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REVIEW

Endothelial actions of atrial and B-type natriuretic peptides

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The cardiac hormone atrial natriuretic peptide (ANP) is critically involved in the maintenance of arterial blood pressure and intravascular volume homeostasis. Its cGMP-producing GC-A receptor is densely expressed in the microvascular endothelium of the lung and systemic circulation, but the functional relevance is controversial. Some studies reported that ANP stimulates endothelial cell permeability, whereas others described that the peptide attenuates endothelial barrier dysfunction provoked by inflammatory agents such as thrombin or histamine. Many studies *in vitro* addressed the effects of ANP on endothelial proliferation and migration. Again, both pro- and anti-angiogenic properties were described. To unravel the role of the endothelial actions of ANP *in vivo*, we inactivated the murine *GC-A* gene selectively in endothelial cells by homologous loxP/Cre-mediated recombination. Our studies in these mice indicate that ANP, via endothelial GC-A, increases endothelial albumin permeability in the microcirculation of the skin and skeletal muscle. This effect is critically involved in the *endocrine* hypovolaemic, hypotensive actions of the cardiac hormone. On the other hand the homologous GC-A-activating B-type NP (BNP), which is produced by cardiac myocytes and many other cell types in response to stressors such as hypoxia, possibly exerts more paracrine than endocrine actions. For instance, within the ischaemic skeletal muscle BNP released from activated satellite cells can improve the regeneration of neighbouring endothelia. This review will focus on recent advancements in our understanding of endothelial NP/GC-A signalling in the pulmonary versus systemic circulation. It will discuss possible mechanisms accounting for the discrepant observations made for the endothelial actions of this hormone-receptor system and distinguish between (patho)physiological and pharmacological actions. Lastly it will emphasize the potential therapeutical implications derived from the actions of NPs on endothelial permeability and regeneration.

Abbreviations

ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide; cGKI, cGMP-dependent protein kinase; VASP, vasodilator-stimulated phosphoprotein

Introduction

The heart secretes two natriuretic peptides (NPs), atrial (ANPs) and B-type NPs (BNPs) (de Bold *et al.*, 1981; Brenner *et al.*, 1990), which activate a common GC-A receptor expressed in a wide variety of tissues and cell types (reviewed by Kuhn, 2003). GC-A [also known as NP receptor A (NPR-A)] is a transmembrane receptor with an intracellular GC domain (Alexander *et al.*, 2011). It synthesizes the second messenger cGMP upon binding of the ligands (ANP with ~10-fold higher affinity than BNP) to its extracellular part (Figure 1) (Drewett and Garbers, 1994). ANP is secreted from atrial granules into the circulation in response to acute or chronic atrial stretch to physiologically act as antihypertensive and antihypervolaemic factor via GC-A in distant organs (de Bold *et al.*, 2001). In contrast, cardiac BNP is constitutively expressed in ventricular myocytes (de Bold *et al.*, 2001). Normally cardiac and plasma BNP levels are very low, but they markedly increase under pathological conditions of pressure or volume overload provoking enhanced cardiac wall tension. A second specific receptor subtype for both NPs is the C receptor (NPR-C, encoded by the *NPR3* gene), a 'clearance' receptor that is devoid of guanylyl cyclase activity and which mediates the cellular internalization and degradation of NPs. Studies conducted in intestinal smooth muscle cells (SMCs) suggested that this receptor may also participate in mediating some of the cellular actions of NPs by means of coupling to G_i proteins and negative modulation of adenylyl cyclase activity

