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Pathophysiology of the Endothelin System – Lessons from Genetically Manipulated Animal Models

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Abstract
Shortly after discovery of ET-1 in 1988, the entire endothelin system was characterized. The endothelin system consists of the three peptides ET-1, ET-2 and ET-3, their G-protein-coupled receptors endothelin receptor A and B (ETRA and ETRB) and the two endothelin-converting enzymes (ECE-1 and ECE-2). Genetically modified animal models are an important tool in biomedical research. Here we describe the key findings obtained from genetically modified animal models either over-expressing compounds of the ET system or lacking these compounds (knockout mice). Results from the different transgenic and knockout models disclose that the ET system plays a major role in embryonic development. Two ET system-dependent neural crest-driven developmental pathways become obvious: one of them being an ET-1/ETAR axis, responsible for cardio-renal function and development as well as cranial development; the other seems to be an ET-3/ETBR mediated signalling pathway. Mutations within this axis are associated with disruptions in epidermal melanocytes and enteric neurons. These findings led to the discovery of similar findings in humans with Hirschsprung disease. In adult life the ET system is most important in the cardiovascular system and plays a role in fibrotic remodelling of the heart, lung and kidney as well as in the regulation of water and salt excretion.

Introduction

Genetically modified animal models are an important part of medical research. By inducing conditions similar to human diseases in animals, pathophysiologic hypotheses and therapeutic strategies can be tested and evaluated.

The endothelin (ET) system plays a complex role in physiologic and pathophysiologic functions of the body. In order to analyse the role of single components of the endothelin system in specific organs and tissues several transgenic and knockout (KO) animal models have been developed.

The ET system consists of the three peptides ET-1, ET-2 and ET-3, their G-protein-coupled receptors endothelin receptor A and B (ETRA and ETRB) and the two endothelin-converting enzymes (ECE-1 and ECE-2) which convert the precursor big-ETs to the biologically active forms. The ET system is expressed in various tissues and cells. Its different components are often counteracting in an autocrine and paracrine way.

Because of its strong vasoconstrictive effect the ET system plays a role in the development of hypertension and atherosclerosis. It is also involved in cardiac diseases because of effects on inotropy and chronotropy. ET has also been shown to be involved in states of portal hypertension and acute and chronic renal failure [1].

In the following, this article outlines the genetically modified animal models, which have been established in order to obtain a better understanding of the ET system.

The Transgenic Animal Models

A model in which the human ET-1 gene under the control of its natural promoter was transferred into the germline of mice was established independently by two research groups [2, 3]. Both groups observed similar results: the overexpression of the ET-1 gene creates a phenotype with characteristic, age-dependent pathologic alterations of the kidney whilst blood pressure remains normal. The most evident effects of the elevated plasma and tissue concentrations of ET-1 are the development of renal interstitial fibrosis, renal cysts and glomerulosclerosis, leading to a progressive decrease in glomerular filtration rate in aged mice. In these transgenic models the profibrotic effect of ET-1 is most ostentatious whereas hypertension can only be induced by salt-loading. This was unexpected since previous studies showed ET-1 to be an potent vasoconstrictor when administered intravenously [4-7].

A bolus injection of the nitric oxide synthesis inhibitor L-NAME induces an exaggerated hypertensive response in ET-1 transgenic mice as well as in ET-2 transgenic rats [8, 9]. This suggests that the nitric oxide system as a vasodilator system is involved.

In an animal model of crossbreds of ET-1 transgenic mice (ET+/+) and mice in which the gene for the endothelial nitric oxide synthase (eNOS) was knocked out [10] this assumption was supported. Compared to the normotensive wild type and ET+/+, the crossbred ET+/+ eNOS KO have a significantly elevated blood pressure. This is also seen in ET+/+ inducible NOS KO crossbreds [11] which corroborates the hypothesis that the NO system counterregu-

lates the ET overexpression in the ET-1 transgenic model leading to a normotensive phenotype.

The ET-1 transgenic model also brought out some interesting findings about the role of endothelin in the etiology of pulmonary diseases. The development of pulmonary fibrosis and chronic lymphocytic inflammation in the absence of pulmonary hypertension was observed in the transgenic animals [12], suggesting ET-1 to be involved in the pathogenesis of lung fibrosis and bronchiolitis.

Endothelium-restricted overexpression of human ET-1 does not result in elevated blood pressure, however altered vascular structure and function in resistance vessels can be observed. This is a sign of blood pressure independent effects of endothelium-generated ET-1 on vasculature [13].

