Chapter 7
Genetic Manipulation of the Endothelin System

Wararat Kittikulsuth and David M. Pollock

Abstract The endothelin (ET) system consists of the three 21-amino acid isopeptides, ET-1, ET-2, and ET-3 synthesized primarily by a family of unique endothelin converting enzymes (ECE). These peptides exert a wide variety of physiological and pathophysiological effects by activating a pair of classical G-protein coupled receptors, ET_A and ET_B. Genetic manipulation of the ET system in rodents has revealed an important role of this system on fetal organ development, blood pressure regulation, and end-organ damage, especially in lung and kidney. Overexpression of ET-1 in mice demonstrates hypertrophic, fibrotic, and inflammatory effects on vasculature, heart, lung and kidney tissues, while overexpression of human ET-2 in rats shows a primary fibrotic effect in glomeruli. Studies from systemic knockout models of the ET system are uniformly lethal, but reveal that the ET-1/ECE-1/ET_A-mediated signaling pathway is necessary for facial and cardiovascular formation, while ET-3/ECE-1/ET_B signaling pathway is important for creation of neural crest-derived enteric neurons and epidermal melanocytes during embryonic development. Furthermore, cell-specific deletion of the ET system in the renal collecting duct leads to impaired water and sodium excretion, increased epithelial sodium channel activity and hypertension. In summary, it is clear that genetic manipulation of the ET system has been, and will continue to be, a powerful tool to aid our understanding of physiological and pathological actions of this complex autocrine and paracrine system.

Keywords Endothelin-1 • Knockout • Transgenic • Collecting duct • Enteric nerves • Cardiac development

W. Kittikulsuth
Department of Pharmacology, School of Medicine, Kagawa University, Kagawa, Japan

D.M. Pollock, Ph.D. (✉)
Division of Nephrology, Department of Medicine, University of Alabama at Birmingham, 720 20th Street South, KAUL 802, Birmingham, AL 35233, USA
e-mail: dpollock@uab.edu

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7.1 Introduction

The endothelin system consists of the three 21-amino acid isopeptides, ET-1, -2 and -3. Synthesis of these three isopeptides occurs from precursors called preproendothelins (prepro-ET-1, -2 and -3). Each prepro-ET undergoes proteolytic cleavage from a furin-like protease to form the big-ET (big ET-1 to big ET-3). Then, each big-ET is cleaved by an isoform of the endothelin-converting enzyme (ECE-1 to ECE-3) to form the active ET peptides. Among the three ET isoforms, ET-1 plays a major role in regulating physiological and pathological effects. Two receptor subtypes, ET_A and ET_B are responsible for the actions of the ET peptides. ET_A receptors are mainly expressed in smooth muscle cells and mediate vasoconstriction. The ET_B receptors are widely expressed in a variety of cell types, including endothelial cells and renal epithelial cells and mediate vasodilation and promote sodium excretion. Both ET receptors also bind their ligands irreversibly and thus can act as clearance receptors (Kohan et al. 2011). This latter function has primarily been attributed to ET_B receptors because specific antagonists consistently raise circulating levels of ET-1, although blockade of ET_A receptors can have a similar effect when ET_B expression is compromised (Elmarakby et al. 2004).

There are several techniques used to investigate the role of ET's actions in healthy or disease states. One way is to use pharmacological agents to increase or inhibit ET effects. By using this approach, it is difficult to control the actions of inhibitors to the specific site-of-interest, which leads to off-target responses. Moreover, antagonists may affect several cell types within a localized region that express ET receptors or ECEs, which does not allow study of ET function in a particular cell type. For these reasons, the genetic manipulation of the ET system was developed to examine the actions of ET peptides and their receptors. This approach has been key to providing a greater understanding of this complex autocrine/paracrine system.

Genetic modification in animals refers to animals containing genetic manipulation of the genes of interest by the germ line. This manipulation causes gain or lack of function of the targeted gene early or later in life. Alteration of gene expression can be induced in a systemic or a tissue-specific manner using either a natural- or cell-specific promoter. Therefore, genetic manipulation is a powerful tool for identifying and understanding physiological or pathological function of the targeted gene *in vivo*. This chapter will review how genetic manipulation of the ET system in rodents affects fetal development and regulation of sodium handling and blood pressure.

