Review

Endothelin as a natriuretic hormone: the case for a paracrine action mediated by nitric oxide

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1. Introduction

There is a widely held view that function of the kidney is the key to understanding human essential hypertension. Indeed, all of the currently recognised human monogenic causes of hypertension reflect abnormalities in the genes that determine ion transport in the distal portion of the nephron [1]. It is also recognised that permanent elevation of blood pressure in experimental models of hypertension, and both primary and secondary hypertension in man, requires the shifting of the natriuretic response curve of the kidney towards higher pressures [2]. However, the mechanism underlying pressure-natriuresis, and its resetting in hypertension, remains poorly understood. In this review we have analysed experiments indicating a role for endothelin (ET)-1 as a key natriuretic hormone and propose a new and testable hypothesis for a paracrine action of ET-1 through NO that accommodates the existing experimental findings.

ET-1 is a 21 amino acid peptide, synthesised mainly by endothelial cells (ECs) and produced from an inactive precursor peptide by several rounds of specific proteolysis. The last step is performed by endothelin converting enzyme (ECE), which releases ET-1 from big ET-1. ET-1 acts in an autocrine and paracrine fashion on two subtypes of ET receptors, termed the ETA (ETAR) and ETB receptors (ETBR). Both ETAR and ETBR are located on vascular smooth muscle cells and binding of ET-1 to these receptors results in sustained vasoconstriction. ETBR are also present on ECs where their activation results in the production of NO and dilator prostanoids, and subsequent vasodilatation. The ET system is now known to play an important role in the regulation of vascular tone and blood pressure [3,4].

However, the role of ET-1 produced and secreted in the kidney may be independent of the systemic vascular effects. Analysis of different tissues found the highest concentrations of immunoreactive ET-1 in the inner medulla of the kidney, while the concentrations in kidney cortex are very low [5]. Kohan was the first to show that high levels of ET-1 are produced by inner medullary collecting duct cells (IMCDs) in culture [6]. Later, ETARs and ETBRs were characterised in IMCD [7]. On this basis, an autocrine model of endothelin action in the kidney was proposed [8]. There is now growing evidence for an intra-renal ET system that is central to the control of water and salt homeostasis, and to the regulation of blood pressure [9]. Abnormalities of this system have been found in experimental forms of hypertension, and may also be important in the pathogenesis of human hypertension, heart failure and kidney failure, in all of which sodium balance is also critical [4]. Indeed, there is evidence that endothelin receptor antagonists effectively reduce blood pressure in human essential hypertension [10].

2. Physiology of the ET system in the kidney medulla: interaction with NO

Extensive functional studies support a natriuretic and diuretic action of ET-1 in the renal medulla, related to sodium transport functions. By intravenous infusion of ET-1 in anaesthetised rats, it has been shown that low doses cause natriuresis due to reduced sodium transport in the proximal and distal nephron segments and that only higher doses result in sodium retention due to glomerular...
vasoconstriction [11]. In experiments conducted on anaesthetised dogs with ETA and ETB specific antagonists, it has been shown that the ETAR predominantly accounts for the renal vasoconstriction, whilst the ETBR is largely responsible for diuresis and natriuresis [12].

ET-1 and anti-diuretic hormone (ADH) have opposing effects in the control of salt and water regulation in the nephron. Nanomolar concentrations of ET-1 reduce ADH-stimulated increases in water permeability of the collecting ducts in rats [13]. Studies in primary cultures of IMCDs also demonstrate that ET-1, acting via ETBRs, downregulates the activity of the epithelial sodium channel (ENaC) and Na/K ATPase [14]. This effect is mediated by an ETBR-stimulated reduction in cAMP levels [15]. The synthesis and secretion of ET-1 from a collecting duct cell line was upregulated by hypertonic extracellular media [16]. In turn, ADH down regulates ETBR, but not ETAR, density in isolated IMCDs [17]. Several studies document increased ET-1 production in IMCDs induced by increased osmolarity, and specifically by increased concentrations of sodium ions [18,19]. In addition, hypoxia can mediate ET-1 secretion in IMCDs [20], and this may suppress renin gene transcription during long-term ischaemia, leading to glomerular vasodilatation. However, the opposite effect was seen following short-term ischaemia. Thus, at present, there probably remains insufficient evidence from which to make any firm conclusions regarding the hypoxia-mediated effects of ET-1 in the kidney [21].

