Complexities of the ET System

There are several unique aspects of the ET system that are worth mentioning because of their unique nature compared with more classical peptide/G-protein–linked receptor systems. Probably the most unusual aspect of this system that has made it difficult to study through the years is the irreversible nature of ET-1 binding. This binding is believed to account in large measure for the prolonged vasoconstrictor actions of ET-1 mediated by the ET\text{A} receptor. Importantly, the same ligand-binding behavior exists for the ET\text{B} receptor such that loss of ET B receptor activity results in exaggerated ET\text{A} receptor activation and does not function as a classic circulating hormone. Given the irreversible nature of ET-1 binding, there can be considerable endogenous ET-1 binding without any change in circulating ET-1 levels. Therefore, plasma ET-1 measurements are not considered a reliable reflection of ET-1 production, but could be a reflection of ET\text{B} receptor availability as explained below.

Loss of ET\text{B} receptor activity, whether by administration of specific receptor antagonists or through genetic deletion or mutation, results in significant increases in plasma ET-1 levels even without noting any change in ET-1 gene expression. This led to initial descriptions of the ET\text{B} receptor as a clearance receptor. However, the extent to which ET\text{B} receptors clear ET-1 compared with functional effects that oppose ET\text{A} receptor activity has not been clearly established.

Furthermore, given that ET\text{A} receptors also bind to ET-1 in an irreversible fashion, one must also consider these receptors in the clearance of ET-1 from the circulation although most studies do not show any increase in circulating ET-1 in conjunction with ET\text{A} receptor blockade.

Although not universally observed, there have been reports of increased plasma ET-1 after specific ET\text{A} receptor blockade, such as in mineralocorticoid-induced hypertension or loss of functional ET\text{A} receptor activity. The most likely explanation is that such increases are only observed when there is insufficient ET\text{B} receptor available to manage endogenous ET-1 production. Within the general circulation, it is fairly clear that ET\text{B} receptors are predominant. This may seem counterintuitive given the powerful vasoconstriction seen with exogenous ET-1 administration, but more revealing information may be gleaned when considering experiments in...
animals that have examined selective ET<sub>A</sub> versus selective ET<sub>B</sub> receptor blockade in otherwise normal animals.<sup>11</sup> ET<sub>B</sub> selective blockade has little effect on baseline blood pressure. In contrast, when an ET<sub>B</sub> antagonist is given, a robust increase in blood pressure is evident.

ET<sub>B</sub> receptors function to protect from overactivity of the ET<sub>A</sub> receptor by removing ET-1 from the circulation as well as stimulating the production of endothelial-dependent relaxing factors such as nitric oxide and prostacyclin. Indeed, the hypertension produced by reduced ET<sub>A</sub> receptor function can be mitigated with an ET<sub>B</sub> antagonist.<sup>11</sup> However, this does not suggest that the only role for ET<sub>B</sub> receptors is to clear ET-1 from the plasma because loss of ET<sub>B</sub> receptor induced hypertension is highly salt dependent as discussed in detail below.

The actions of ET-1 in the kidney have been a major focus of our laboratory and others. When considering renal ET-1, there is considerable evidence that urinary ET-1 is derived exclusively from intrarenal production and not derived from the circulation.<sup>7</sup> First of all, as already stated, both ET<sub>A</sub> and ET<sub>B</sub> receptors bind ET-1 irreversibly, and so it would be difficult to traverse the entire renal circulation without being cleared. Second, intravenous infusion of radiolabeled ET-1 results in accumulation within vascular structures, but not epithelial cells or urine.<sup>12</sup> Although ET-1 is synthesized by most all segments of the nephron, principal cells of the inner medullary collecting duct far exceed production from other cell types in the kidney. Selective gene deletion of ET-1 from collecting duct cells reduces urinary ET-1 excretion by ≥50%, suggesting that the majority of urinary ET-1 is from this cell type. Furthermore, whereas plasma levels of ET-1 are influenced by receptor availability, receptor antagonists do not influence urinary ET-1 excretion, suggesting that the urinary ET-1 had limited exposure to ET<sub>A</sub> or ET<sub>B</sub> receptors or that receptor antagonists do not have access to receptors on the luminal surface of tubular epithelium.<sup>9,11</sup>