7.2 Transgenic Models of the Endothelin System

Creation of transgenic animals is a way to study "gain-of-function" of the gene of interest that can be expressed in specific cell types or a broad spectrum of tissues *in vivo*. It involves a process of introducing foreign DNA into the host's genome. Successful integration of DNA leads to an increase in protein expression of the transgene along with basal protein expression of the native gene. For the ET system, this technique has allowed us to understand the potential pathological effect of increased ET-1 levels in many tissues (Table 7.1).

Mice non-specifically overexpressing the human ET-1 gene have increased ET-1 peptide concentrations in plasma and tissues, particularly in lung, liver and kidney (Hocher et al. 1997, 2000). This chronic elevation of ET-1 in the transgenic mice leads to lung inflammation but with normal pulmonary pressure (Hocher et al. 2000). Moreover, the transgenic mice display renal interstitial fibrosis, renal cyst formation, glomerulosclerosis and reduced glomerular filtration rate, which is an age- and androgen-dependent effect (Hocher et al. 1997; Kalk et al. 2009). The ET-1 transgenic mice have a normal systemic arterial pressure; however, as the animals age, salt-sensitive hypertension develops (Shindo et al. 2002).

Several lines of evidence suggest that an increase in nitric oxide may be involved in maintaining the normal blood pressure observed in ET-1 transgenic mice. ET-1 transgenic mice have increased urinary nitrate-nitrite excretion (Hocher et al. 2004). Endothelium-dependent relaxation is enhanced in aorta from ET-1 transgenic mice, which can be inhibited by the nitric oxide (NO) synthase inhibitor L-NAME (Quaschning et al. 2003). Moreover, the administration of L-NAME produces a greater increase in blood pressure in ET-1 transgenic compared to genetic control mice (Hocher et al. 2004). Cross-breeding between ET-1 transgenic mice and mice lacking the gene for endothelial NO synthase (NOS3 KO) have a further increase in blood pressure as compared to either ET-1 transgenic or NOS3 KO mice alone (Quaschning et al. 2007). Similarly, crossing ET-1 transgenic and NOS2 (or iNOS) knockout (KO) mice have significantly elevated blood pressure (Quaschning et al. 2008).

Mice overexpressing human ET-1 in endothelial cells using the tie-2 promoter exhibit a threefold elevation in preproET-1 mRNA in aorta and a sevenfold increase in plasma ET-1. These mice also express vascular endothelial dysfunction and altered vascular structure of resistance vessels, yet have normal blood pressure (Amiri et al. 2004).

Overexpression of human ET-1 specifically in cardiomyocytes using the α-myosin heavy chain (α-MHC) promoter results in a tenfold increase in ET-1 levels in the heart, with no change in ET-1 concentrations in plasma or other tissues. These mice have normal appearance at birth; however, they rapidly develop pulmonary and hepatic congestion, and die at 5 weeks of age. These findings suggest that ET-1 can promote inflammation and cytokine expression in the heart (Yang et al. 2004).

Table 7.1 Summary of phenotype results for genetic modification of endothelin system