Conditional cardiac overexpression of endothelin-1 induces inflammation and dilated cardiomyopathy in mice indicating that ET-1 acts as a proinflammatory molecule in the heart [14].

In rats overexpressing the human ET-2 gene under control of its natural promoter a blood pressure-independent fibrotic remodelling of the kidney is seen but is mainly restricted to the glomeruli. This is most probably due to the preferential expression of the transgene within the glomeruli in ET-2 transgenic animals [15], whereas the transgene is ubiquitously expressed within the entire kidney of ET-1 transgenic mice [2].

ET-3 plays an important role in the development of epidermal melanocytes [16]. A tetracyclin-responsive melanocyte-specific lineage transgenic system was created and used to conditionally regulate the overexpression of ET-3 in different stages of melanocyte development. Investigations showed that ET-3 interacts with precursors and differentiated melanocytes [17]. The activation of the transgene in mice leads to a hyperpigmented phenotype both in embryonic development and in mature mice.

The Knockout Animal Models

Homozygous ET-1 KO mice die within 15-30 minutes after birth due to craniofacial abnormalities resulting in an inability to breath normally [18]. Furthermore they display malformations of cardiovascular system [19], thymus and thyroid [20]. These affected tissues and organs are predominantly formed by cranial neural crest-derived ectomesenchymal cells. No abnormalities in other organs such as the lung, kidney and central nervous system can be observed.

Similar craniofacial and cardiovascular malformations are seen in mice with disruption of the ETAR gene. The important role of ET-1 in skeletal development during intrauterine life was recently confirmed by using prepro-ET-1-lacZ-transgenic mice [21].

The examination of expression patterns of ETARs and ET-1 suggests that ET-1/ETAR interaction is essential in cranial bone and connective tissue formation as well as in the development of the heart and its outflow tract [22].

Heterozygous ET-1 KO mice which have lower serum levels of ET-1 than wild type mice paradoxically develop elevated blood pressure [18]. Enhanced basal and hypercapnia-induced renal sympathetic nerve activity in the heterozygous ET-1 KO mice seem to be involved in elevation of blood pressure [23, 24]. Some other causes that might contribute to blood pressure elevation in heterozygous ET-1 KO mice have been excluded, such as salt sensitivity or respiratory abnormalities [25, 26].

Collecting duct (CD)-specific ET-1 KO mice do not have gross morphologic abnormalities. Plasma ET-1 levels are not affected, however urinary excretion of ET-1 is reduced. CD ET-1 KO mice are hypertensive but show no differences in body weight, urine volume, creatinine clearance, sodium and potassium excretion, urine and plasma osmolality, plasma aldosteron concentration and renin activity. CD ET-1 KO mice are salt-sensitive and exhibit reduced sodium excretion during the first three days of high salt diet. Amiloride and furosemide are able to prevent sodium retention and exacerbation of hypertension. However, they do not reduce blood pressure in CD ET-1 KO mice on a normal sodium diet [27].

Plasma vasopressin (AVP) concentrations are substantially reduced in CD ET-1 KO mice, despite all other aspects of water metabolism being similar. The response to continuous infusion of 1-deamin-8-D-arginine vasopressin is stronger in CD ET-1 KO mice. There seems to be an increased renal sensitivity to the effects of AVP suggesting that ET-1 acts as a physiological autocrine regulator of AVP action in the collecting duct [28].

Thus, CD-derived ET-1 seems to be an important physiological regulator of renal salt and water handling and systemic blood pressure.

CD-specific KO of the ETAR has no effect on blood pressure or urinary sodium excretion in mice, independently of salt intake. Those mice have increased plasma AVP levels but do not show differences of renal water excretion under baseline conditions. However they have a more rapid decrease in urine osmolality following an acute water loading. During exogenous AVP infusion CD ETAR KO mice increase urine osmolality similar to WT mice but have a more rapid subsequent fall in urine osmolality during sustained AVP administration. The lower AVP responsiveness in CD ETAR KO mice is contradictory to the results seen in CD ET-1 KO mice. These animals have elevated AVP sensitivity, and impaired ability to excrete an acute water load. This implies that ET-1 must exert its influence on vasopressin through CD ETBR and/or through paracrine effects [29].

