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Bioactive Signaling in Next-Generation Pharmacotherapies for Heart Failure
A Review

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IMPORTANCE The standard pharmacotherapy for heart failure (HF), particularly HF with reduced ejection fraction (HFrEF), is primarily through the use of receptor antagonists, notably inhibition of the renin-angiotensin system by either angiotensin-converting enzyme inhibition or angiotensin II receptor blockade (ARB). However, the completed Prospective Comparison of ARNI With an ACE-Inhibitor to Determine Impact on Global Mortality and Morbidity in Heart Failure (PARADIGM-HF) trial identified that the use of a single molecule (sacubitril/valsartan), which is an ARB and the neutral endopeptidase inhibitor (NEPi) neprilysin, yielded improved clinical outcomes in HFrEF compared with angiotensin-converting enzyme inhibition alone.

OBSERVATIONS This review examined specific bioactive signaling pathways that would be potentiated by NEPi and how these would affect key cardiovascular processes relevant to HFrEF. It also addressed potential additive/synergistic effects of ARB. A number of biological signaling pathways that may be potentiated by sacubitril/valsartan were identified, including some novel candidate molecules, which will act in a synergistic manner to favorably alter the natural history of HFrEF.

CONCLUSIONS AND RELEVANCE This review identified that activation rather than inhibition of specific receptor pathways provided favorable cardiovascular effects that cannot be achieved by renin-angiotensin system inhibition alone. Thus, an entirely new avenue of translational and clinical research lies ahead in which HF pharmacotherapies will move beyond receptor antagonist strategies.

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Heart failure (HF) is a leading cause of morbidity and mortality, and a large proportion of patients present with a reduced left ventricular (LV) ejection fraction less than 50%, termed *HF with reduced ejection fraction* (HFrEF). However, effective HFrEF pharmacotherapies appear to have plateaued,[1-4] and most of these therapeutic strategies involve receptor inhibition. A notable exception was the use of a single molecule with a duality of function, sacubitril/valsartan, which combines the neutral endopeptidase inhibitor (NEPi) neprilysin with an angiotensin II receptor blocker (ARB).[5,6] In a large study of patients with HFrEF, the Prospective Comparison of ARNI With an ACE-Inhibitor to Determine Impact on Global Mortality and Morbidity in Heart Failure (PARADIGM-HF) trial[6] reported a significant decrease in a composite end point of death from cardiovascular cause or hospitalization for HFrEF with sacubitril/valsartan compared with the angiotensin-converting enzyme (ACE) inhibitor enalapril. These results provide the basis for generating a guiding hypothesis that next-generation pharmacotherapies will be multifunctional, in which receptor inhibition is coupled with potentiation of other biological signaling pathways, and act in a synergistic manner to favorably alter the natural history of HFrEF.

The goals of this review are several. First, we examine some of the better-characterized bioactive molecules potentially relevant to HFrEF. Second, we introduce some additional bioactive signaling pathways that have a strong likelihood of relevance to the pathophysiology of HFrEF. Third, while more exploratory in nature, some more novel bioactive molecules of potential relevance to cardiovascular performance and NEPi are examined. Fourth, based on the level of scientific evidence provided, we present new directions and questions for both translational and clinical research.

Sacubitril/Valsartan Structure and Cardiovascular Physiology

Sacubitril/valsartan is a single molecule that provides both NEPi as well as ARB and thus is multifunctional and is commonly known as a dual-acting angiotensin receptor neprilysin inhibitor (**Figure**, A).[5] The ARB contained within sacubitril/valsartan is structurally that of valsartan, which has been shown to reduce cardiovascular outcomes in HFrEF.[7] However, the results of the PARADIGM-HF trial[6]

suggest that the second function of sacubitril/valsartan, that of NEPi, yielded additional favorable effects. Neutral endopeptidase, a zinc metalloendopeptidase, degrades peptides that regulate the cardiovascular, nervous, inflammatory, and immune systems.[8,9]

Left Ventricular Afterload

Atrial natriuretic peptides (ANPs) and brain natriuretic peptides (BNPs) reduce LV afterload by regulating volume status and opposing angiotensin II–mediated vasoconstriction.[10-12] In addition, C-type natriuretic peptides (CNPs) regulate vascular tone and attenuates adverse vascular remodeling.[12] Collectively, these actions reduce blood pressure through favorable effects on arterial stiffness and resistance, key determinants of LV afterload, which in turn would be favorable in HFrEF.[13] In clinical studies, NEPi elevates plasma ANP levels and natriuresis with a corresponding reduction in blood pressure.[13,14] However, while increased ANP levels and subsequent natriuresis with NEPi occur,[14] these effects appear to be transient.[15-19] Using the NEPi candoxatril, an initial but nonsustained reduction in blood pressure was observed.[18] A potential explanation for this observation is that NEP also degrades angiotensin II; therefore, with long-term NEPi use, the potentiation by angiotensin II would create an opposing vasopressor effect.[19] Thus, with sacubitril/valsartan, the increased levels of angiotensin II that may occur with long-term NEPi use are offset by the ARB component of this molecule (Figure, A). Neutral endopeptidase inhibitor use has been shown to favorably alter vascular extracellular matrix structure and medial thickness in preclinical models of hypertension.[20] Elevated levels of angiotensin II in HFrEF bind to the angiotensin II type 1 receptors within the smooth muscle vasculature and cause vasoconstriction[21]; with prolonged receptor activation, elevated levels can cause adverse vascular remodeling.[22,23] Angiotensin II receptor blockers counteract these effects by reducing blood pressure[24,25] and improving indices of arterial stiffness.[26] In clinical studies with sacubitril/valsartan, a sustained reduction in blood pressure and arterial stiffness in patients with hypertension was observed.[27-29] Thus, sacubitril/valsartan is likely to produce positive effects on LV afterload through both NEPi and ARB effects, both by initially reducing smooth muscle vascular tone and circulating volumes and also by reducing and reversing adverse vascular remodeling over time.