**Industry Response to ET-1 Discovery**

It did not take long after the discovery of ET-1 for several companies to develop immunoassays for measurement of ET-1 peptide concentrations in plasma, urine, and tissues. One of the problems with many of these initial assays is that they had varying degrees of sensitivity and cross-reactivity with the less biologically active peptides (eg, ET-3, big ET-1, etc.). Nonetheless, there were reports of elevated plasma ET-1 levels in hypertensive subjects. Of note, these levels were even higher in black subjects.<sup>13</sup>

The time to develop highly selective, potent, and orally active ET<sub>A</sub> or combined ET<sub>A</sub>/ET<sub>B</sub> receptor antagonists was incredibly fast, only a few years after the discovery of ET-1.<sup>14–16</sup> The first antagonists were cyclic pentapeptides discovered through natural product screening. The most common of these peptides was BQ-123, which is highly selective for the ET<sub>A</sub> receptor, whereas another peptide, BQ-788, has clear ET<sub>B</sub> selectivity. Both of these commercially available peptide antagonists have a high degree of receptor specificity but are limited in their use because of a lack of oral bioavailability. However, it did not take long for the development of orally active small molecular weight organic compounds with variable degrees of selectivity for the 2 receptors. Bosentan was the first such compound to be developed and has been used for several years in the treatment of pulmonary hypertension.<sup>17</sup> Developed by Actelion, bosentan is referred to as a mixed or combined antagonist because it has similar affinity for both ET<sub>A</sub> and ET<sub>B</sub> receptors. In October 2013, Actelion received approval from the US Food and Drug Administration for marketing their next-generation antagonist, macitentan, for pulmonary hypertension that is again a mixed antagonist but chemically modified to be more lipophilic and thus concentrate in tissues more effectively. The only other antagonist approved for human use in the United States is ambrisentan,<sup>18</sup> a product of Gilead Sciences, which has a fairly high degree of ET<sub>A</sub> receptor selectivity. Several other companies have developed similar compounds targeting other cardiovascular-related disease, but most of the efforts to develop these compounds have been abandoned because of lack of efficacy and concern over waning patent life, side effects, lack of efficacy, and the high cost of new trials. Nonetheless, there remains some interest in these antagonists for disorders beyond pulmonary hypertension including essential hypertension and various forms of nephropathy, in particular, diabetic nephropathy.

**ET Antagonists in Human Hypertension**

The most significant study during the initial years of development with these drugs was that published by Krum et al,<sup>19</sup> in the New England Journal of Medicine. In this study, 293 subjects with identified hypertension were required to withdraw from all current medication for a 4-week period, then were treated with 1 of 3 doses of bosentan. An additional subgroup was given the angiotensin-converting enzyme inhibitor, enalapril, as a comparator. Interestingly, the degree of blood pressure lowering was identical between bosentan and enalapril. However, Actelion did not pursue use of bosentan as an antihypertensive agent further because the company decided to develop the drug for use in primary pulmonary hypertension.

Several years later, Gilead Sciences sponsored several clinical trials targeting resistant hypertension with their highly selective ET<sub>A</sub> antagonist, darusentan.<sup>20</sup> This trial included subjects with resistant hypertension as defined by inadequate blood pressure control despite continued treatment with at least 3 distinct antihypertensive medications. After 14 weeks of treatment, the darusentan-treated group had an average of ≈10 mm Hg lower 24-hour ambulatory blood pressure compared with that of the placebo group, an effect that was highly significant (Figure 1). Unfortunately, the primary end point in the trial was clinic blood pressure that was not statistically different from the placebo group. Therefore, although there is a consensus among hypertension specialists that ambulatory blood pressure is a more reliable measure of hypertension and predictor of complications, the company decided not to pursue development of this drug.

**ET-1 Control of Sodium and Water Excretion**

Because of the incredible potency and prolonged vasoconstrictor effects of exogenous ET-1, for a long time, investigators believed that the physiological role of ET-1 must be related to this effect. This is why one of the first studies to carefully examine renal effects of ET-1 was largely ignored as a probable artifact. Time has proven the study by Schmermann...
et al\textsuperscript{21} published in 1992 to be highly relevant. In anesthetized rats, these investigators infused a relatively high dose of ET-1 and observed a reduction in urine flow rate along with a decrease in glomerular filtration rate. However, when infusion was switched to a low dose that did not reduce glomerular filtration rate, there was an increase in urine flow rate without any associated increase in systemic arterial pressure. These are the first indications that ET-1 may function to promote the excretion of water.