2.1. Lessons from experimental models

Recent work utilising transgenic animal models with dysfunctional ETBR genes has confirmed that the predominant role of the ETBR in the kidney is one of tonic inhibition of sodium reabsorption, possibly via inhibition of ENaC [22]. A naturally occurring 301 base pair deletion of the coding sequence of the ETBR gene is found in spotting lethal rats (sl) [23]. Gariepy and colleagues ‘rescued’ homozygous sl rats by expression of an ETBR transgene under a dopamine-β-hydroxylase promoter (DβH) [24]. The resultant DβH-ETB; sl/sl rats only express functional ETBRs in ganglionic cells of the intestine (preventing the development of fatal intestinal obstruction) and in adrenergic tissues. The lack of functional vascular ETBRs in DβH-ETB; sl/sl rats is supported by an absence of the normal early ETBR-mediated transient hypotensive response following bolus ET-1 administration [22]. On either a normal or high (8% NaCl) salt diet, DβH-ETB; sl/sl rats were found to develop severe hypertension (170 mmHg systolic). The rise in blood pressure was reversed to normal by the specific ENaC inhibitor, amiloride. ETAR blockade with the selective antagonist FR139317 only modestly reduced blood pressure. Although it is most likely, that ETBR deficiency in this model is translated into reduced ability to excrete sodium, normalisation of blood pressure by amiloride of itself does not prove it. Also, elevated blood pressure caused by volume expansion or systemic vasoconstriction can be normalised by diuretics like amiloride. In addition, increased plasma ET-1 concentrations in rescued ETBR−/− mutants complicates interpretation of the results. Indeed, plasma ET-1 concentrations in DβH-ETB; sl/sl rats were between 5 and 6 times higher than those of DβH-ETB; ETB+/+ rats. The authors attribute this to the absence of ‘scavenging’ endothelial ETBRs in DβH-ETB; sl/sl rats. Nevertheless, such a rise in plasma ET-1 concentrations may have caused a significant increase in vascular resistance through activation of ETAR on vascular smooth muscle cells, including those of the glomerulus. Indeed, the observed partial normalisation of blood pressure in DβH-ETB; sl/sl rats by acute injection of an ETAR antagonist supports this possibility. In further support of this interpretation, it was recently reported that chronic treatment with ABT-627, an ET(A) receptor antagonist, completely suppresses DOCA-salt-induced hypertension in DβH-ETB; sl/sl and wild type rats [25].

Two mouse models of ETBR deficiency exist. One is similar to the DβH-ETB; sl/sl rat, but with knocked out ETB alleles. Salt sensitive hypertension was reported in DβH-ETB; ETB−/− mice. No data on plasma ET-1 concentrations are available [26]. Another mouse model was produced by breeding ETBR+/− animals to piebald (s) mutants, which are a mouse model similar to the sl rat. This resulted in 1/8 of the normal expression of ETBR in s/− animals. Mean BP in ETB s/+ animals was 20 mm higher than in s/+ or wild type controls on a normal salt diet. Plasma concentrations of ET-1 in s/− and s/+ animals were not significantly different but were higher than in wild type controls [27].

2.2. Functional interactions of ETBR, nNOS and eNOS in renal medulla

A potent inhibitory action of NO on tubular sodium reabsorption is well described [28]. Given that components of the ET and NO systems are present within the renal tubular system, and that an important interaction between ET and NO is already known to exist in the peripheral vasculature, it would not be unreasonable to propose an interaction between these systems in the kidney. Indeed, a number of possible interactions between the renal ET and NO system were recently summarised by Plato and Garvin [29]. The same authors have demonstrated inhibition of chloride transport in the thick ascending limb of Henle by endogenous nitric oxide [30]. Since then, important new data have been published showing that the natriuretic action of ET-1 is mediated by NO in the renal medulla. In this study, the NO synthase inhibitor, N-nitro-l-arginine methyl ester (l-NAME), and the specific ETBR antagonist, A-192621, both acted independently to abolish the diuretic and natriuretic effects of big ET-1 in the kidneys of anaesthetised rats [31]. Previously the same group has...
shown that ET-1 increases renal medullary blood flow in NO dependent manner [32].