Fibrotic Pathways and Structure

While the factors that drive the fibrotic process are multifactorial, a key profibrotic signaling molecule is transforming growth factor β (TGF-β), which can be regulated by natriuretic peptides. Increased BNP levels facilitate degradation of extracellular matrix (collagen) accumulation and dampens the expression of TGF-β.[12,30] In rodent models, with a loss of BNP signaling, blood pressure–independent cardiac fibrosis and hypertrophy occurred, presumably driven in part through increased TGF-β levels.[31,32] Further, CNP infusion in animals attenuated fibrosis and hypertrophy.[33] Treatment with a NEPi, which likely potentiated local BNP/CNP levels, reduced the magnitude of LV adverse remodeling in animal models of hypertension and myocardial infarction.[34,35] The increased levels of angiotensin II with developing HFrEF would cause abnormal matrix synthesis and degradation within the myocardium and vasculature through complementary pathways. First, increased LV afterload induced by angiotensin II will induce a mechanical stimulus and cause activation and proliferation of fibroblasts.[36,37] Second, direct activation of the angiotensin II type 1 receptors will cause increased matrix synthesis. In addition, stimulation of the angiotensin II type 1 receptor induces the expression of TGF-β and thus amplifies the profibrotic effects of angiotensin II. In animal models,[38,39] sacubitril/valsartan was shown to reduce cardiac fibrosis through the effects of both NEPi and ARB.[40] The antifibrotic effects of sacubitril/valsartan are likely realized through the attenuation of TGF-β by increasing local natriuretic peptide levels as well as interference of angiotensin II–mediated signaling.

Key Points

Question Has the time arrived for new heart failure therapeutic pathways over and above receptor antagonism to be considered?

Findings This review identified that regulating angiotensin II receptor activation as well as activation of specific receptor pathways can provide favorable cardiovascular effects in the context of heart failure, which cannot be achieved by receptor inhibition alone.

Meaning The positive results of the PARADIGM-HF trial using a drug with both neutral endopeptidase and angiotensin II receptor inhibition properties heralds a shift in conventional concepts from solely using receptor inhibition in heart failure towards simultaneously potentiating novel signaling pathways.

