

Endothelins

Myocardial Actions of a New Class of Cytokines

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It has long been assumed that the primary influences regulating cardiac contractility are the extent of mechanical loading of muscle fibers and the activity of the autonomic nervous system. However, the vascular endothelium within the large epicardial and penetrating coronary arteries is well known to release vasodilator and vasoconstrictor agents that regulate coronary blood flow and, by this mechanism, indirectly affect cardiac contractility. In response to a number of hormonal, metabolic, and mechanical stimuli, vascular endothelium of the coronary arteries has been shown to synthesize and secrete compounds of disparate biological activity, including mitogens, prothrombogens, antithrombogens, and vasoactive substances. However, there is growing evidence that locally elaborated paracrine factors, perhaps derived from the microvascular endothelium or the endocardium, also are important in modifying cardiac function. Within the heart, the role of the endocardial endothelium in normal cardiac physiology has been examined in recent reports by Brutsaert and colleagues^{1,2} documenting that in vitro this endothelium regulates the inotropic response of subjacent cardiac myofibrils to changes in extracellular Ca^{2+} .

Among the biologically active autocoids released by vascular endothelium are a recently described group of peptide autocoids or cytokines known as endothelins. Vascular endothelial cells have been known since the mid 1980s to elaborate a vasoconstrictor activity, as well as vasodilators such as endothelium-derived relaxing factor and prostacyclins.³⁻⁶ However, it was not until 1988 that Yanagisawa and colleagues,⁷ working in Tomoh Masaki's laboratory,

reported the isolation of a potent 21-amino acid vasoconstrictor peptide from the supernatant of cultured porcine endothelial cells, which they termed "endothelin." At least three isoforms of endothelin have since been described, each with 21 amino acids and four cysteine residues forming two disulfide bonds (Figure 1).⁸ In addition to the three isoforms of endothelin described by Masaki and colleagues, a peptide autocoid recently identified in the murine intestinal tract termed vasoactive intestinal contractor (VIC) differs from endothelin-2 by a single amino acid substitution.⁹⁻¹¹ Although VIC may possibly represent a fourth isoform, a recent report suggests that VIC is the murine (and rat) form of endothelin-2 because of the identical sizes of the human and rat mRNAs for the precursor form of endothelin-2 known as preproendothelin-2, which differ markedly from the larger mRNAs for preproendothelin-1 and preproendothelin-3.¹² The different endothelin isoforms are presumed to have arisen from a common ancestor initially by exon duplication followed by gene duplication with dispersion of the three known isoform genes among three different chromosomes (at least in humans). Interestingly, the venom of the burrowing asp *Atractaspis engaddensis* contains peptides termed "sarafotoxins" that were identified coincidentally with the original description of endothelin-1 and were noted to have remarkable homology to the endothelins (Figure 1).¹³

Since the original description of endothelin, it has been found to be a nearly ubiquitous peptide autocoid released from many endothelial cells, including porcine aortic,^{4,14-17} bovine pulmonary,¹⁸ bovine carotid,^{19,20} bovine adrenal cortical capillary,²¹ and human umbilical vein²² and human mesenteric artery²³ endothelial cells. Endothelin has also been identified in numerous nonendothelial tissue sources, including several renal cell lines,^{24,25} canine airway epithelial cells,²⁶ and rabbit endometrial cells in primary culture.²⁷ Using immunohistochemical and in situ hybridization techniques, it has also been found in relatively high levels in the central and peripheral nervous systems, where its role has yet to be determined. In addition to their paracrine actions, endothelins may also act as autocrine peptide regulatory factors, as in the recent description of endothelin-1's