Although these experiments demonstrate an NO mediated diuretic and natriuretic action of ET-1 in the kidney, it is not possible to conclude which cell type is the major source of NO, and which isoform of NO synthase is responsible for these effects. All three isoforms — endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) — are present in the kidney. iNOS plays an important role during inflammation but, at present, there is no evidence that it can be activated by ET-1 or other vasoactive hormones, and, because it is not regulated, we have not considered iNOS further. It is, however, important to distinguish whether ETBR are linked to eNOS or nNOS. As there are no highly specific inhibitors of these isoforms, pharmacological approaches are unlikely to be helpful.

There is substantial evidence that, in the cortical collecting ducts (CCD), NO inhibits ADH stimulated sodium reabsorption [33] and hydro-osmotic water permeability [34]. There is also a preliminary report that ET-1 stimulates NO release from IMCDs [35] and the only published work, showing increased ET-1/ETBR stimulated cGMP production in rat IMCDs, provides indirect support for this link [17]. The group that discovered high NOS activity in the IMCD [36], has also demonstrated that intra-medullary injection of antisense oligonucleotides specific to nNOS promotes a modest salt dependent rise in blood pressure in rats [37]. Given that the ETBR is expressed in IMCDs, nNOS in the same location could be the target for autocrine activation by ET-1. However, involvement of nNOS in the regulation of extracellular volume and blood pressure is not supported by knock out experiments [38,39]. In fact, mean arterial pressure in anaesthetised nNOS knock out mice (nNOS−/−) is slightly lower then in wild type controls [40]. In contrast, eNOS−/− mice are hypertensive [41–43], but no direct data on salt dependence of blood pressure in either knock out has been published. However, indirect evidence suggests that eNOS−/− animals, but not nNOS knock outs or wild type controls, accumulate salt, reflected in their lower mRNA levels and renal renin content. In addition, l-NAME reduced renal renin mRNA levels in wild type and nNOS−/− mice, but not in eNOS−/− mice [44]. The relative importance of eNOS over nNOS in sodium excretion by the thick ascending limb was also demonstrated in another study using knock out strains. Here, the NO synthase substrate, l-arginine, inhibited chloride transport in the isolated thick ascending limb in wild type as well as nNOS−/− mice, whereas in eNOS−/− mice, l-arginine has no effect [40]. Taken together, these data indicate that eNOS is likely to be the major regulator of renal sodium reabsorption, potentially under the regulation of ET-1. As with all G-protein coupled receptors ETBR can activate the effector enzyme (nNOS or eNOS) only when they are expressed in the same cell. The important question is: what are the target cells for ET-1 action that respond by major NO production in the renal medulla?

3. Localisation of the ET system and NOS in the kidney

To answer this question, we have compared cellular expression patterns of the elements of ET system and NOS. The results are summarised in Table 1. The major source of ET-1 in the renal medulla is IMCDs [5,8,45]. Detailed light and electron microscopic (EM) investigation of radioactively labelled ET-1 binding to ETAR and ETBR comes from two independent laboratories [46,47]. Both groups report predominant expression of ETAR over ETBR in renal medulla, with intense staining of the inner stripes of medulla and papillae. Binding sites were in the glomeruli and in the vasa recta, the peritubular capillaries that penetrate deep into the medulla [47]. Using in vivo [125I]ET-1 binding and EM-autoradiography, ETBR specific binding was localised almost exclusively to the fenestrated ECs of glomerular capillaries and peritubular capillaries, with no appreciable binding in cells of