Several studies have shown that urinary ET-1 excretion is positively correlated with sodium excretion as well as other prionatriuretic systems such as nitric oxide and cGMP\textsuperscript{7} In 2000, work by Plato et al\textsuperscript{22} demonstrated that ET-1 can directly inhibit Cl\textsuperscript{-} flux in isolated thick ascending limb segments indicating a direct effect independent of any hemodynamic influence. These investigators demonstrated that this is mediated by ET\textsubscript{B} receptor-dependent NO production. In isolated split open collecting ducts, work from Stockand’s laboratory demonstrated a direct effect of ET-1 via the ET\textsubscript{B} receptor to reduce the open probability of epithelial sodium channels.\textsuperscript{23} These investigators went on to show that mice lacking the ET\textsubscript{B} receptor in the collecting duct do not have suppressed epithelial sodium channel activity when placed on a high salt diet.\textsuperscript{24} These findings provide evidence not only that ET-1 suppresses epithelial sodium channel activity but actually participates in the regulation of activity in response to changes in dietary salt intake.

Kohan and colleagues\textsuperscript{25–28} published a series of studies demonstrating that the collecting duct ET-1/ET\textsubscript{B} autocrine pathway is important for regulating blood pressure. Using a now common cre-lox approach, these investigators knocked out ET-1 from the collecting duct, which resulted in an elevated baseline blood pressure and exaggerated hypertensive response to a high salt diet (Figure 2). Loss of collecting duct ET-1 also reduced urinary NO metabolite excretion. They went on to show that ET-1 seems to function in an autocrine manner because knocking out the ET\textsubscript{B} receptor or both ET\textsubscript{A} and ET\textsubscript{B} receptors from the collecting duct results in a similar salt-sensitive hypertensive phenotype.

ET-1 stimulates NO release from cultured collecting duct cells, an effect that can be blocked by a specific inhibitor of neuronal nitric oxide synthase (NOS1, or nNOS).\textsuperscript{29} This led our laboratory to perform studies examining intramedullary ET\textsubscript{B} agonist infusion, which revealed that stimulation of ET\textsubscript{B} receptors with the selective agonist, sarafotoxin 6c, produced a natriuresis that could also be blocked by specific NOS1 inhibition.\textsuperscript{30} These findings clearly suggested that ET\textsubscript{B} inhibition of sodium reabsorption in the collecting duct was mediated by NOS1-generated NO. However, more definitive evidence came from a recent study by Hyndman et al, who showed that similar to the ET-1 or ET\textsubscript{A} receptor gene deletion studies, knockout of NOS1 specifically from the collecting duct also led to reduced NO excretion and a salt-sensitive blood pressure phenotype.\textsuperscript{31} Stockand and colleagues have gone on to show that ET-1 does not reduce epithelial sodium channel activity in collecting ducts from collecting duct–specific NOS1 knockout mice (unpublished observations, article under revision).

Knowing that salt intake is a powerful stimulus of ET-1 production in the kidney, we now have a fairly clear picture of the physiological role of ET-1, that is, to facilitate the excretion of salt and water (see discussion below). This effect is primarily through an autocrine action of ET-1 stimulating ET\textsubscript{B}-dependent NOS1-dependent NO in the collecting duct, but may also involve thick ascending limb NOS3 activation as well as effects within the renal microcirculation.

**High-Salt Diet Stimulates Renal ET-1**

As has been reported many times, a high-salt diet increases renal medullary ET-1 production (Figure 3).\textsuperscript{7} However, the mechanisms by which this stimulus occurs are not completely clear. Herrera and Garvin\textsuperscript{32} showed that renal medullary interstitial osmolality in rats on a high-salt diet is elevated. They also observed that increasing osmolality in the culture media stimulates ET-1 release from isolated thick ascending limbs. This raises the question also of whether increased tubular fluid flow and sodium delivery to the distal nephron could be enhanced by ET-1.
Figure 3. Hypothetical scheme for endothelin (ET)-1 and ET<sub>A</sub> and ET<sub>B</sub> receptor involvement in the physiological response to high salt. Sites where angiotensin II may interfere to induce salt-sensitivity are indicated by the red asterisk. ENaC indicates epithelial sodium channel; GFR, glomerular filtration rate; NOS1, neuronal nitric oxide synthase; NOS3, endothelial nitric oxide synthase; and UNaV, sodium excretion.