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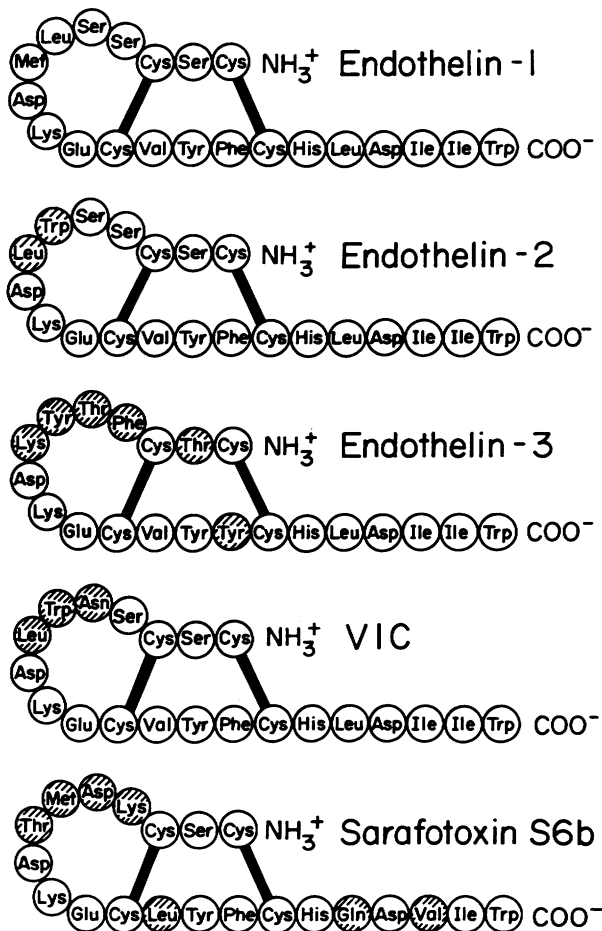


FIGURE 1. Illustration of amino acid sequences of endothelin-1, endothelin-2, vasoactive intestinal constrictor (VIC, possibly the murine/rat analogue of endothelin-2), and endothelin-3, as well as one of the peptide constituents of the venom of the burrowing asp *Atractaspis engaddensis*, termed sarafotoxins, which, along with other members of this peptide family, have a remarkable degree of homology to the endothelins. Specific toxin illustrated is sarafotoxin S6b.

action in normal parathyroid tissue³¹ in a number of transformed cell lines^{28–30} and in normal parathyroid tissue.³¹

Given the widespread distribution of endothelins throughout many different tissues, their expression during ontogeny and into adult life, their primary role as intercellular signaling factors, and the apparent complexity of their effects including promoting proliferation or cellular hypertrophy, the appellation “cytokine” is appropriate to describe this class of autocrine/paracrine peptide signaling factors. As recently reviewed by Nathan and Sporn,³² most cytokines arise from apparently unrelated cell types with often unpredictable effects on an untested tissue or cell type. They argue that the actions of cytokines on a given cell at a given point in development is determined largely by the context, including the specific cell type involved, the composition of the extracellular matrix, and the mix of other peptide

regulatory factors also present in the intercellular milieu, several of which may have partially redundant actions.³² Although not included in their review, the local myocardial and intravascular release of endothelins, and perhaps also of angiotensins, qualifies both classes of peptides as cytokines as well. An important conclusion drawn by Nathan and Sporn, based on their argument that the actions of cytokines (i.e., interleukins, interferons, colony-stimulating factors, peptide growth factors—and perhaps endothelins and angiotensins) are “contextual,” is that much of the work examining the effects of a specific cytokine on individual cells or isolated cell populations in vitro may be misleading, “leading to the description of countless, often contradictory bioactivities.”³² Although ultimately the physiological or pathophysiological role of a given cytokine must be determined in the intact organism, possibly by using soluble cytokine receptor complexes, specific receptor antagonists, or other techniques, in vitro modeling of cytokine effects must address the biological complexity of the relevant intercellular milieu. Although most of the work that we and others have done on the intramyocardial actions of endothelins has concentrated on primary cultures of neonatal myocytes or on individual adult ventricular myocytes, in vitro systems are being developed that more accurately model the context of endothelin’s actions, including primary cocultures of cardiac myocytes with selected nonmyocyte cells such as microvascular endothelium and the use of extracellular matrixes that more closely reflect those found in the cardiac interstitium.