Animal model	Phenotype	References
Transgenic mice		
ET-1$^{+/+}$	Lung: chronic inflammation; normal pulmonary pressure Kidney: increased renal cyst formation; renal interstitial fibrosis; glomerulosclerosis; age-dependent salt-sensitive hypertension	Hocher et al. (1997, 2000), Shindo et al. (2002), Kalk et al. (2009)
ET-1$^{+/+}$/NOS2$^{-/-}$	Increased blood pressure	Quaschning et al. (2008)
ET-1$^{+/+}$/NOS3$^{-/-}$	Elevated blood pressure	Quaschning et al. (2007)
Endothelial ET-1$^{+/+}$	Vascular endothelial dysfunction; vascular remodeling	Amiri et al. (2004)
Cardiomyocyte ET1$^{+/+}$	Pulmonary and hepatic congestion	Yang et al. (2004)
Knockout mice		
ET-1$^{-/-}$	Abnormalities in craniofacial and cardiovascular system, thyroid and thymus gland; death after birth	Kurihara et al. (1994, 1995a, b)
ET-1$^{-/+}$	Mild blood pressure elevation; increased resting renal sympathetic nerve activity	Kurihara et al. (1994), Kuwaki et al. (1996, 1999), Ling et al. (1998), Morita et al. (1998)
Cardiomyocyte ET-1$^{-/-}$	Shorter lifespan; age-associated reduction in cardiac function	Zhao et al. (2006)
Collecting duct ET-1$^{-/-}$	Increased blood pressure; salt-sensitive hypertension; impaired ability to excrete water	Ahn et al. (2004), Ge et al. (2005a)
ET-2$^{-/-}$	Growth retardation; internal starvation; severe hypothermia; lung dysfunction; death at an early age	Chang et al. (2013)
ET-2$^{-/-}$ at adulthood	Reduced weight gain; reduced lipid deposition	Chang et al. (2013)
Intestinal epithelium ET-2$^{-/-}$	Normal growth and blood glucose level	Chang et al. (2013)
Neuron ET-2$^{-/-}$	Normal core temperature	Chang et al. (2013)
ET-3$^{-/-}$	Aganglionic megacolon; coat color spotting; death at an early age	Baynash et al. (1994), Kuwaki et al. (2002)
$ET_A^{-/-}$	Craniofacial and cardiovascular defects (similar phenotype to ET-1$^{-/-}$)	Clouthier et al. (1998)
Cardiomyocyte $ET_A^{-/-}$	Normal development and cardiovascular function; Angiotensin II or isoproterenol-induced myocardial hypertrophy	Kedzierski et al. (2003)
Smooth muscle $ET_A^{-/-}$	Defects of arterial network, mandibular and thymus structure	Donato et al. (2014)
Collecting duct $ET_A^{-/-}$	Normal blood pressure; impaired ability to excrete water	Ge et al. (2005b)

(continued)

Table 7.1 (continued)

Animal model	Phenotype	References
Whole nephron $ET_A^{-/-}$	Normal blood pressure; fluid retention during high salt intake	Stuart et al. (2012, 2013)
$ET_B^{-/-}$	Aganglionic megacolon; coat color spotting (similar phenotype to ET-$3^{-/-}$)	Hosoda et al. (1994)
Endothelial $ET_B^{-/-}$	Endothelial dysfunction	Bagnall et al. (2006)
Collecting duct $ET_B^{-/-}$	Elevated blood pressure; increased ENaC activity; salt-sensitive hypertension	Ge et al. (2006), Bugaj et al. (2012)
Collecting duct $ET_A^{-/-}$/ $ET_B^{-/-}$	Elevated blood pressure; increased ENaC activity; salt-sensitive hypertension	Ge et al. (2008), Bugaj et al. (2012)
ECE-$1^{-/-}$	Craniofacial and cardiovascular defects (similar phenotype to ET-$1^{-/-}$); aganglionic megacolon and coat color spotting (similar phenotype to ET-$3^{-/-}$)	Yanagisawa et al. (1998)
ECE-$2^{-/-}$	No detectable abnormalities	Yanagisawa et al. (2000)
Transgenic rat		
ET-$2^{+/+}$	Glomerulosclerosis	Liefeldt et al. (1995, 1999)
Dysfunctional ET system in rat		
Homozygous spotting lethal (*sl*/*sl*)	Aganglionic megacolon; white coat color	Gariepy et al. (1996)
Rescued ET_B-deficient	Coat color spotting; salt-sensitive hypertension	Gariepy et al. (1998, 2000)

Overexpression of human ET-2 in the rat causes a marked elevation in ET-2 levels in kidney, intestines, lung, and brain (Liefeldt et al. 1995, 1999). In the kidney, human ET-2 is predominantly expressed in glomeruli so that renal fibrosis in the ET-2 transgenic rats occurs strictly in glomeruli. Moreover, ET-2 transgenic rats have increased urinary protein excretion and reduced glomerular filtration rate with no change in blood pressure. It is not clear whether these findings reflect any known problem related to renal disease, but suggest that further investigation is needed.