Table 1

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<th>Inner Medulla</th>
<th>Endothelial Cells</th>
<th>IMCDs</th>
<th>Loop of Henle</th>
<th>RMIC</th>
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<tr>
<td>n+eNOS</td>
<td>ND</td>
<td>NP , IHC [55]</td>
<td>++ + , RT [36]</td>
<td>++ + , RT [36]</td>
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n+eNOS ++ + , enzyme activity [36]

++ + , high; ++ , moderate; + , low; NP , not present; ND , not determined. IHC, immunohistochemistry; RIA, radioimmunoassay; RL, radioligand binding; RLEM, radioligand electron microscopy; RMIC, renal medullary interstitial cells; RT, reverse transcriptase polymerisation chain reaction; WB, Western blotting.
glomerular mesangium, renal tubules and the interstitium [48]. In vitro labelling also demonstrated ETBR specific binding to the glomeruli, proximal tubules and renal medulla interstitial cells [49]. Nevertheless, radioligand binding studies do show ETBR on IMCDs, and functional responses to ET-1 in cell culture [17]. There is close contact between the basolateral membrane of the IMCDs and the abluminal plasma membrane of ECs of the vasa recta, separated only by a basal membrane. Interestingly, the ETBR are localised exclusively on the abluminal surface of the capillaries, facing the IMCDs. In vitro binding detected ETBR in the basal infoldings of IMCDs, but only at levels 7-fold lower than in cells of the vasa recta [47].

As noted earlier, the highest levels of expression of ETBR are found in ECs, particularly in the vasa recta and peri-tubular capillaries of the renal medulla. Biochemical analysis of micro-dissected renal vessels reveals the highest levels of calmodulin-dependent NO synthase activity in vasa recta. These are 3-fold greater than in glomeruli or afferent arterioles and more than 20-fold higher than in arcuate and interlobular arteries. Both nNOS and eNOS mRNA were identified in each vascular segment by RT-PCR [50]. These data correlate well with strong histological staining in transgenic animals expressing a beta-galactosidase histological marker under a 5.2 kb eNOS promoter [51]. It should be noted here that eNOS expression is usually present where there is an interface between ECs and smooth muscle cells and, therefore, usually absent in the capillaries. In this regard, the vasa recta are a notable exception in showing strong eNOS expression. In contrast, beta-galactosidase staining was not found in the renal tubular elements of the medulla. A very similar pattern of staining has been shown using antibodies for eNOS [52], but with this technique it is difficult to distinguish exact localisation of the binding.

Evidence of expression of eNOS and nNOS in the IMCDs, the second strongest site of expression of ETBR in the renal medulla, is limited. Little evidence has been found of NO synthases in the collecting ducts by diaphorase staining, whereas it was detected in thick ascending limbs by this technique and by anti-eNOS antibody. nNOS was detected in nerve fibres and tubular epithelium by anti-nNOS antibody, although staining was much less intense in the latter [53]. By contrast, nNOS has been clearly shown, whereas eNOS was not detected, in isolated CCD of the rat [54]. No eNOS has been detected in the renal tubules of the rat [55], whereas there is strong staining with an anti-eNOS antibody in vessels of the kidney. In the same study, nNOS staining was present in macula densa and in the thick ascending limb of the nephron. These data are consistent with the negative staining in renal tubules and collecting ducts with a beta-galactosidase marker driven by an eNOS promoter [51].

An extensive biochemical analysis of NO synthase activity in micro-dissected segments of the nephron and kidney vasculature has been performed [36]. The highest NOS activity per mm length of the segment was found in IMCDs and in vasa recta, with very low activities in CCD, outer medullary collecting duct, and thick and thin segments of the loop of Henle. Both nNOS and eNOS mRNA was detected by RT-PCR in IMCD and vasa recta preparations. However, nNOS was the predominant isoenzyme in IMCDs, and eNOS in vasa recta. It has to be taken into account that cross-contamination cannot be excluded in this type of micro-dissection procedure and preparation of collecting ducts uncontaminated with adjacent cells was found to be impossible even with laser capture microdissection (LCM) [56]. Fluorescent immunostaining specific for nNOS was demonstrated in isolated IMCDs of the rat and an increase in nNOS protein was detected by Western blot analysis after exposure to a high sodium diet [57].