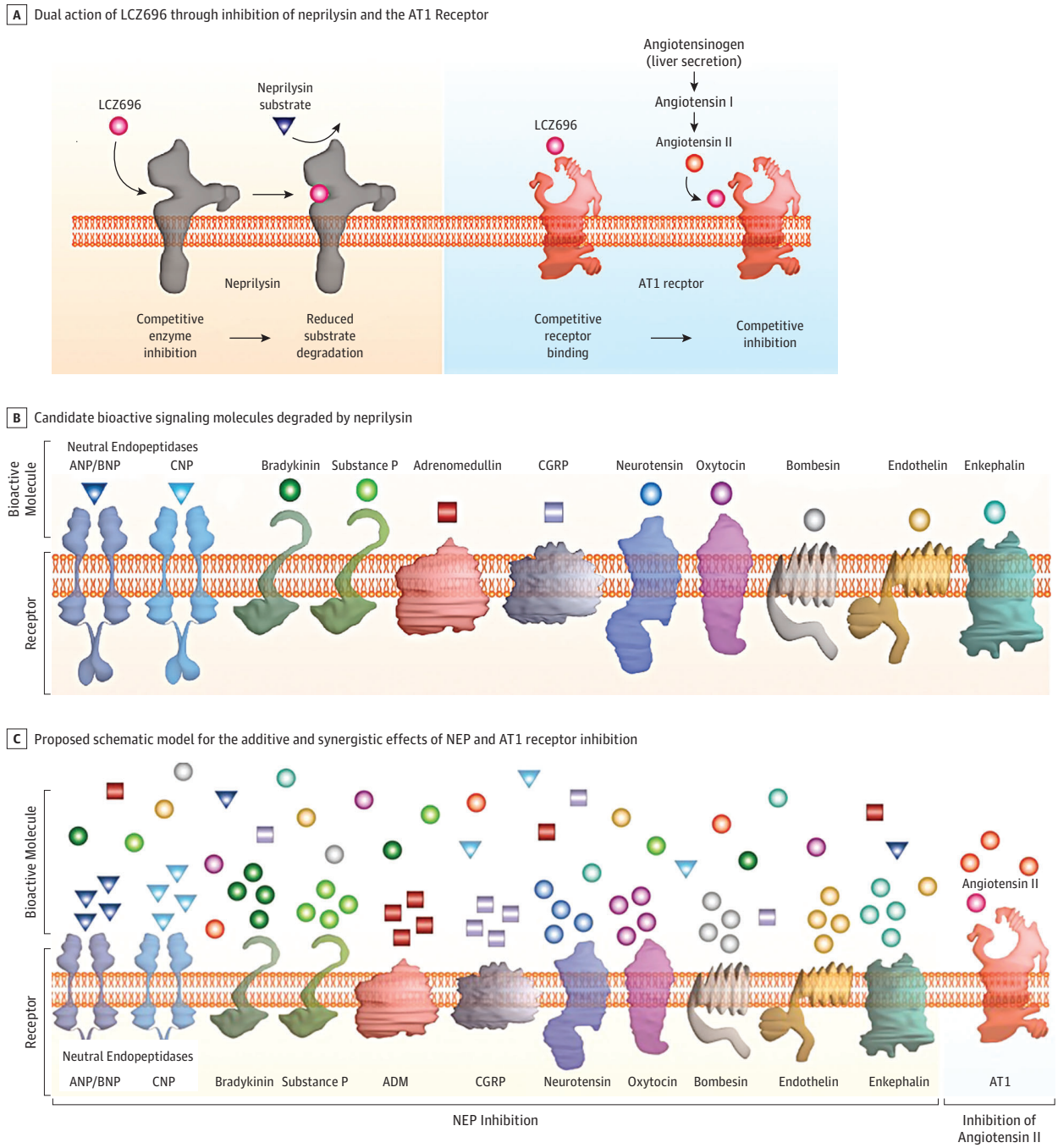
Cardiorenal Function

In clinical studies, ARBs attenuate the development of renal dysfunction in cardiovascular disease, essential hypertension, and diabetes.[41-43] Atrial natriuretic peptides also dampen the release of renin, causing sodium and water excretion.[13,30] For example, ANP potentiation in an animal model of HFrEF improved the natriuretic response.[44] Additional preclinical studies suggest that NEPi may enhance renal function in mild HFrEF and provide protection during acute renal failure.[45,46] Analysis of PARADIGM-HF trial data[47] determined that sacubitril/valsartan slows renal decline in patients with HFrEF. The ongoing United Kingdom Heart and Renal Protection trial[48] evaluating sacubitril/valsartan vs the ARB irbesartan in patients with chronic kidney disease may further elucidate operative mechanisms influencing renal physiology.

Sympathetic Adrenergic Activity

Chronically elevated sympathetic activity in HFrEF contributes to the deterioration of systolic performance, arrhythmias, and sudden cardiac death.[49,50] Angiotensin II receptor blockers reduce levels of catecholamines and central sympathetic outflow in HFrEF.[51,52] Preclinical models using BNPs and CNPs reduced heart rate via enhanced vagal input.[53,54] Moreover, transgenic mice lacking natriuretic peptide receptors demonstrate elevated heart rates and increased frequency of sinus arrhythmias.[55] Seemingly contradictory, a clinical study using healthy patients reported that NEPi increased plasma epinephrine levels.[56] However, these patients were unable to trigger a reflexive tachycardia, suggesting NEPi also inhibited sympathetic pathways. Thus, while further study is necessary, the above data suggest sacubitril/valsartan may dampen sympathetic activity.

Figure. Influence of Dual Actions of Sacubitril/Valsartan on Multiple Biological Pathways and on Cardiovascular Function and Structure



A, The dual actions of sacubitril/valsartan are shown and identify that the inhibition of neprilysin, a neutral endopeptidase (NEP), will increase local bioactive molecule concentrations through slowing of the degradation process. The potentiation of these biomolecules is accompanied by simultaneous angiotensin II type 1 (AT1) receptor inhibition. B, Candidate bioactive molecules that are substrates for neprilysin are shown and are paired with illustrations of the cognate transmembrane receptors. A number of these pathways are attenuated in heart failure with reduced ejection fraction, presumably in part by enhanced NEP-mediated degradation. C, Proposed schematic model for the additive and synergistic effects of combined NEP and AT1 receptor inhibition in the context of heart failure with reduced ejection fraction. While an oversimplification, the generalized effects of NEP inhibition would be to increase local bioactive molecule concentrations by reducing the rate of degradation, which in turn would amplify respective signaling activity and, ultimately, cardiovascular effects. In turn, sacubitril/valsartan provides for potent inhibition of the AT1 receptor. The summation of these effects would yield important changes within the cardiovascular system and, in turn, provide a mechanistic basis for the effects of sacubitril/valsartan in the context of heart failure with reduced ejection fraction. Neutral endopeptidase inhibition potentiates multiple bioactive molecules and signaling. Combined with AT1 inhibition, this leads to improved left ventricular loading conditions and vascular structure, favorable changes in myocardial structure and viability, and neurovascular signaling with chronotropic and inotropic effects. ANP indicates atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; CGRP, calcitonin gene-related peptide.