In summary, studies from rodents overexpressing human ET-1 and ET-2 suggest deleterious effects of ET-1 and ET-2 in many organs, but are particularly evident in kidney. ET-1 has hypertrophic, fibrotic, and inflammatory effects on vasculature, heart, lung and kidney, while ET-2 has a major fibrotic effect in glomeruli. These deleterious effects of both ET-1 and ET-2 occur independent from blood pressure elevation, which may appear surprising given the potent vasoconstrictor effects of exogenously administered ET-1. However, these findings are consistent with studies where exogenous ET-1 was infused chronically without any change in blood pressure, yet there are clear signs of inflammation and renal dysfunction (Saleh

et al. 2010). We speculate that the lack of hypertension is due to efficient clearance of ET peptides by the ET_B receptor and the vasodilator effects that oppose ET_A dependent vasoconstriction.

7.3 Knockout Models of the Endothelin System

Loss-of-function of the protein of interest is another way to study the role of specific gene products. This approach can be achieved through gene targeting by eliminating a specific gene or deleting a portion of the gene that results in the absence of the functional domain of the protein of interest. Homologous recombination is an important step to completely remove gene loci resulting in the production of a mutated or truncated protein, or no protein production at all. Gene deletion can occur in all or specific cell types. The latter is generated by using site-specific recombination technology, such as Cre-lox. The Cre-lox system is composed of (1) Cre recombinase, an enzyme that induces the recombination between two loxP sites on the gene of interest driven by a cell-specific promoter, and (2) loxP, where the recombination occurs within the gene of interest (Kohan 2008). Normally, the process of gene inactivation occurs during the embryogenic state. However, it also can be induced in adulthood or a certain time period using inducers, such as tetracycline or tamoxifen that can activate specific promoters (Kohan 2008). Using this technique, the importance of the ET system in embryonic development and sodium homeostasis has been revealed.

7.3.1 The Endothelin System in Embryonic Development

Homozygous ET-1 KO mice die immediately after birth due to respiratory failure, which is caused by craniofacial developmental abnormalities (Kurihara et al. 1994). ET-1 KO mice also have abnormalities of cardiovascular system (Kurihara et al. 1995b), thyroid and thymus glands (Kurihara et al. 1995a); however, no abnormalities in the lung, kidney, and central nervous system could be found (Kurihara et al. 1994). Moreover, ET_A receptor or ECE-1 KO mice show craniofacial deformities and defects in the cardiovascular systems leading to death soon after birth (Clouthier et al. 1998; Yanagisawa et al. 1998). These phenotypes from ET_A or ECE-1 KO mice are nearly identical to those found in ET-1 KO mice.

Similar to ET-1 or ET_A receptor KO mice, pharmacological inhibition of the ET_A receptor with either a mixed ET_A and ET_B (Spence et al. 1999) or selective ET_A receptor antagonist (Cross et al. 2012) produces teratogenicity, including malformations of the head, mouth, face, and large blood vessels. Furthermore, neutralizing antibodies to ET-1 or an ET_A antagonist leads to cardiovascular defects in the pups from pregnant heterozygous ET-1 KO mice (Kurihara et al. 1995b). Thus, these results from both genetic deletion and pharmacological inhibition

demonstrate an important role of ET-1/ECE-1/ET_A-mediated signaling in facial and cardiovascular formation, and control of the respiratory system after birth.

Heterozygous ET-1 KO mice appear to have normal development and are fertile. Unexpectedly, heterozygous ET-1 KO mice, which have reduced plasma and renal ET-1 levels, display a mild elevation in resting blood pressure suggesting a loss of ET_B receptor activation (Kurihara et al. 1994; Morita et al. 1998), but is not exacerbated by salt (see discussion below on ET_B receptor function) (Morita et al. 1998). The mechanism of blood pressure elevation in these mice is not due to impaired nitric oxide activity (Kurihara et al. 1994). One possible explanation is that these mice have increased resting renal sympathetic nerve activity (RSNA) and maximum RSNA during basal conditions (Kuwaki et al. 1996; Ling et al. 1998; Kuwaki et al. 1999). ET-1 and both its receptors are expressed in the central and peripheral nervous system, but little is known about their physiological role.

Cardiomyocyte specific deletion of ET-1 does not affect cardiac structure or function in young mice (Zhao et al. 2006). However, aged cardiomyocyte ET-1 KO mice display a significant reduction in fractional shortening, reduced left ventricle systolic function, and a dilated left heart ventricle (Zhao et al. 2006). For these reasons, these KO mice have a shorter lifespan than controls (median life expectancy: 11 months for KO mice vs. 2 years for wild-type) (Zhao et al. 2006).