A stimulus for ET-1 production and thus facilitating sodium excretion. In cultured collecting duct cells, Lyon-Roberts et al. have reported that extracellular sodium can stimulate ET-1 production. In a more intact preparation, Boesen infused hypertonic NaCl into the renal medullary interstitium and reported that higher osmolality increased urinary excretion rate of ET-1.

Renal Hemodynamics

The renal circulation, similar to most vascular beds, contains both ET<sub>A</sub> and ET<sub>B</sub> receptors. Intravenous infusion of exogenous ET-1 reveals a profound vasoconstrictor action that seems to be primarily mediated by ET<sub>A</sub> receptors but does involve ET<sub>B</sub>-dependent constriction at higher doses. Endothelial-dependent vasodilation mediated by the ET<sub>B</sub> receptor is evident, although the prolonged vasoconstrictor effects of ET-1 over-ride this effect when exogenous peptide is administered. There also seems to be a heterogeneous distribution of ET<sub>A</sub> and ET<sub>B</sub> receptors over the length of the renal arterial system as revealed in isolated vascular preparations. In general, the relative proportion of ET<sub>A</sub> to ET<sub>B</sub> receptors decreases as one moves along the renal arterial tree, with more prominent ET<sub>B</sub>-dependent vasodilation being more evident in the efferent arteriole. ET<sub>B</sub>-dependent vasconstriction is only seen at higher concentrations of ET-1, suggesting weaker binding affinity for ET<sub>B</sub> receptors on vascular smooth muscle. Little is known about the renal hemodynamic effects of ET-1 in humans. There have been few studies to evaluate the receptor-specific effects of exogenous ET-1 on renal hemodynamics in humans. Kaasjager et al. demonstrated that ET-1 produced a profound decrease in renal plasma flow and glomerular filtration rate, although the ET<sub>B</sub> selective ligand, ET-3, had no effect, thus suggesting a predominance of the ET<sub>A</sub> receptor in humans.

More than 10 years ago, our laboratory observed that intravenous infusion of the ET-1 precursor, big ET-1, reduces renal cortical blood flow in rats, whereas medullary blood flow remains relatively unchanged. Knowing that the renal medulla is where ET-1 and ET<sub>A</sub> receptor expression is most prevalent, we hypothesized that the actions of ET-1 within the renal medullary circulation could function to influence renal medullary control of salt and water excretion. Infusion of big ET-1 into the renal medulla of rats on a high-salt diet resulted in a significant decrease in renal cortical blood flow as in rats on a normal salt diet. However, renal medullary blood flow was actually increased by systemic infusion of big ET-1 in rats on a high salt diet. Once again, ET-1 would seem to be behaving as a pronatriuretic factor.

Our collaborators have further investigated the influence of salt diet on renal hemodynamics in a series of preliminary, unpublished experiments. Insco and colleagues have observed that a high-salt diet reduces renal blood flow autoregulatory efficiency whether it is in vivo or in the in vitro blood perfused juxta-medullary nephron preparation. Autoregulation was normalized in preparations where the ET<sub>B</sub> receptor was inhibited, consistent with ET-1 also having important hemodynamic effects under conditions of chronic elevations in salt intake. In other words, efforts to maintain a high glomerular filtration rate under conditions of high salt intake help to facilitate high tubular flow rates and elevated renal medullary perfusion, both of which are pronatriuretic and complement the renal tubular actions of this system.

ET and the Sympathetic Nervous System

Attention to the renal sympathetic nerves in control of blood pressure has taken something of a roller coaster ride during the past century but, in recent years, renewed interest has sprung from promising clinical trials using new methods for renal nerve ablation. Involvement of the ET-1 system with central and peripheral nervous system components has received limited attention, but represent a fertile area for future investigation. One of the most notable observations developed from studies where the ET<sub>B</sub> receptor gene was knocked out of mice resulting in a lethal phenotype. The lethality is because of the lack of enteric nervous system development, thus leading to agangliosis and megacolon characteristic of Hirschsprung disease. Indeed, the lack of a functional ET<sub>B</sub> receptor has been identified in this patient population.