Table 1. Neprilysin Substrates in the Cardiovascular System and Potential Effects in Heart Failure With Reduced Ejection Fraction (HFrEF)

Neprilysin Substrate	Biological Activity Relevant to Cardiovascular System	Proposed Effects of LCZ696	Potential Effects in HFrEF
ANP/BNP[12,13,18,19,30-32,53,67,68]	Vasodilation; reduce arterial stiffness; promote natriuresis and diuresis; and promote antifibrotic pathways	Increased	Reduce LV afterload; attenuate endothelial dysfunction; attenuate cardiac remodeling; and attenuate renal dysfunction
CNP[11,12,33,64,69]	Vasodilation; inhibit smooth muscle proliferation; and promote antifibrotic pathways	Increased	Reduce LV afterload; attenuate endothelial dysfunction; and attenuate vascular and cardiac remodeling
Bradykinin[70-81]	Vasodilation; inhibit collagen deposition; and modulate ischemic preconditioning	Increased	Reduce LV afterload; and attenuate cardiac remodeling
Substance P[76,81-86]	Vasodilation; promote profibrotic pathways; upregulate inflammation; and modulate ischemic preconditioning	Increased	Reduce LV afterload; and alter cardiac remodeling
Adrenomedullin[67,81,87-94]	Vasodilation; reduce arterial stiffness; promote natriuresis and diuresis; and promote positive inotropic effects on the myocardium	Increased by indirect mechanism	Reduce LV afterload; promote hemodynamic regulation; increase demand on the myocardium; and attenuate renal dysfunction
CGRP[95]	Vasodilation	Increased	Reduce LV afterload
Endothelin[11,68,69]	Vasoconstriction and/or vasodilation; and simulate collagen synthesis and myocardial cell growth	Increased by indirect mechanism	Alter LV afterload; and alter cardiac remodeling
Bombesin[96-98]	Vasoconstriction; and increase sympathetic nervous activity	Increased	Increase LV afterload; increase demand on the myocardium; and alter cardiac remodeling
Neurotensin[99-110]	Promote species-dependent effects on blood pressure and coronary vascular tone; and increase heart rate and contractility	Increased	Promote species-dependent effects on LV afterload and ischemic event outcomes; and increase demand on the myocardium
Enkephalin[111-118]	Vasoconstriction; and modulate ischemic preconditioning	Increased	Alter LV afterload
Oxytocin[119-124]	Vasodilation; promote cell viability after ischemic or reperfusion injury; promote antifibrotic pathways; and oppose myocardial growth	Increased	Reduce LV afterload; and attenuate cardiac remodeling

Abbreviations: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CGRP, calcitonin gene-related peptide; CNP, C-type natriuretic peptide; LV, left ventricular.

Endothelial Dysfunction

Endothelial dysfunction is a common complication of cardiovascular disease and is an independent risk factor for future cardiovascular events.[57] The endothelium releases local vasodilators, such as nitric oxide (NO).[58] Forearm resistance studies demonstrate that ARBs improve endothelial function by increasing local levels of NO.[58-60] Natriuretic peptides activate NO synthase, increasing NO production.[61-63] A loss of CNP signaling in animal models results in vascular dysfunction despite active NO pathways.[64] In preclinical studies, NEPi preserves endothelium-dependent relaxation and reduces plaque formation in models of atherosclerosis.[65] Additionally, sacubitril/valsartan was found to attenuate endothelial dysfunction to at least an equivalent degree to that of valsartan alone in an animal model of hypertension.[66]

Potentiation of Neprilysin Substrates and Effects on the Cardiovascular System

As shown in **Table 1**, NEP proteolytically processes a number of bioactive molecules that influence cardiovascular physiology. Based on this level of scientific evidence, these bioactive molecules activate receptors and cause a summation of cardiovascular effects (Figure, B).

Bradykinin

Classically considered an inflammatory mediator, bradykinin acts on its receptor to induce vasodilation through release of endothelial NO and prostaglandins.[70,71] Preclinical studies have demonstrated that NEPi elevates bradykinin levels and enhances NO-mediated vasodilation.[72-76] Bradykinin may also modulate ischemic preconditioning through actions on sensory nerves.[77,78] This postulate is further supported by a preclinical study[79] that reported NEPi administration to be cardioprotective in ischemic preconditioned rabbits via bradykinin pathways. Moreover, bradykinin can inhibit collagen synthesis and alter extracellular matrix remodeling pathways.[71] However, peripheral increases of bradykinin levels cause vascular permeability, which may contribute to angioedema.[80] In the PARADIGM-HF trial,[6] increased angioedema with sacubitril/valsartan compared with enalapril alone was reported. In a clinical study using a combined NEPi/ACE inhibitor (omapatrilat), the incidence of angioedema was quite severe.[125] Because both ACE inhibition and NEPi both prevent the degradation of bradykinin, it can be postulated that this was the cause for increased angioedema. Thus, while remaining circumstantial, sacubitril/valsartan may likely potentiate bradykinin levels in the context of HFrEF.

