will help confirm the mechanism proposed above.

It is logical to assume that long-term therapeutic use of NO donors in the setting of cardiovascular disease may have off target effects. NO-induced reduction in ECE-1 expression and shedding could in turn reduce ET-1 production. This can help enhance the vasodilator effects of NO, thereby further reducing blood pressure. Given the possible role of ET (and therefore ECE) in cancer progression, (Smollich et al. 2007; Lambert et al. 2008; Smollich and Wulfing 2008; Hong et al. 2011), inhibition of ECE-1 by NO may lead to beneficial effects in the setting of cancer.

In conclusion, ET-1 production occurs extracellularly. A rate-limiting step in the production of ET-1 is the expression and localization of ECE-1. Research conducted by us and others indicate that NO can inhibit the production of soluble ECE-1 as well as the expression of ECE-1 on the cell surface. Therefore, inhibition of ECE-1 expression can be one mechanism by which NO inhibits ET-1 production. ECE-1 plays a significant role in the pathogenesis of cardiovascular and neurodegenerative disease, as well as cancer. In this context, a vasoactive factor such as NO can be a potential tool for the therapeutic manipulation of ECE-1 expression. Understanding the specific mechanism(s) by which NO regulates ECE-1 would facilitate the translation of this finding to the clinic while adding new knowledge to the field of endothelial biology.

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