Morphological analysis indicates that ECs of vasa recta are a major site of expression of ETBR and eNOS (Table 1). Vasa recta ECs are in close proximity to, and are likely to be a major target of, ET-1 produced and secreted by IMCD. Physiological experiments in knock out animals, discussed earlier, suggest that inactivation of either ETBR or of the eNOS gene, but not nNOS or nNOS, have major effects on blood pressure and ion transport in the nephron. Current evidence suggests that ET-1 produced locally within the kidney is involved in the regulation of sodium reabsorption, with ET-1 producing both natriuresis and diuresis. Taken together these facts prompted us to formulate the following hypothesis.

4. Hypothesis and its implications

We hypothesise (Fig. 1) that ET-1 is a paracrine natriuretic and diuretic hormone, produced by IMCDs and acting mainly on ETBR of ECs of the vasa recta to activate eNOS and generate NO. In turn, NO acts simultaneously on IMCD cells to reduce sodium transport and on vasa recta to stimulate blood flow and inhibit the counter-current mechanism. Together, these effects act to reduce sodium reabsorption and enhance sodium excretion.

The novelty of this hypothesis constitutes the assumption that ET-1 is a paracrine hormone secreted by IMCDs that activates ETBR mainly on the ECs of vasa recta. This view contrasts with an autocrine model in which ET-1 is secreted by and activates ETBR of IMCD cells, while ETBR of the renal medullary microvasculature are activated by ET-1 produced by ECs. It also implies that the capillary bed is the major source of NO, acting as an intercellular mediator between ECs of renal medullary capillaries and the tubular epithelium. This hypothesis fits with the observation that the ligand (ET-1) is primarily generated by IMCDs and that the greatest expression of ETBR is on the adjacent capillary ECs. It is also consistent with the phenotypes of NOS knock out animals.
Fig. 1. In the kidney, ET-1 is produced by inner medullary collecting duct cells (IMCDs) and acts mainly on ETB receptors (ETBR) of the endothelial cells (ECs) of vasa recta capillaries, where it stimulates endothelial nitric oxide synthase (eNOS) to generate NO from l-arginine (L-Arg). NO acts on guanylate cyclase (GC) in IMCDs to produce cyclic GMP (cGMP) that acts in turn to reduce ENaC activity, leading to enhanced sodium and water excretion. Simultaneously, NO acts on smooth muscle cells and pericytes surrounding vasa recta (not shown) to enhance blood flow. This alteration of blood flow, in turn, reduces the axial gradient of sodium chloride (NaCl) in the renal medulla and leads to natriuresis and diuresis.

Excretion of salt and water can be augmented by two processes that reduce urinary sodium reabsorption; one, a reduction in tubular sodium transport and the other, an increase in blood flow to the vasa recta. The ability of the kidney to excrete a concentrated urine crucially depends on an axial gradient of osmolarity in the renal medulla. This gradient is produced by a counter-current mechanism of guanylate cyclase Ð in adjacent cells and simultaneously affect ion transport in different segments of the nephron as well as influence medullary blood flow. This unique property of NO makes it an ideal local inter-cellular mediator, and would be superfluous in the autocrine signalling model.