Substance P

The traditional role of substance P is to modulate nociception via sensory nerve fibers.[82] Substance P also activates NO pathways in the endothelium, and infusion in patients with hypertension causes vasodilation.[83] Additionally, animal models of ischemic or reperfusion injury have demonstrated the release of substance P in the coronary[82] and carotid[84,85] vasculature. The release of substance P in response to hypoxia is thought to be cardioprotective by inducing coronary vasodilation and activating chemoreceptors in the carotid body.[82,84,85] However, substance P may promote maladap-

tive cardiac remodeling, as observed in a mouse model of HFrEF with substance P deletion.[86] Furthermore, cardiac inflammatory cells have been observed to release renin, angiotensin II, and cytokines in response to substance P.[82] While animal models using a NEPi reported potentiation of substance P,[84,85] a clinical study[75] reported NEPi had no effect on the vascular actions of substance P.

Adrenomedullin

Adrenomedullin and proadrenomedullin N-terminal 20 peptide are bioactive products of the same precursor molecule pre-proadrenomedullin.[87] Proadrenomedullin N-terminal 20 peptide induces weak vasodilation in humans, likely via direct effects, and attenuates peripheral catecholamine release.[88,89] On the other hand, adrenomedullin induces potent vasodilation through direct actions on smooth muscle and endothelial NO pathways.[88,89] Furthermore, adrenomedullin reduces pulse wave velocity in clinical studies[90] and increases natriuresis while reducing proteinuria in rats subjected to ischemic or reperfusion injury.[126] However, adrenomedullin also increases sympathetic outflow and has direct positive inotropic effects on the myocardium.[90-92] Adrenomedullin can influence cardiomyocyte contraction,[91] and infusion in humans increases heart rate[90] and myocardial contractility.[92] While NEP has been observed to rapidly cleave proadrenomedullin N-terminal 20 peptide, the same study concluded that NEP did not cleave adrenomedullin under similar conditions.[87] However, preclinical models of HFrEF have demonstrated potentiation of adrenomedullin concentrations and natriuresis after NEPi administration.[93,94] Additionally, a clinical study reported that local NEPi administration potentiated adrenomedullin-mediated vasodilation in the human forearm.[89] Although adrenomedullin is unlikely to be processed by NEP, several NEP substrates are known to upregulate the secretion of adrenomedullin. For instance, ANP infusion in humans increased circulating adrenomedullin levels,[67] and bradykinin and substance P increased adrenomedullin production in vascular smooth muscle cells in vitro.[81] Taken together, it is reasonable to speculate that NEPi and sacubitril/valsartan increase adrenomedullin levels through upstream bioactive pathways.

Calcitonin Gene-Related Peptide

Calcitonin gene-related peptide (CGRP) is a product of alternative splicing of the calcitonin gene.[95] At the time of discovery, CGRP was identified as modulating both nociception and efferent pathways in the nervous system.[95] Calcitonin gene-related peptide has since been recognized as a potent vasodilator via attenuation of sympathetic outflow.[95] One clinical trial[127] using a NEPi in patients with HFrEF reported increased CGRP levels with NEPi administration. However, a small number of clinical and preclinical studies have reported that NEPi may not increase CGRP levels or produce effects on the vasculature.[75,128] These observations may be because of the slow metabolic rate of degradation of CGRP by NEP.[129] Because the importance of neurovascular control, particularly that of sympathetic and parasympathetic efferent activity, is well recognized in HFrEF, clarifying the potential effects of sacubitril/valsartan on CGRP levels is warranted.

Endothelin

Produced in the vasculature, the effects of endothelin vary depending on the tissue and receptor subtype. Binding to endothelin B receptors in the endothelium promotes endothelin clearance and vasodilation via NO and prostaglandin pathways.[130] Conversely, binding to the endothelin A and endothelin B receptors on vascular smooth muscle induces vasoconstriction.[130] In the myocardium, endothelin stimulates fibroblast synthesis of collagen[131] and promotes cardiac hypertrophy.[130,131] High endothelin levels are also correlated with disease severity in patients with HFrEF.[132,133] Therefore, it would first appear that NEPi or, by extension, sacubitril/valsartan would increase circulating endothelin levels and contribute to negative HFrEF outcomes. However, clinical trials using NEPi have reported conflicting effects of endothelin potentiation on hemodynamic regulation.[56,134,135] Neutral endopeptidase inhibitor potentiation of endothelin was reported to increase systolic blood pressure in healthy patients[56] and induce vasoconstriction in healthy patients and patients with hypertension.[134] On the contrary, elevated endothelin levels after NEPi administration in patients with heart transplants reported no adverse effects on systemic or renal hemodynamics.[135] Adding to the complexity, sacubitril/valsartan decreased plasma endothelin levels in patients with HFrEF over the course of 21 days.[55] Although seemingly contradictory, these data merely suggest that the association of sacubitril/valsartan with endothelin pathways is more complex than substrate potentiation alone. As an example, the reduction in plasma endothelin levels observed with sacubitril/valsartan could be attributed to changes in receptor distribution or dampening of stimuli for endothelin release.