Whole body ET-2 KO mice exhibit severe growth retardation and die at the age of 3–4 weeks. These mice display internal starvation, severe hypothermia, and lung dysfunction. Deletion of ET-2 function at adulthood cause diminished weight gain and reduced lipid deposition (Chang et al. 2013). Unlike whole body ET-2 KO mice, intestinal epithelium–specific ET-2 KO mice have normal growth and blood glucose levels, suggesting the internal starvation cannot be explained by intestinal absorption. Similarly, severe hypothermia in ET-2 KO mice cannot be explained by the lack of ET-2 function in the enteric nervous system since neuron-specific ET-2 KO mice had normal core temperature (Chang et al. 2013). These results indicate that ET-2 is essential for growth regulation and survival of postnatal mice and the maintenance of energy homeostasis even in adulthood. However, the precise origin and localized activity remains somewhat unclear. Interesting, ET-2 has been described in some of the early literature as vasoactive intestinal constrictor (VIC) prior to discovery of its amino acid sequence (Bloch et al. 1991).

Disruption of the gene for ET-3 in mice displays aganglionic megacolon, coat color spotting and no melanin pigment in choroidal layer of the retina. Most of the KO mice became sick and die at about 4 weeks after birth (Baynash et al. 1994). These similar phenotypes can be found in ET_B receptor or ECE-1 KO mice (see below) (Hosoda et al. 1994; Yanagisawa et al. 1998). These findings suggest the role of the ET-3/ECE-1/ET_B signaling pathway in neural crest-derived enteric neurons and epidermal melanocytes. Deletion of the ET-3 gene does not affect blood pressure and heart rate in infant mice (Kuwaki et al. 2002).

As described above, ET_A receptor KO mice die immediately after birth with defects of craniofacial and cardiovascular formation (Clouthier et al. 1998). However, ET_A KO mice using the α-MHC-Cre promoter to target cardiomyocytes specifically are viable and have normal development and function of cardiovascular

system. These mice develop myocardial hypertrophy after angiotensin II or isoproterenol infusion. Because cardiomyocyte-specific ET_A KO mice have a twofold increase in ET_B receptor binding in cardiac tissue (Kedzierski et al. 2003), it is possible that ET_B receptors may have hypertrophic effects in cardiomyocytes during pathological situations when ET_A receptors are absent.

Recently, smooth muscle (SM)-specific ET_A KO mice have been generated using the SM22-Cre promoter to drive Cre-recombinase. SM ET_A KO mice display developmental abnormalities of the arterial network mandibular and thymus structure, leading to reduced survival. SM ET_A KO mice also exhibit attenuated ET-1-induced vasoconstriction and blood pressure elevation when given exogenously. Moreover, these mice have lower blood pressure during high salt intake as compared to the controls (Donato et al. 2014). These data again confirm a role for ET-1 and ET_A signaling in SM in vascular, mandibular, neural crest and thymus development as well as a less well-understood influence on blood pressure.

Similar to ET-3 KO animals, mice lacking ET_B receptors are born with white spotting of the coat as a result of abnormal melanocyte function. These mice stay healthy in the first week after birth; however, they become severely ill with aganglionic megacolon (Hosoda et al. 1994).

The homozygous spotting lethal (*sl*/*sl*) rat was originally maintained as a model for Hirschsprung's Disease due to a lack of enteric nerve development. Gariepy and colleagues discovered that this rat has a naturally occurring deletion in exon 1 of ET_B receptor (*EDNRB*) gene, which encodes a transmembrane portion of the ET_B receptor. This results in a non-functional ET_B receptor being expressed and so this model is referred to as the ET_B-deficient rat. These rats also display a white coat color, aganglionic megacolon, and die within the first few weeks after birth (Gariepy et al. 1996). Transgene expression of the full, intact ET_B receptor using human dopamine-β-hydroxylase (DβH) promoter increases the expression of *EDNRB* gene in enteric neurons and the putative embryonic neuroblast (Kapur et al. 1991; Mercer et al. 1991); however, non-adrenergic tissues, such as kidneys, do not have functional ET_B receptors. The transgene increases survival and rescues these animals from the development of megacolon. However, *DβH-ENDRB* transgene is not expressed in vascular endothelial cells or renal tubular epithelium and does not prevent coat color spotting phenotype (Gariepy et al. 1998).