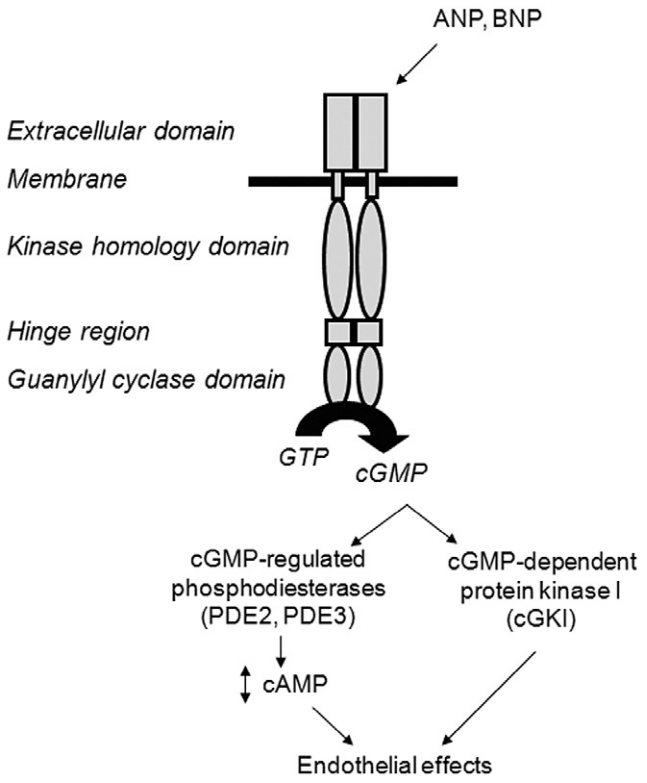

Figure 1

Basic topology of the transmembrane GC-A receptor for ANP and BNP. A single transmembrane segment separates an extracellular domain from intracellular protein kinase – like, hinge, and cyclase catalytic domains. GC-A forms homodimers or higher ordered structures. In general, three cGMP-modulated proteins (as third messengers) are expressed in endothelial cells: cGMP-stimulated PDE 2, a dual substrate esterase, which appears to hydrolyse cGMP under resting conditions but targets cAMP in the presence of stimulation; cGMP-inhibited PDE 3; and cGMP-dependent protein kinase I (cGKI).

(Murthy *et al.*, 2000). However, the hypotensive and hypovolaemic phenotype of mice with ablated NPR-C indicates that at least within the cardiovascular system this receptor mainly functions to regulate circulating and local NP concentrations (Matsukawa *et al.*, 1999). A third 'natriuretic' peptide, C-type NP (CNP), acting via its GC-B receptor (also known as NPR-B), probably is not involved in body fluid control and blood pressure, at least under physiological conditions. Instead, the CNP/GC-B system activates growth plate chondrocyte proliferation and differentiation within the bone, as well as cell proliferation/differentiation within the neuronal and vascular systems (Chusho *et al.*, 2001; Tamura *et al.*, 2004). Here, CNP is secreted from endothelial cells and possibly exerts auto/paracrine actions on vascular remodelling and tone (Doi *et al.*, 2001). Importantly, NPs are the only agonists able to stimulate GC-A (ANP and BNP) and GC-B (CNP). For the lack of space, this review will focus on the endothelial actions of ANP/BNP and their shared GC-A receptor.

The important physiological role of the ANP/GC-A system in the maintenace of arterial blood pressure and intravascular volume homeostasis was emphasized by the phenotype of monogenetic mouse models. Targeted deletion of the murine genes encoding for the peptide ($ANP^{-/-}$) or its receptor ($GC\text{-}A^{-/-}$), leads to chronic arterial hypertension and hypervolaemia (John *et al.*, 1995; Lopez *et al.*, 1995). Remarkably, hypervolaemic hypertension in GC-$A^{-/-}$ mice is apparent even under conditions of normal dietary salt intake (Lopez *et al.*, 1995). The physiological role of BNP is less clear. Intriguingly, although both NPs signal through the same receptor, mice without BNP exhibit a phenotype different from that of ANP-deficient mice. Whereas BNP-deficient mice do not have hypertension or cardiac hypertrophy, they are susceptible to cardiac fibrosis (Tamura *et al.*, 2000). Thus, it is possible that BNP mainly acts as a local paracrine factor modulating cellular proliferation and tissue remodelling within the heart as well as in other tissues (where it is expressed, for instance, in response to hypoxia, as described in the following).

The relevance of these experimental observations to normal human physiology has been elegantly established by recent genetic studies that examined the association of common variants at the *ANP*, *BNP* and *NPR-C gene* loci with circulating concentrations of ANP/BNP and arterial blood pressure (Newton-Cheh *et al.*, 2009; Ehret and the International Consortium for Blood Pressure Genome-Wide Association Studies, 2011). The results demonstrate that genetically determined small variations in NP concentrations correlate with significant inverse changes in blood pressure. Of note, these studies could not distinguish whether ANP and/or BNP are linked to blood pressure regulation because both human genes (*NPPA* and *NPPB*) are concomitantly regulated.

The main known GC-A/cGMP-mediated hypotensive and hypovolaemic actions of ANP include: stimulation of renal function, leading to increased natriuresis and diuresis; vasodilatation; modulation of transvascular fluid exchange; increased fluid efflux from the intravascular to the lymphatic system within the spleen; inhibition of the renin-angiotensin-aldosterone system by direct actions on juxtaglomerular cells and the adrenal glomerulosa; and central nervous effects to decrease sympathetic tone, salt appetite and water drinking (comprehensively reviewed by Brenner *et al.*, 1990). However, the relative contribution of these different action sites to the critical physiological role of ANP in the acute and chronic regulation of arterial blood pressure/blood volume remains controversial.