Bombesin

Bombesin traditionally modulates nociception yet has additional effects on cardiovascular physiology.[96] As a regulator of resting sympathetic tone, systemic administration of bombesin has demonstrated vasopressor, hypertensive, and tachycardic effects in animal models.[97,98] In addition to increasing LV afterload, long-standing elevations in sympathetic tone are known to increase demand on the myocardium and contribute to maladaptive cardiac remodeling.[49,50,136,137] The association of NEPi or sacubitril/valsartan with neurotensin bioactivity remains to be established, but the role of bombesin in the HFrEF process is an area that warrants investigation.

Neurotensin

Primarily involved in nociception pathways, neurotensin is also widely expressed in the cardiovascular system.[99] While neurotensin increases basal heart rate and myocardial contractility in various animal models,[100-104] other physiologic effects appear to be species dependent. In rats, neurotensin reduces blood pressure and increases coronary vascular tone.[105,106] However, the opposite has been reported in guinea pigs, as neurotensin increases blood pressure and reduces coronary vascular tone.[103,107] It is difficult to extrapolate these results to human physiology, as, to our knowledge, the function of neurotensin in the human cardiovascular system is unknown. However, in vitro studies suggest a vasodilatory effect, as isolated human endothelial cells release prostaglandins in response to neurotensin.[108] Furthermore, preclinical studies using NEPi to study neurotensin have focused predominantly on the central nervous system.[109,110] For example, intracerebroventricular injection of a NEPi in rats decreased locomotion via neurotensin pathways[109] and potentiated the analgesic effects of neurotensin in mice.[110] While these studies put forward the concept that NEPi or sacubitril/

Table 2. Additional Neprilysin Substrate Candidates and Bioactivity in the Cardiovascular System

Substrate Candidate	Biological Activity Relevant to Cardiovascular System	Potential Effects in HFrEF	Evidence for Substrate Potentiation by NEPi
Physalaemin (tachykinin)[18,19,65,139,140]	Vasodilation	Reduce LV afterload	Potentiated by NEPi in isolated human bronchial smooth muscle cells
Dynorphin (opioid)[18,65,111,112,139,140]	Reduce blood pressure and heart rate; vasodilation during ischemic events; and alter cardiac electrical activity	Reduce LV afterload; reduce demand on the myocardium; and influence arrhythmias	Potentiated by NEPi in various animal tissues in vitro
Corticotropin (ACTH)[141,142]	Alter blood pressure; increase sympathetic outflow; stimulate cortisol release; increase blood pressure; and salt and water retention	Alter LV afterload; increase demand on the myocardium; and alter cardiac remodeling	Potentiated by NEPi in isolated human endothelial cell membranes
Melanin-concentrating hormone[143]	Reduce MAP and heart rate; reduce sympathetic outflow and cardiac reflexes; and reduce cortisol levels	Reduce LV afterload; decrease demand on the myocardium; and attenuate cardiac remodeling	Unknown
Melanin-stimulating hormone (melanotropin)[13,141-155]	Acutely increase blood pressure and heart rate; chronically decrease blood pressure via NO-induced vasodilation; and increase cortisol release	Alter LV afterload; attenuate endothelial dysfunction; and alter demand on the myocardium	Potentiated in vitro by NEPi in human endothelial cell membranes and human melanocytes
Glucagon[156-158]	Vasodilation; increase cardiac index, MAP, and heart rate; attenuate cardiac injury after ischemic or reperfusion injury; alter cardiac electrical activity; and increase RPF and GFR	Reduce LV afterload; increase cardiac performance and demand on the myocardium; influence arrhythmias; and influence renal function	Unknown
Gastrin[159-161]	Increase coronary blood flow; influence NO pathways; and alter β-adrenergic input on the myocardium	Alter LV afterload; influence endothelial function; and alter demand on the myocardium	Potentiated by NEPi in clinical imaging studies using radiolabeled gastin; NEPi inhibited gastrin secretion in animal models via GRH potentiation
Cholecystokinin 8[159,160,162-165]	Dose-dependent effects on blood pressure and heart rate	Dose-dependent effects on LV afterload and demand on the myocardium	Weakly potentiated by NEPi in vitro
Caspase 9 precursor[166,167]	Increase myocardial apoptosis after ischemic or reperfusion injury	Influence cardiac remodeling following ischemic events	LCZ696 decreased apoptosis in doxorubicin-induced cardiomyopathy, likely via upstream pathways that regulate caspases
Vasoactive intestinal peptide[163,168,169]	Vasodilation; increase GFR; chronotropic effects; and inotropic effects	Natriuresis; increased coronary flow; and reduced LV afterload	Potentiated by NEPi

Abbreviations: ACTH, corticotropin; GFR, glomerular filtration rate; GRH, growth hormone–releasing hormone; HFrEF, heart failure with reduced ejection fraction; LV, left ventricle; MAP, mean arterial pressure; NEPi, neutral endopeptidase inhibitor; NO, nitric oxide; RPF, renal plasma flow.

valsartan may potentiate neurotensin, the relevance of this in the context of HFrEF remains to be fully established.