Coronary Flow, Endothelial Cells, and Myocardial Function

Shortly after endothelin had been identified as an endothelium-derived vasoconstrictor, Masaki and colleagues³³ also reported that endothelin-1 had a potent inotropic activity in guinea pig atrial strips. Subsequently, high-affinity receptors were identified in mammalian atria and ventricles,^{34–36} and endothelin was also found to act as a potent secretagogue for atrial natriuretic peptide.^{37–43} A number of laboratories have now confirmed these original reports, documenting endothelin to be one of the most potent inotropic agents yet described in mammalian myocardium.

The relevant site of origin of intracardiac endothelins is unknown. However, the work of Brutsaert and colleagues^{44,45} suggests that release of endothelin or other cardiotropic autocrine by the endocardial endothelium may be of physiological or pathophysiological importance in the regulation of myocardial function. In addition, several lines of evidence point to an appreciable role for coronary artery endothelium, including microvascular endothelium, in modifying cardiac contractile state. For example, the importance of coronary flow rate per se as a determinant of cardiac contractile state was originally identified by the cardiac physiologist Gregg, as detailed in a comprehensive review by Feigl.⁴⁶ This concept has since been a source of continuing con-

trovery.^{47,48} By carefully demonstrating that the relation between coronary flow rates and cardiac contractility was not due to relative myocardial ischemia at low flow rates and that there was no evidence for increased sarcomere length at high flow rates (the "garden hose" effect), Kitakaze and Marban⁴⁸ recently excluded two trivial explanations often raised to explain the somewhat counterintuitive notion that changing coronary flow rate per se modifies cardiac contractile state. One remaining explanation is the possibility that the coronary microvasculature senses the changes in flow rate and releases paracrine-acting mediators that alter the contractile state of the myocardium.

Further evidence for the possible control of myocardial function by endothelium has come from pharmacological studies of peptide cytokines and other autocoids of presumed endothelial origin such as angiotensin II and endothelin. The role of angiotensin II in modifying cardiac function is being reexamined with the recent detection by biochemical, immunological, and *in situ* molecular biology techniques of components of the renin-angiotensin system within the heart (i.e., renin, angiotensinogen, and both angiotensin converting enzyme (ACE) and the recently described angiotensin I-specific, neutral serine protease⁴⁹). At present, however, the role of the intracardiac renin-angiotensin system in normal and abnormal physiology remains unclear.⁵⁰

Evidence from *in vitro* studies indicates that transcriptional regulation of preproendothelin messenger RNA may occur by a dynamic interaction between cardiac myocytes and adjacent endothelial cells. In preliminary data from this laboratory, preproendothelin mRNA was markedly induced *in vitro* in isolated primary cultures of microvascular endothelial cells, isolated from adult rat myocardium only when these cells were cocultured with adult rat ventricular myocytes ($n=3$). This differs from the constitutive production of preproendothelin mRNA observed in large-vessel (bovine aortic) endothelial cells.⁵¹ In contrast, no preproendothelin mRNA was detected in cardiac microvascular endothelial cells cultured alone (i.e., in monoculture) under similar conditions, although cardiac myocyte-conditioned media could increase endothelin precursor mRNA in monocultures of these microvascular endothelial cells.