5. Opportunities for further experiments

As was discussed earlier, ET-1 can increase blood flow in the renal medulla, and this is NO dependent [31,32]. It was suggested that contractile properties of renal medullary interstitial cells regulate blood flow in medullary capillaries [59]. As well as regulating sodium excretion, there is a growing body of evidence that the extraglomerular microcirculation of the kidney plays an important role in pressure-natriuresis [2,60]. An important advantage of the paracrine hypothesis is that it explains a reciprocal feedback mechanism between blood flow in the vasa recta and sodium reabsorption in the tubular epithelium. Indeed, both systems can react coherently if they respond to mutual signals; that is, ET-1 secreted by IMCD cells and NO produced by ECs. The primary signal for ET-1 secretion from IMCDs is an increased osmolarity of the interstitial fluid [16]. The main effect of ET-1 secretion in renal medulla will be on ETBR of vasa recta to generate sufficient NO to block sodium reabsorption. On the other hand, increased blood flow in the vasa recta caused by increased systemic blood pressure could be a primary signal for the activation of capillary eNOS, NO dependent blockade of ENaC and consequent natriuresis. A distinctive feature of NO is its fast diffusion rate and high permeability. This means that NO produced in endothelial or epithelial cells can efficiently reach its target — guanylate cyclase — in adjacent cells and simultaneously affect ion transport in different segments of the nephron as well as influence medullary blood flow. This unique property of NO makes it an ideal local inter-cellular mediator, and would be superfluous in the autocrine signalling model.
Yanagisawa’s group [63]. A line expressing Cre recombinase in a tissue-specific manner in IMCDs (Aquaporin-2 Cre; AQPN2) has been generated by Professor D Kohan [64]. AQPN2 Cre enzyme expression is highly specific and restricted to principal cells of the collecting duct. Efficient deletion of sequenced flanked with lox P sites by AQPN2-Cre enzyme in the renal medulla has been confirmed using specific histological markers [65]. By cross-breeding these lines, one can selectively delete functional ETBR in a tissue-specific manner; in the case of the Tie-2 and AQPN2 models, this would delete ETBR in ECs and IMCDs respectively. These models will allow us to address the relative contribution of ETBR expressed in ECs and IMCDs to the regulation of ET-1 stimulated natriuresis.

Comparison of three transgenic lines will be required: mice with ETBR expression down regulated in ECs only; mice with ETBR expression down regulated in IMCDs only; and the ‘rescued’ ΔβH-ETB−/− mice. Simple biochemical measurement of ET-1 stimulated NOS activity in these lines will demonstrate the partial contribution of each cell type to NO production. In addition, if the paracrine model is correct, then down regulation of ETBR in ECs will have a major effect on big ET-1 stimulated, L-NAME sensitive sodium excretion [31]. If, on the contrary, the autocrine model is correct, then NO produced by eNOS within ECs of the vasa recta will not be involved in ET-1 mediated inhibition of tubular sodium transport. In this case, mice with reduced expression of ETBR in the collecting duct will exhibit impaired ET-1 stimulated sodium excretion.

If the paracrine theory holds, then the important question will be of the role of ETBR on IMCDs. It is possible that these receptors regulate ET-1 secretion. In transgenic animals with deleted ETBR in IMCDs, EC ETBRs will be expressed normally and hence we would not anticipate an increase in plasma ET-1 concentrations, as occurs in transgenic ΔβH-ETB; sl/sl rats and ‘rescued’ piebald mice. Here, EC and smooth muscle cell ETBR in the vasculature will remain intact, allowing us to investigate the effect of tubular ETBRs on salt and water excretion without the confounding effects of deletion or blockade of vascular ETBR. We can also monitor modulation of ET-1 levels, as a measure of ET-1 secretion, in plasma, urine and renal medulla during normal and high salt consumption [66]. Analysis of pressure-natriuresis relationships in these lines might allow evaluation of the relative contribution of ETBR expressed in IMCDs and ECs in regulation of the acute response to volume expansion and to increased systemic blood pressure.

If NO synthases from the vasa recta ECs are critical for sodium homeostasis, then it will be very important to analyse salt sensitivity of blood pressure in eNOS−/− and nNOS−/− animals, as well as in double knock out animals, which have already been generated [44], particularly because inhibition of kidney nNOS with antisense oligonucleotides suggests that nNOS deficient animals may manifest salt sensitive hypertension [37]. In addition, crossing tissue-specific ETBR knock out animals to eNOS−/− and nNOS−/− animals will provide a further tool to determine the precise role of these isoforms in ET-1 mediated control of sodium excretion.

The mechanism of maintenance of salt and water homeostasis was developed by an evolutionary process, and discordant and superfluous responses shall have been eliminated by the process of the natural selection. The challenge now is to understand how coherence is achieved, at least in relation to the function of three genes (nNOS, eNOS and ETBR) acting in two interacting cell types.

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References