As mentioned above, mice that lack ECE-1 expression display abnormalities of craniofacial tissues, great vessel and cardiac outflow structures, which are also observed in ET-1 and ET_A receptor KO mice. Moreover, ECE-1 KO mice show defects of epidermal and choroidal melanocytes, and enteric formation, which is similar to ET-3 and ET_B receptor KO mice. These data suggest the importance of ECE-1 in the conversion of the ET precursor peptides, big ET-1 and big ET-3, to active ET-1 and ET-3. Even though tissue ET-3 levels are dramatically reduced in ECE-1 KO mice, ET-1 and ET-2 levels in the KO mice are only reduced by about 50 %, suggesting a role for other enzymes, such as ECE-2, in conversion of big ETs to ETs (Yanagisawa et al. 1998).

The deletion of ECE-2 in mice results in no detectable defects in embryonic development. Adult ECE-2 KO mice are healthy and fertile. ECE-2 does not appear to play a role in converting big ETs to active ETs under basal conditions since tissue ET-1 and ET-2 levels are comparable between ECE-2 KO and WT mice. Furthermore, tissue ET-1 and ET-2 levels are comparable between double ECE-1/ECE-2 and ECE-1 KO mice (Yanagisawa et al. 2000).

7.4 The Endothelin System in Water and Sodium Homeostasis

7.4.1 Collecting Duct System and Sodium Homeostasis

Among the three ET peptides, ET-1 is a major isoform that is produced and has actions in the kidney (Kohan and Fiedorek 1991; Ujiie et al. 1992). ET-1 tissue content is the highest in the renal medulla, which is mainly driven by inner medullary collecting duct (CD) expression (Kohan and Fiedorek 1991; Ujiie et al. 1992). Several *in vitro* studies demonstrate that ET-1 enhances sodium excretion in the thick ascending limb (TAL) (Plato et al. 2000; Herrera and Garvin 2004; Herrera et al. 2009) and CD (Edwards et al. 1993; Kohan et al. 1993; Gallego and Ling 1996), which is inhibited by an ET_B receptor antagonist. Similarly, an increase in ET_B activation in the renal medulla using a specific ET_B agonist increases sodium and water excretion in rodents, which is independent in changes in medullary blood flow (Nakano et al. 2008; Nakano and Pollock 2009; Kittikulsuth et al. 2011, 2012). These data suggest that ET_B-induced natriuresis is from renal tubular action. However, these studies do not provide direct evidence that ET-1 regulates sodium excretion in TAL or CD in animals. Therefore, mice lacking ET-1 and its receptors were generated using tissue specific KO by Cre-lox methodology. The cre-specific promotor for TAL (Tamms-Horsfall) (Stricklett et al. 2003) and the CD principal cell (AQP2) (Nelson et al. 1998) have each been developed. To date, however, only mice with CD principal cell KO of the ET system have been generated.

As mentioned above, ET_B deficient rats only express ET_B receptors in adrenergic tissues and lack ET_B receptor expression in other tissues such as kidneys, lung, endothelial cells. These rats have elevated plasma ET-1 because ET_B receptors in the vasculature function to clear circulating ET-1 through their irreversible binding properties (Kohan et al. 2011). ET_B deficient rats have slightly increased blood pressure when on a normal salt diet, which increases further during high salt feeding. The mechanism of salt-induced blood pressure elevation in these rats can be partially explained by the over-activation of ET_A receptors since administration of an ET_A receptor antagonist reduces blood pressure and ameliorates renal injury. Interestingly, amiloride, which can inhibit ENaC, blunts blood pressure elevation in these rats during high salt intake (Gariepy et al. 2000). These data are consistent