Mechanism of Positive Inotropic Effect of Endothelin-1

The positive inotropic effect of endothelin has been documented in isolated hearts,^{52,53} in isolated cardiac muscle preparations,^{33,54-62} and in isolated adult rat and rabbit ventricular cells.^{63,64} As with the vasoconstrictor effects of the peptide, calcium channel blockers shift the dose-response curve to the right, and a variety of other neurohumoral and autocoid antagonists have no effect.^{33,63} Similarly, the kinetics of endothelin-1's action on heart muscle resemble those in vascular smooth muscle, with a delayed onset of action and a maximal effect at 5-8

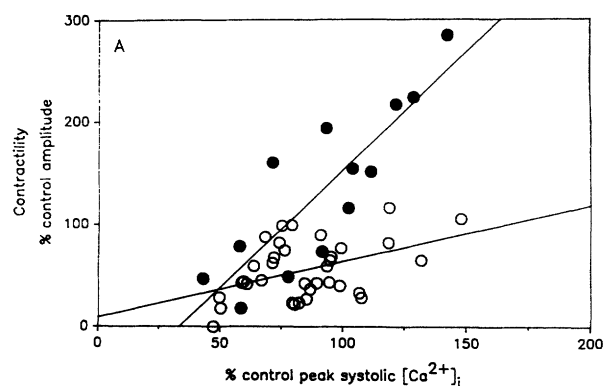


FIGURE 2. Graph shows values for contractile amplitude and peak systolic intracellular calcium activity $[Ca^{2+}]_i$ given for six cells loaded with the Ca^{2+} -selective fluorescent probe fura-2. Cells were initially exposed to 0.9 mM external Ca^{2+} in physiological buffer and were then gradually stepped down in five successive increments to 0.1 mM Ca^{2+} followed by a stepwise return back up to 0.9 mM extracellular Ca^{2+} , with sufficient time at each step to allow contractile amplitude and $[Ca^{2+}]_i$ to stabilize. Cells were exposed to this protocol in the absence (open circles) or presence (closed circles) of 100 pM endothelin-1. Note that for any given level of systolic $[Ca^{2+}]_i$, expressed as a percentage of initial control values for systolic $[Ca^{2+}]_i$ at 0.9 mM Ca^{2+} outside, contractile amplitude was always higher in endothelin-treated cells. (From Reference 63, with permission.)

minutes in isolated cell preparations. The positive inotropic effect is quite prolonged, lasting 5-15 minutes after washout of the peptide.

In addition to the high potency, delayed onset of action, and prolonged inotropic effect of endothelin noted above, there is another unusual characteristic of endothelin's mechanism of action as a positive inotropic agent. We reported that in isolated adult rat ventricular myocytes paced at 1.5 Hz, endothelin-1 at concentrations below 1 nM yielded a maximal inotropic response of about 100% above baseline.⁶³ However, despite this appreciable increase in contractile amplitude, we could not detect an increase in cytosolic calcium as reported by fluorescence ratio measurements by using the calcium-specific intracellular probe fura-2.⁶³ Endothelin thus appeared to be acting to sensitize the myofilaments to calcium, at least in isolated adult rat ventricular myocytes. In contrast, Lauer et al⁶⁴ did find an increase in calcium transients and transmembrane calcium current in isolated rabbit cells at 20 nM endothelin, although contractility was not measured. We also noted a variable increase in cytosolic calcium in isolated rat cardiocytes at concentrations of endothelin in the 10-100 nM range, but this was well above the concentration eliciting a maximal inotropic effect of the peptide (i.e., 0.5-1.0 nM).⁶³ As shown in Figure 2, when the calcium concentration of the buffer superfusing isolated single fura-2-loaded myocytes paced at 1.5 Hz was varied from 0.1 to 0.9 mM Ca^{2+} outside, 100 pM endothelin increased contractile amplitude