Studies of ET<sub>A</sub> and ET<sub>B</sub> receptor function in the sympathetic nervous system have been limited to only a few groups of investigators despite clear functional involvement. A series of studies from investigators at Michigan State University (Kreulen, Galligan, Watts, and Fink) has demonstrated functional ET<sub>A</sub> receptors in sympathetic ganglia that account for hypertension produced by chronic infusion of an ET<sub>B</sub> selective agonist. Studies from our own laboratory also support a sympathoactivation role for ET<sub>B</sub> receptors in the hypertensive response to acute stress. Using acute (3 minutes) air jet stress in restrained rats, we observed that combined ET<sub>A</sub>/ET<sub>B</sub> receptor blockade, but not ET<sub>A</sub> selective blockade, severely inhibited the pressor response in Dahl salt–sensitive rats. In contrast, under conditions of ET<sub>A</sub> receptor deficiency where ET-1 levels are chronically elevated, ET<sub>B</sub> receptors function to inhibit acute stress-induced pressor responses. In both human and animal studies, acute stress results in transient increases in circulating ET-1 within 1 to 2 minutes, suggesting
either a rapid release of ET-1 or a reduction in ET\textsubscript{B} receptor availability, or both\textsuperscript{13,45}.

Our group has reported that blacks have higher plasma concentrations of ET-1, suggesting a relative reduction in ET receptor number or availability, and most likely the ET\textsubscript{B} receptor given their more pronounced influence on circulating ET-1 levels and the higher level of salt-sensitivity in the black population.\textsuperscript{13} We have also reported differences in the response to acute environmental stress among races that could possibly be explained by differences in ET\textsubscript{A} or ET\textsubscript{B} receptor function. The pressor response to acute stress is more pronounced in obese subjects carrying a specific polymorphism in the ET-1 gene, suggesting that a combination of genetic and environmental factors may play a role in ET-1 involvement in acute blood pressure responses mediated by the sympathetic nervous system, but specific mechanisms have yet to be clarified.\textsuperscript{46} Furthermore, whether the actions within the sympathetic nervous system have any relation to the overall physiological role in regulating fluid-electrolyte balance is unknown.

Another area where ET\textsubscript{A} and ET\textsubscript{B} receptors may also influence sodium excretion is by modulating the activity of renal sensory nerves within the renal pelvis. Kopp et al\textsuperscript{47} have shown that ET-1 enhances the activation of renal sensory nerves in rats on a high sodium diet via ET\textsubscript{B} receptors. In contrast, on a low sodium diet, ET-1 suppresses the activation of renal sensory nerves by stimulation of ET\textsubscript{A} receptors. The effect on renal sensory nerves is important because they have a profound influence on renal effluent nerve activity.

**Skin as a Buffer for Extracellular Sodium**

A recent and provocative hypothesis has emerged in the last few years regarding fluid-electrolyte homeostasis. This is the concept that sodium can be stored in the interstitial matrix, in particular, within the skin as a means of sodium conservation. This work, primarily driven by Jens Titze at Vanderbilt University, suggests that sodium can be stored and then cleared through the lymphatic system to serve as a secondary clearance system for sodium in the body.\textsuperscript{48} Deposition of sodium in the interstitial space requires activation of a series of inflammatory signaling systems, such as monocyte chemoattractant protein-1 and intracellular cell adhesion molecule-1.\textsuperscript{49} These are the same factors activated by suppressor doses of ET-1.\textsuperscript{50} Although the role of ET-1 in this storage mechanism has yet to be determined, it is compelling that ET-1 is increased in extrarenal tissues in response to high sodium intake.\textsuperscript{51} In cross-transplantation studies, Ohkita et al\textsuperscript{52} provided evidence that extrarenal ET-1 plays a significant role in the cardiovascular response to increases in dietary sodium intake. Our working hypothesis for ongoing experiments is that ET-1 functions as a physiological modulator in movement of sodium through extrarenal tissue in a fashion similar to that in the kidney.