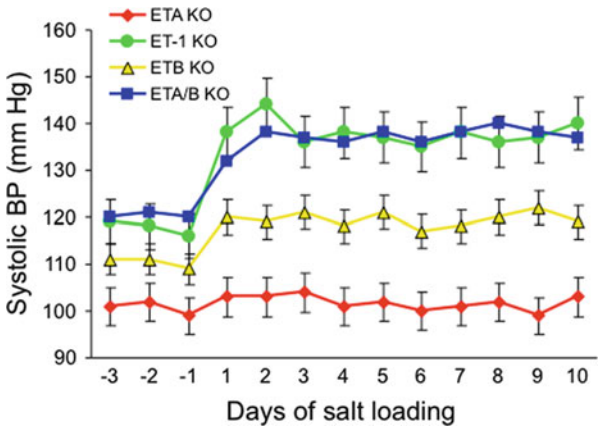

Fig. 7.1 Effect of collecting duct specific deletion of ET-1 system on systolic blood pressure during a normal or high salt diet [with permission from Pollock (2014)]

with observations that ET_B-induced inhibition of ENaC activity (Bugaj et al. 2008) is involved in sodium handling and blood pressure control (Kohan et al. 2011).

Kohan's laboratory has generated a series of mice where components of the ET-1 system have been specifically knocked out of CD principal cells using the Cre-lox system with the AQP2 promoter used to drive Cre-recombinase (Nelson et al. 1998). Mice lacking the ET-1 gene in the CD (CD ET-1 KO) have increased blood pressure during a normal salt diet that increases even more during high salt intake (Fig. 7.1). These mice exhibit an impaired ability to excrete sodium on the first 2 days of a high salt diet. Moreover, administration of diuretics, amiloride or furosemide, ameliorate blood pressure elevation during high salt diet (Ahn et al. 2004). These data suggest the lack of CD ET-1 leads to salt-sensitive hypertension, which is caused by a lack of ability to excrete sodium.

It is quite clear that ET-1 acts in an autocrine or paracrine manner and both ET receptor subtypes, ET_A and ET_B, are expressed in the CD. The Kohan laboratory has further examined which ET receptor subtype is responsible for ET-1-induced sodium excretion. Using similar gene targeting techniques, deletion of ET_A receptors in the CD (CD ET_A KO) does not alter blood pressure (Fig. 7.1) or sodium excretion during normal or high salt feeding (Ge et al. 2005b). Similarly, whole nephron KO mice of ET_A using PAX-8 Cre-promoter have normal blood pressure and sodium excretion during normal and high salt intake, although these mice display mild fluid retention during a high salt diet (Stuart et al. 2012, 2013). Unlike the CD ET_A KO, mice with CD-specific disruption of the ET_B receptor gene (CD ET_B KO) have blood pressure elevation during a normal and high salt diet (Fig. 7.1). CD ET_B KO mice have no change in sodium excretion during chronic sodium load; however, these mice display a reduced ability to excrete an acute sodium load (Ge et al. 2006).

The regulation of sodium excretion is regulated by the balance between tubular transport pathways and renal hemodynamics. ET_B receptors are highly expressed in vascular endothelial cells and cause vasodilation, which helps to excrete sodium (Kohan et al. 2011). Deletion of ET_B receptor in endothelial cells (EC) in mice

causes endothelial dysfunction, but how the endothelial ET_B receptor may influence sodium excretion and blood pressure is a bit unclear (Bagnall et al. 2006). When placed on a high salt diet, blood pressure was increased in the EC ET_B KO mouse, but to a similar degree as the control strain, which was salt-sensitive. Thus, further exploration is needed to uncover the full role of EC ET_B receptors.

The degree of blood pressure elevation in CD ET_B KO mice is roughly one-half that observed in CD ET-1 KO mice during a normal and high salt diet (Fig. 7.1). These data suggest that the hypertensive effect in CD ET-1 KO mice is only partially mediated by the lack of CD ET_B receptor action. It is possible that there is a compensatory effect from ET_A receptors in CD ET_B KO mice. For this reason, mice with double deletion of ET_A and ET_B receptor gene in the CD (CD $ET_{A/B}$ KO) were generated. These animals have an identical degree of blood pressure elevation as compared to CD ET-1 KO during a normal and high salt diet (Fig. 7.1) suggesting some sort of receptor crosstalk or cooperation. Since KO of the ET_A receptor from the CD does not affect blood pressure, these data suggest that ET_A receptors also play a role in blood pressure regulation only when ET_B receptors are absent. Unlike CD ET-1 KO mice, however, CD $ET_{A/B}$ KO mice show a slower progression of blood pressure increase (Ge et al. 2008).