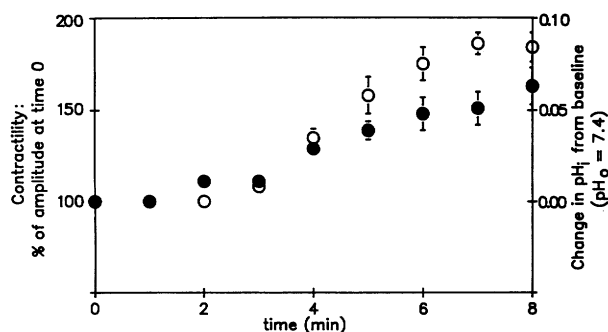


FIGURE 3. Plot depicts time course of changes in pH_i and contractile amplitude with endothelin. Freshly isolated ventricular myocytes superfused with physiological buffer at pH 7.4, loaded with the pH-sensitive dye 2',7'-bis(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF) and stimulated at 1.5 Hz, were exposed to 100 pM endothelin-1 at time 0. A change in contractile amplitude (open symbols) or pH_i (closed symbols) was not apparent for about 4 minutes ($p < 0.05$ at 4 minutes; $n = 6$), with a maximal effect at 7 minutes. (From Reference 72, with permission.)

with little or no change in the systolic level of Ca^{2+} transients as monitored concurrently in each paced myocyte by changes in the fura-2 fluorescence ratio.⁶³

Prior exposure of isolated myocytes to pertussis toxin completely abolished the positive inotropic effect of the peptide,⁶³ implicating either a G_o or G_i GTP binding protein in the signal transduction steps after endothelin's binding to its sarcolemmal receptor. The intermediary role of GTP-binding proteins is reflected in the deduced amino acid sequences of the three endothelin receptors cloned to date.⁶⁵⁻⁶⁷

In contrast to our findings in freshly isolated adult rat ventricular myocytes, pertussis toxin had no effect on the endothelin-induced rise in inositol trisphosphate (EC_{50} , 10 nM) in isolated adult rat atrial myocytes.⁶⁸ Significant stimulation of phospholipase C resulting in phosphoinositide hydrolysis, by either pertussis toxin- or cholera toxin-sensitive mechanisms, has been documented as well in neonatal ventricular myocytes, but again using concentrations of endothelins and sarafotoxins (1 μ M) well above that which resulted in maximal increases in contractility in isolated adult ventricular myocytes.⁶⁹

A rise in intracellular pH (pH_i) has been well documented to increase contractile amplitude with little or no change in $[Ca^{2+}]_i$ ^{70,71}; therefore, we also investigated whether this apparent sensitization of the myofilaments to intracellular calcium with endothelin could be attributed to a rise in pH_i . As shown in Figure 3, 100 pM endothelin increased pH_i by 0.07 ± 0.02 units ($n = 8$) and contractile amplitude to $190 \pm 26\%$ of baseline, whereas 1 nM endothelin increased pH_i by 0.13 ± 0.03 units with little further increase in contractility.⁷² Amiloride, an inhibitor of sarcolemmal Na^+-H^+ exchange that had no effect on baseline contractile amplitude in isolated adult rat myocytes, completely prevented the intracellular alkalization response to endothelin and blunted the

inotropic effect. Nonspecific inhibitors of protein kinase C, such as H-7 and sphingosine, also diminished or abolished the rise in pH_i with endothelin.⁷² However, neither amiloride nor inhibitors of protein kinase C could completely suppress the positive inotropic response. Conversely, pretreatment with pertussis toxin, which as mentioned above had been documented to abolish the inotropic response to endothelin,⁶³ only partially diminished the intracellular alkalization in isolated myocytes in response to endothelin-1.⁷² Intracellular alkalization as a consequence of stimulation of the sarcolemmal Na^+-H^+ antiporter has also been implicated recently in the positive inotropic action of the α -adrenergic agonist phenylephrine. As in the case of endothelin, an amiloride congener reduced the maximal phenylephrine-mediated increase in myocardial contractility by about 50%.⁷³