Enkephalin

Enkephalin is an endogenous opioid that functions to modulate pain sensation[111]; however, opioid receptors are also present on the myocardium. Endogenous opioids are released in response to ischemia and likely contribute to ischemic preconditioning.[112] This concept was supported in an animal model of ischemic preconditioning that demonstrated a marked reduction of cardioprotective effects in the presence of naloxone.[112,113] Additionally, enkephalin increased myocyte viability following ischemic or reperfusion injury in vitro.[114] However, enkephalin administration has also demonstrated vasopressor effects in both rat and rabbit models.[115,116] Additionally, NEPi elevated enkephalin levels in mice[117] and potentiated the vasopressor response of enkephalin in a rat model of hypertension.[118] Taken together, increased levels of enkephalin through the effects of sacubitril/valsartan may influence HFrEF outcomes through myocardial protection as well as neurovascular-mediated effects.

Oxytocin

Released from the hypothalamus, oxytocin is essential for regulating parturition and lactation.[119] In addition, synthesis of oxytocin and the presence of oxytocin receptors have been discovered within the vasculature of various animal species.[120] Binding of oxytocin to vasculature smooth muscle produces weak vasoconstriction as well as potent vasodilation in skeletal muscle, hepatic, renal, and splanchnic vasculatures.[121] Furthermore, the presence of oxytocin in the myocardium is thought to activate prosurvival pathways that reduce fibrosis and hypertrophy.[122,123] In models of diabetic mice, oxytocin treatment prevented cardiomyopathy[122] and attenuated cardiomyocyte death from ischemic or reperfusion injury.[123] Although NEPi and the effects of oxytocin have not been evaluated in the cardiovascular system, uterine strips from pregnant rats display a marked increase in oxytocin-induced contractions when exposed to a NEPi.[124] Therefore, it can be postulated that sacubitril/valsartan would increase oxytocin and cause beneficial effects of oxytocin in the cardiovascular system.

Additional Neprilysin Substrate Candidates and Bioactivity in the Cardiovascular System

The NEP substrates described in the preceding sections are likely to be far from complete, and there are likely to be more substrates and pathways relevant to the effects of sacubitril/valsartan in HFrEF. An in silico approach for NEP substrate mapping was performed and revealed more than 60 NEP substrates potentially relevant to human biology.[138] A subset of these substrates' bioactivity relevant to cardiovascular physiology are provided in **Table 2**.

Neutral endopeptidase degrades what have been generically termed *stress hormones*, which will alter sympathetic outflow and regulate cortisol-mediated effects on blood pressure.[141,142,144-154] Furthermore, melanin-concentrating hormone has been correlated with declining cardiac function in preclinical models of HFrEF.[143] Glucagon, a NEP substrate once pursued as a therapeutic for HFrEF, improves parameters of cardiac performance and renal function.[156-158] Neutral endopeptidase also cleaves the gastrointestinal signaling molecules cholecystokinin and gastrin.[159,160] These neuropeptides are expressed in the cardiovascular system and influence blood pressure,[161-163,170] heart rate,[161-163,170] and autonomic input.[161-164,170] Cholecystokinin levels in older women may also predict cardiovascular mortality.[165] Vasoactive intestinal peptide was initially identified as a neurotransmitter and neuromodulator but can regulate cardiovascular function primarily through a cyclic guanosine monophosphate–mediated vasodilation of both the systemic and coronary vasculature.[171] This peptide may also influence chronotropy as well as inotropy, but these effects have been primarily identified in vitro.[171] Neutral endopeptidase inhibition has been shown to augment vasoactive intestinal peptide,[168] most interestingly in patients with HFrEF.[169] Lastly, NEP processes the precursor of caspase 9 into its biologically active form.[138] Caspase 9 promotes cardiomyocyte apoptosis and is upregulated following ischemic or reperfusion injury.[172] While the association of NEPi with caspase 9 is unclear, sacubitril/valsartan reduced cardiomyocyte apoptosis in preclinical doxorubicin-induced cardiomyopathy via caspase regulatory pathways.[166,167]

This list of potential NEP substrates, and its relevance to sacubitril/valsartan, is likely to be incomplete and is intended to be hypothesis generating. Nevertheless, the results from the PARADIGM-HF study[6] and substrate mapping methodology would support basic and translational investigations into how these novel NEP substrates would alter the pathophysiology of HFrEF.