Administration of exogenous ET-1 has been shown to inhibit ENaC open probability in the cortical CD, which can be prevented by pharmacological inhibition of the ET_B receptor (Bugaj et al. 2008). Moreover, the patch-clamp technique on isolated split-open cortical CD revealed that ET-1 inhibits ENaC activity in control and CD ET_A KO mice; however, ENaC activity remains after ET-1 stimulation in CD KO mice lacking ET_B or $ET_{A/B}$ (Bugaj et al. 2012). It is well known that ENaC activity is inversely correlated to the amount of salt intake and is a function of circulating aldosterone levels (Stockand et al. 2010; Mironova et al. 2011). ENaC activity in CD ET_A KO mice is low during high salt feeding; however, ENaC activity is inappropriately elevated in CD ET_B or CD $ET_{A/B}$ KO mice (Bugaj et al. 2012).

The full range of studies using CD KO mice of the ET-1 system confirm that ET-1 through ET_B receptors play an important role in control of sodium excretion and blood pressure during high salt intake by inhibiting ENaC activity. Furthermore, ET_A receptors may be involved in sodium handling during high salt feeding if ET_B receptors are dysfunctional, which may account for some of the fluid retention problems observed during administration of ET_A antagonists to subjects with impaired renal function (Mann et al. 2010; Andress et al. 2012).

7.4.2 Collecting Duct System and Water Homeostasis

ET-1 also plays a specific role in regulating water excretion in the CD. CD ET-1 KO mice have reduced plasma vasopressin (AVP) levels with no change in water excretion during normal water intake. Furthermore, CD ET-1 KO mice have an impaired ability to excrete water following an acute water load. Infusion of the AVP

receptor 2 agonist, [deamino-Cys1, D-Arg8]-Vasopressin (DDAVP) increases urine osmolality and AQP2 expression in CD ET-1 KO mice as compared to controls. In addition, AVP-stimulated cAMP production in CD ET-1 KO mice is enhanced in the inner medulla compared to controls. These data suggest that the absence of CD ET-1 reduces the ability to excrete water during an acute water load, which may be due to increases in AVP responsiveness (Ge et al. 2005a). It is possible that the diuretic effect of ET-1 on the CD may be a result of ET_B receptor activation since ET_B receptor antagonist inhibits AVP action in rat inner medullary CDs (Edwards et al. 1993).

Knockout of the ET_A receptor from the CD increases plasma AVP with no change in water excretion during a normal water intake. These mice have a modestly enhanced ability to excrete an acute water load. AVP responsiveness is reduced in inner medullary CDs isolated from CD ET_A KO mice (Ge et al. 2005b). These data suggest that while ET-1 induces diuresis via ET_B receptor activation in the CD, data from these KO animals suggest that ET_A receptors may enhance AVP action in the CDs to cause water retention. However, CD ET_A KO mice do not display fluid retention in response to ET_A receptor blockade as do control mice (Stuart et al. 2013) so the precise mechanisms have not been clarified.

7.5 Perspectives

Genetic manipulation of the ET system reveals important developmental, physiological, and pathological actions of this system in many organs. The interaction of ET peptides and its receptors are important in embryonic development. ET-1 via ET_A receptors is critically essential for facial and cardiovascular formation and ET-3 via ET_B signaling pathway is involved in the generation of neural crest-derived enteric neurons and epidermal melanocytes. During the postnatal period, ET-1, possibly through ET_A receptors, has a pro-fibrotic effect on lung, heart and kidney tissue.

In terms of its physiological role, ET-1, primarily via ET_B receptor activation, displays favorable effects on increasing sodium excretion and lowering blood pressure. Cell specific knockouts have been instrumental in elucidating this physiological role. ET_B receptors also appear to provide protection against the profound vasoconstrictor actions that occur with ET_A activation in the vascular system. Disruption of the balance between these two receptor systems can lead to localized tissue inflammation and organ damage that results from unchecked ET_A activation. There is much that has yet to be learned about this complex system and so these and the next generation of genetically manipulated animals will continue to provide insights into this critically important system.

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