Endothelin's Role in Cardiac Myocyte Cell Biology and Function

Intracellular alkalization could also have other roles in normal myocyte physiology aside from the effects on contractility described above. A rise in pH_i is clearly associated with a mitogenic response in many cells and with hypertrophy in vascular smooth muscle.⁷⁴⁻⁷⁸ In addition, endothelin has been shown to have mitogenic effects in vascular smooth muscle cells⁷⁹ as well as in other cell types.⁸⁰⁻⁸⁴ Importantly, the mitogenic effects of endothelin and its effects on the expression of certain proto-oncogenes such as *c-fos* and *c-jun*, both of which are associated with some forms of hypertrophic growth in cardiac myocytes,⁸⁵ occur at relatively low concentrations of endothelin (i.e., <1 nM), concentrations that also result in maximal increases in contractile amplitude and pH_i in isolated adult rat ventricular myocytes.^{63,72} Shubeita et al⁸⁶ have recently demonstrated that endothelin can cause hypertrophy directly in serum-starved neonatal rat ventricular myocytes, as determined by increases in cell size and by the assembly of myosin light chain-2 and other myofibrillar proteins. This is accompanied by the activation of "immediate early" gene expression (e.g., *c-fos* and *erg-1*) and by the expression of atrial natriuretic peptide, with a maximal response at 1 nM.⁸⁶ Activation of phospholipase C by endothelin has been well documented in both established cell lines and primary cultures of vascular smooth muscle cells,⁸⁷ endothelial cells,⁸¹ and glial cells.⁸⁸

The response of a variety of cell lines and primary cultures pretreated with pertussis toxin and then exposed to endothelin also implicates complex and perhaps tissue-specific signal transduction mechanisms for this peptide, comprising several independently regulated pathways. A dissociation between phospholipase C and phospholipase A_2 activation in vascular smooth muscle cells after endothelin administration has been noted, based on their response to pertussis toxin and phorbol esters.⁸⁹ As noted above for atrial and neonatal rat myocytes, however, these

effects have been observed only at higher concentrations, typically with an EC_{50} of 10 nM or higher; for example, a large increase in phospholipase A_2 activation with endothelin was seen in mesangial cells, but only at concentrations near 0.1 μ M.⁸³ However, Shubeita et al⁸⁶ have demonstrated in neonatal rat myocyte cultures maximal production of diacylglycerol and phosphoinositides at 10 nM with an EC_{50} of about 0.5 nM.

Although it is quite possible that different actions of the endothelin peptides on ventricular myocytes may be manifest at concentrations below and above 1 nM, it is unknown what local tissue concentrations of endothelin are relevant. The reported K_d for receptor binding of endothelin-1 in cardiac tissue is in the subnanomolar range,^{34–36} but higher concentrations could occur locally, especially under pathological conditions. Interestingly, plasma endothelin levels were found to double in dogs with congestive heart failure induced by rapid ventricular pacing.⁹⁰ It is unclear whether the elevated plasma levels of endothelin-1 were contributing to an elevated systemic vascular resistance or simply reflecting the activation of a number of neurohumoral and paracrine regulatory systems in advanced heart failure. The answers to these questions will require the development of specific receptor antagonists. Nevertheless, the data reported by Shubeita et al⁸⁶ and evidence from our own laboratory^{63,72} indicate that endothelin, in amounts well below 1 nM, will result in increased contractile function and an intracellular alkalinization coupled with increased transcription of selected genes that could facilitate the development of myocyte hypertrophy.

Summary

There is growing evidence to support the existence of a dynamic interaction in vivo between cardiac myocytes and adjacent microvascular endothelial cells in the regulation of both cardiac myocyte and possibly endothelial cell phenotype and function. Endothelins may be only one of several endogenous cytokines or autoids that are released by the cardiac microvascular and/or endocardial endothelium and transported vectorially to adjacent myocytes that could modify cardiac contractile state, perhaps in response to changes in microvascular blood flow. Similarly, cardiac myocytes themselves could release cytokines that could directly affect endothelial cell proliferation or angiogenesis and indirectly elicit or modify the release of endothelium-derived cytokines and autoids. Thus, in addition to modifying function, endothelial cell–cardiac myocyte interactions may also be of importance in the dynamic events that lead to myocardial wall remodeling and angiogenesis during hypertrophic growth and in the response to cardiac injury.

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KEY WORDS • inotropic agent • vasoconstrictor • endothelium • myocardium • contractility • cytokine • Point of View