**ET-1 Interaction With Angiotensin II**

One of the early preclinical observations with ET antagonists in models of systemic hypertension is their effectiveness seemed to be somewhat limited to salt-dependent forms of hypertension including mineralocorticoid and angiotensin II infused models, but not spontaneously hypertensive rats. Chronic angiotensin II (Ang II) produces a prolonged, sustained hypertension that can be completely prevented or reversed by either ET\textsubscript{A} selective or ET\textsubscript{A}/ET\textsubscript{B} receptor antagonists.\textsuperscript{53-55} This seems to be a direct effect of Ang II on ET-1 production as demonstrated in cultured endothelial cells.\textsuperscript{56}

Given the important role of the ET\textsubscript{A} receptor in mediating sodium excretion, we hypothesized that the ET\textsubscript{B} receptor system was dysfunctional in Ang II–dependent hypertension leaving the ET\textsubscript{A} receptor unchecked in contributing to blood pressure elevation as a means of facilitating pressure natriuresis by the kidney. As discussed above, intramedullary infusion of the ET\textsubscript{B} selective agonist, sarafotoxin 6c (S6c) in the rat, results in a natriuretic response. In rats that were chronically infused with Ang II, the natriuretic response to S6c was absent.\textsuperscript{57} The lack of a response to intramedullary S6c infusion seems most likely because of an effect of Ang II to reduce ET\textsubscript{B} receptor expression as evidenced by reduced ET\textsubscript{B} specific binding in membrane preparations from the inner medulla of Ang II infused rats. These studies are consistent with an overall autocrine/paracrine function in the inner medulla that is impaired during Ang II exposure and can explain, at least to some degree, the salt-sensitivity associated with this model (Figure 4).

To determine whether the effect on the ET\textsubscript{B} natriuretic pathway is a function of physiological regulation by endogenous Ang II alone or whether this effect is perhaps a consequence of hypertension induced injury, we performed similar intramedullary S6c infusion experiments in rats with elevated endogenous Ang II by feeding them a low sodium diet for 1 week.\textsuperscript{58} Interestingly, the natriuretic response to S6c was...
absent in animals maintained on a low sodium diet. In addition, in animals given a low-sodium diet along with the AT1 receptor antagonist, candesartan, the natriuretic response to S6c was restored. These findings provide clear evidence that Ang II is an important physiological regulator of ETB-dependent natriuresis.

Another interesting aspect of this relationship between the ET and Ang II systems is that female rats seem to be slightly different from males. First of all, it is well known that Ang II infusion does not produce nearly as strong of a hypertensive response in females compared with males. In addition, we observed that chronic Ang II does not completely block ETB-dependent natriuresis as it does in the males.57 Evidence to date also suggests that in the rat, the ETB receptor can contribute to some of the natriuretic response to ET-1, but more work needs to be performed to determine whether this occurs in humans, and whether this could explain some of the fluid retention issues reported in humans taking ETA antagonists.

**Summary**

The accumulation of evidence during the last 10 to 20 years has led to a fairly clear picture that one of the major physiological roles of ET-1 is to participate in the regulation of fluid and electrolyte balance and that derangements of this system lead to salt-sensitive hypertension. This role covers a full range of aspects that were not covered in this particular review, but all point toward a wide range of actions within the kidney, vasculature, and even the peripheral nervous system (Figure 5).

The fundamental mechanism for ET-1 participation in hypertension seems to be a loss of epithelial ETB receptor function in the kidney and perhaps beyond to potentially account for loss of endothelial-dependent NO production. This results in an inappropriate level of ETA receptor activation that, although facilitating natriuresis, results in elevated renal perfusion pressure and risk for hypertensive end-organ damage. The rationale for use of ETA receptor antagonists in resistant hypertension is strong, but issues related to drug development and risk for side effects have led to the major pharmaceutical houses to withdraw interest in this area. Indeed, fluid retention is reported as the most prominent complication, especially in patients with some level of renal dysfunction.59,60 It is not completely clear whether this is related to some degree of ETA receptor blockade even with ETB-specific antagonists or it could involve ETB-dependent effects on fluid-electrolyte handling that have heretofore been underestimated. There also remain additional therapeutic targets within the spectrum of hypertension including that induced by tyrosine kinase inhibitors.61 Nonetheless, promising studies related to the use of these antagonists in diabetic nephropathy may help aid in our understanding how and when these drugs may be more effectively prescribed for cardiovascular-related disease and perhaps provide a means of managing the fluid retention.62

**Sources of Funding**

Dr Pollock’s research is supported by grants from the National Heart, Lung, and Blood Institute (P01 HL69999, P01 HL95499, and U01 HL117684).

**Disclosures**

None.

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Research Clarifying the Physiology of Endothelin

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Hypertension. 2014;63:e110-e117; originally published online March 10, 2014;
doi: 10.1161/HYPERTENSIONAHA.114.02441

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