At 6 month of age mice with disruption of the ET-1 gene in cardiomyocytes (CM ET-1 KO) have a significantly lower fractional shortening and develop a dilated left ventricle (LV). During the next month the left ventricle systolic function of CM ET-1 KO mice declines and ultimately, they die at much younger ages (median life expectancy of CM ET-1 KO: 11 months, WT: 2 years). Histological characterization of the CM ET-1 KO hearts reveal dilated heart chambers with heterogeneity of myocyte size, increased fibrosis and raised apoptosis.

At the age of 2 months, aortic banding leads to dilated cardiomyopathy in CM ET-1 KO mice but to LV hypertrophy in WT mice [30].

Transcriptional and Western blot analyses suggest that increased apoptosis in CM ET-1 KO is mediated by enhanced activity of tumor necrosis factor (TNF). CM ET-1 KO hearts also have diminished NF-κB activity, amounting to diminution of downstream inhibitors of TNF signaling [30].

CM ET-1 KO mice are resistant to the hypertrophic stress of treatment with thyroid hormone. While mice with intact ET-1 gene develop a pronounced LV hypertrophy, CM ET-1 KO mice show only a marginal increase in LV mass.

These findings indicate that the interaction of locally produced ET-1 with thyroid hormone, is essential in the development of thyroid hormone induced myocardial hypertrophy [31].

There are no ET-2 knockout models described in the published literature.

ET-3 KO mice exhibit aganglionic megacolon and pigmentary disorders of the skin and choroidal layer of the retina. These findings indicate that ET-3 is essential in the development of two neural crest-derived cell lineages, epidermal and choroidal melanozytes and enteric ganglion neurons [16, 30, 32].

Blood pressure and heart rate of infant ET-3 KO mice are not different from those in age matched WT mice. These results suggest that ET-3 is not involved in cardiovascular regulation at least in early life before they die of complications of the megacolon [33].

Phenotypes of animals with natural mutations or with a targeted disruption of the ETBR gene are similar to that of ET-3 KO animals [16, 34]. This underlines the importance of ET-3/ETBR-mediated signaling in the development of melanocytes and enteric neurons.

A naturally occurring rodent model of the described phenotype is the spotting lethal (sl/sl) rat which carries a deletion of the ETBR gene.

To establish a viable animal model it was necessary to support a normal enteric nervous system development. ETBR deficient sl/sl rats were rescued by breeding them with rats harboring a dopamine-beta-hydroxylase (DBH)-ETBR transgene. This transgene leads to the development of a normal enteric nervous system. The resulting transgenic rats (DBH-ETBR; ETBRsl/sl) are healthy but present with total absence of ETBR in all non-adrenergic tissues including the kidneys [35]. Those ETBR-deficient rats are normotensive on a sodium-deficient diet, mildly hypertensive on a standard diet and exhibit severe hypertension on a high-sodium diet [36].

Their endothelial function is impaired but it was shown that it is independent of the salt-enriched diet and therefore not responsible for the hypertension [37].

In young sl/sl rats fractional sodium excretion is markedly reduced. When treated with the specific epithelial sodium channel blocker amiloride the animals show increased excretion of sodium [38]. This seems to be due to a lack of the inhibitory property of the ETBR on the epithelial sodium channel activity. This data indicates that a reduced renal ETBR activity might contribute to a salt-sensitive hypertension.

The effect of the ETBR on the progression of diabetic nephropathy was analyzed by rendering the transgenic animals diabetic with streptozotocin. These animals develop severe hypertension and show an enhanced functional renal impairment compared to diabetic WT animals. Further analyses suggested the elevated plasma levels of ET-1 to be responsible for the hypertension and not the renin-angiotensin system or the NO system [39].

In a model of endothelial cell-specific ETBR KO, the animals showed endothelial dysfunction but no hypertension in response to a high salt diet [40]. This points out that the salt-induced hypertension seen in the model of rescued ETBR-deficient rats is not mediated by endothelial cells.

A CD-specific ETBR KO causes salt sensitive hypertension and sodium retention [41]. Taken together with the results from the CD-specific ET-1 KO, these findings point out an ET-1/ETBR axis which has influence on systemic blood pressure at least partially mediated through autocrine inhibition of CD sodium reabsorption. Yet it is not only the renal ETBR which is crucial for the hypertension since ETBR deficient mice with transplanted normal kidneys are hypertensive as well [42].

The highly specific ET converting-enzymes (ECEs) are membrane-bound metalloproteases which catalyse the cleavage of a Trp21 bond in the precursor big-endothelins resulting in active endothelins. Two isoenzymes ECE-1 and ECE-2 have been identified [43, 44] and their role in the ET system was investigated by creating KO models.