Conclusions and Future Directions

The interactive effects of sacubitril/valsartan are summarized in schematic form in Figure, C. Specifically, the reduced degradation of bioactive molecules through NEPi will, in turn, increase receptor binding and activation while at the same time preventing the adverse consequences of angiotensin II. For example, combined NEPi/ARB will decrease LV afterload through several mechanisms, initially through inhibiting vasoconstriction, potentiating vasodilation, and enhancing natriuresis.[20,24-26] Evidence to suggest that sacubitril/valsartan produces additive or synergistic effects with respect to LV afterload was the increased incidence of symptomatic hypotension reported in the PARADIGM-HF study.[6] Most certainly, the association with LV loading conditions and the effects of sacubitril/valsartan in the context of HFrEF are multifactorial and may also include important interactive effects of NEPi and ARB through a reduction in sympathetic tone/outflow.[51-54,56] While the results from the PARADIGM-HF study[6] provide the basis of a renewed and provocative examination of novel NEP substrates and pathways, the potency and relevance of ARB cannot be minimized. Specifically, the net systemic exposure of valsartan following administration of sacubitril/valsartan in healthy participants is 40% greater than that of the equivalent dose of valsartan alone.[5] Furthermore, pharmacokinetic studies in patients with HFrEF have observed a reduction in the clearance of sacubitril/valsartan compared with healthy participants.[55] These findings suggest that the ARB physiologic effects may be greater for the valsartan moiety contained within sacubitril/valsartan compared with valsartan alone.

Most certainly, the hypothesized effects of sacubitril/valsartan (Figure, C) is an oversimplification, as a number of bioactive peptides have multiple effects and interactions. Furthermore, it is important to recognize that many substrates potentiated by NEPi likely exert both positive and negative effects on cardiovascular physiology. These actions are likely dependent on dose, duration, and context. Moreover, a number of studies that were postulated to be influenced by sacubitril/valsartan were derived from animal models, and extrapolation of these results to the complex HF syndrome can be problematic. Nevertheless, these basic science studies coupled with the clinical observations currently available for sacubitril/valsartan can form the impetus for developing a set of high-level unanswered questions, which are summarized in the **Box**. While Figure,

Box. Unanswered Questions Toward Identifying Novel Mechanisms and Pathways of Combined Neutral Endopeptidase Inhibition (NEPi) and Angiotensin II Receptor Blockade (ARB) in Heart Failure With Reduced Ejection Fraction (HFrEF)[a,b]

Are the effects of combined NEPi/ARB additive or synergistic with respect to key physiological processes in HFrEF, such as LV afterload, chronotropy, and inotropy?

How does combined NEPi/ARB influence cardiovascular structure and function, such as myocardial and vascular hypertrophy?

Can high throughput and sensitive multiplex-based protein assays identify unique bioactive molecules and signaling pathways operative with combined NEPi/ARB?

Can specific pharmacological targeting of candidate molecules affected by combined NEPi/ARB open up new combinatorial strategies for HFrEF?

How does combined NEPi/ARB affect proinflammatory processes over and above monotherapy in developing HFrEF?

Does combined NEPi/ARB influence indices of cardiovascular fibrosis, such as myocardial fibrosis, and would this facilitate reversal of adverse changes in HFrEF?

Does combined NEPi/ARB favorably influence nervous system outflow, such as sympathetic efferent pathways, in a synergistic manner compared with monotherapy?

Can combined NEPi/ARB improve myocardial structure and viability in nonischemic causes of HF, such as chemotherapy-induced cardiomyopathy?

To what degree can combined NEPi/ARB alter the course of a distinctly different HF phenotype, such as HF with preserved ejection fraction, and what would be the mechanisms?

Can the outcomes of combined NEPi/ARB guide the development of other novel combinatorial molecules directed as specific subtypes of HF?

Abbreviation: LV, left ventricle.

[a] The generic term *combined NEPi/ARB* is used here, but the impetus for putting these questions forward was the result from using the combined NEPi/ARB LCZ696.

[b] The term *heart failure* is confined in this context to HFrEF in which LCZ696 was shown to impart beneficial effects on clinical outcomes.

C, suggests that favorable cardiovascular effects of sacubitril/valsartan will occur in terms of LV loading, chronotropy, and inotropy, to our knowledge, these have not been carefully dissected as of yet in patients with HFrEF.

While this review has put forward some lead candidate molecules that are likely affected by sacubitril/valsartan, comprehensive biomarker platforms to determine the relative levels of these candidate molecules remains to be accomplished. Along the same lines of inquiry, using appropriate biomarker panels and imaging, how sacubitril/valsartan influences fibrotic and inflammatory pathways would be important lines of investigation. It also must be emphasized that the PARADIGM-HF trial,[6] and hence the focus of this review, was with HFrEF; whether and to what degree these findings can be extended to other forms of HF remains to be established. Finally, the use of sacubitril/valsartan may be only the first of a new generation of multifunctional molecules that target unique and complementary pathways. Nevertheless, the results from the PARADIGM-HF trial[6] and some of the biological pathways outlined herein will hopefully not only move us beyond conventional concepts regarding inhibition of signaling pathways as therapeutics for HF but also towards targeting the potentiation of novel biological signaling pathways.

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