Most of the ECE-1 KO animals die in utero because of an embryonic lethal phenotype of cardiac abnormalities. Those severe defects of patterning of the great vessels and formation of the outflow tract are not found to this extent in other KO models of the endothelin system [45].

The surviving mice have craniofacial abnormalities strikingly similar to those of the ET-1 KO and ETAR KO animals. The animals also present with an absence of epidermal melanocytes and enteric neurons of the distal gut, the phenotype known from the ET-3 KO and ETBR KO mice [18, 22]. This combined phenotype corroborates the assumption that ECE-1 by activating both ET-1 and ET-3 in vivo has an influence on the development of distinct subsets of neural crest cell lineages.

The tissue levels of ET-1, ET-2 and ET-3 were measured as whole embryo extracts and compared to those of WT animals. ET-3 is markedly reduced whereas the levels of ET-1 and ET-2 are still about 50% of the WT mice. However, biologically active ET seems not to be sufficiently produced at the sites crucial for normal embryonic development.

Unlike the ECE-1 KO mice, animals with a homozygous KO of ECE-2 show no embryonic developmental disorders. They are healthy, fertile and have a normal life span [46].

A cross breeding of the two knockout lines brought out animals with a lack of both ECE genes [46]. These ECE1 -/-; ECE2 -/- animals show a similar but more profound embryonic ECE-1 KO phenotype with the same amount of ET-1 and ET-2 as in the ECE-1 KO suggesting yet another protease to be also responsible for the activation of ETs.

Transgenic Models	Phenotype	References
ET-1+/+	kidney: renal cysts, renal interstitial fibrosis, glomerulosclerosis lung: fibrosis and chronic inflammation	[2, 3, 12]
endothelium specific ET+/+	vascular remodelling and endothelial dysfunction	[13]
cardiac specific ET+/+	inflammatory cardiomyopathy	[14]
ET-2+/+	glomerulosclerosis	[15]
ET-3+/+	hyperpigmentation	[17]
ET-1+/+eNOS-/-	elevated blood pressure	[11]

Knockout Models	Phenotype	References
ET-1-/-	craniopharyngeal and cardiovascular malformations	[18, 19, 20]
ET-1+/-	elevated blood pressure	[18, 23, 24, 25]
collecting duct-specific ET-1-/-	elevated blood pressure	[27, 28]
cardiomyocyte-specific ET-1-/-	dilatative cardiomyopathy	[30, 31]
ET-3-/-	aganglionic megacolon, pigmentary disorders	[16, 32]
ETAR-/-	craniopharyngeal and cardiovascular malformations	[21]
ETBR-/-	aganglionic megacolon, pigmentary disorders	[16, 34]
rescued ETBR-/-	salt-sensitive hypertension	[35, 36, 38]
diabetic rescued ETBR-/-	low-renin hypertension, progressive renal failure	[39]
endothelial cell-specific ETBR-/-	endothelial dysfunction, normotensive on high-salt diet	[40]
collecting duct-specific ETBR-/-	elevated blood pressure, increasing blood pressure and impaired sodium excretion on high-salt diet	[41]
ECE-1-/-	severe cardiac developmental disorders, craniofacial abnormalities, aganglionic megacolon, pigmentary disorders	[45]
ECE-2-/-	healthy phenotype	[46]
ECE-1-/-;ECE-2-/-	worsened ECE-1-/- embryonic phenotype	[46]

Conclusion

Taken together, results from the different transgenic and knockout models disclose that the ET system plays a major role in embryonic development. Two ET system-dependent neural crest-driven developmental pathways become obvious: one of them being an ET-1/ETAR axis, responsible for normal cardiac and cranial development [47]; the other seems to be a ET-3/ETBR mediated signalling pathway. Mutations within this axis are associated with disruptions in epidermal melanocytes and enteric neurons. These findings led to the discovery of similar findings in humans with Hirschsprung disease [48, 49].

In adult life the ET system is most important in the renal and cardiovascular system as shown by a chronically activated ET system which results in a blood pressure-independent fibrosis of kidneys and lungs. Especially collecting duct-derived ET-1 seems to have influence on the physiologic regulation of renal salt and water handling and systemic blood pressure via ETBR.

The creation and investigation of genetically modified animals has contributed significantly to the understanding of ET function in vivo.

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