Systemic endothelial hyperpermeability actions of ANP are essential for the maintenance of intravascular volume homeostasis

Already very early investigations in human volunteers and intact animals noted that administration of synthetic, *exogenous* ANP at very low doses causes an acute, immediate contraction of intravascular volume, which appeared to occur well before the ANP-induced urinary losses of fluid and electrolytes (Trippodo and Barbee, 1987; Tucker *et al.*, 1992). Even more, this effect was fully preserved in rats with both the kidney and spleen removed and was accompanied by capillary escape of plasma protein, suggesting that ANP regulates transvascular fluid balance via changes in endothelial permeability (Almeida *et al.*, 1986; Flückiger *et al.*, 1986; Curry *et al.*, 2010;

reviewed by Curry, 2005). Indeed, our intravital microscopy studies in mice showed that synthetic ANP mildly stimulates the extravasation of fluorescently labelled albumin from postcapillary venules within the skin (Schreier *et al.*, 2008) and the cremaster muscle (Chen *et al.*, 2012). This effect is due to real increases in microvascular endothelial permeability, and not to increased clearance due to changes in microvascular perfusion related to ANP-induced vasodilatation (Chen *et al.*, 2012). It is much milder than the effect of inflammatory hormones such as histamine or bradykinin. To dissect the contribution of the vasodilatating versus endothelial permeability effects of ANP to the regulation of systemic blood pressure/volume, we generated two genetic mouse models with conditional, selective deletion of the GC-A receptor either in vascular SMCs or in endothelia (EC). Remarkably, *smooth muscle*-restricted deletion of GC-A in mice (SMC GC-A KO) completely abolished the direct vasodilatating effects of ANP but did not affect resting arterial blood pressure and plasma volume (Holtwick *et al.*, 2002). In contrast, *endothelial*-restricted GC-A deletion preserved ANP vasodilatation but caused mild *chronic*, salt-resistant hypervolaemic hypertension (Sabrane *et al.*, 2005). Physiological and Doppler-echocardiography studies showed that the total intravascular volume of EC *GC-A* KO mice was expanded by 12–14%, despite unaltered renal function. By comparison, in mice of the same genetic background harboring a global, systemic GC-A deletion (GC-A$^{-/-}$) plasma volume was chronically increased by ~30% (Skryabin *et al.*, 2004). The *acute* hypotensive responses to infusion of synthetic ANP were abolished in mice of the three genotypes (with global or conditional deletion of GC-A in EC or SMC). Furthermore, acute intravascular volume expansion, causing a sudden release of *endogenou*s cardiac ANP, did not alter blood pressure levels of control mice, but provoked acute hypertensive reactions in both SMC GC-A KO and EC GC-A KO mice (Holtwick *et al.*, 2002; Sabrane *et al.*, 2005; Schreier *et al.*, 2008). Together, these observations indicate that the heart communicates with the systemic vasculature through the ANP/GC-A signalling pathway. The phenotype of mice with endothelial GC-A deletion suggests that the mild endothelial hyperpermeability actions of ANP are critically involved both in the *acute* and *chronic* maintenance of arterial blood pressure and intravascular volume homeostasis. In contrast, the vasodilatating effect of the peptide seems to be more important for the resetting of *acute* alterations in blood pressure. Is there a general effect of ANP on permeability of the systemic endothelium or does this effect involve the endothelium of specific organs? Magnetic resonance imaging was combined with the double-tracer method for comparison of ANP effects on albumin blood-to-tissue clearances in different mouse tissues (Curry *et al.*, 2010). The results indicate that the microvasculatures of skeletal muscle and skin are the most responsive to ANP modulation of endothelial albumin permeability. Hence, the interstitial space in these organs possibly acts as reversible sink for plasma protein and fluid exchange from blood-to-tissue, thereby continuously contributing to adjust intravascular fluid volume.

Together, these observations corroborate the notion that concerted renal and endothelial effects of ANP cooperate in the regulation of intravascular volume. ANP induced renal fluid loss, initially from the vascular space during renal water excretion concentrates the plasma proteins in the vascular space. Simultaneously, the hormone increases microvascular albumin extravasation (Sabrane *et al.*, 2005; Schreier *et al.*, 2008; Curry *et al.*, 2010; Chen *et al.*, 2012). There is a shift of the concentrated plasma protein from the vascular to the interstitial space mainly in skin and skeletal muscle (Curry *et al.*, 2010), reduced rate of plasma protein concentration, reduced reabsorption from the extravascular space and preferential loss of fluid from the intravascular space. Our observations in EC GC-A KO mice suggest that this unique coordination of renal and endothelial actions of ANP is essential for the chronic maintenance of intravascular volume homeostasis but also contributes to the acute hypovolaemic actions of this cardiac hormone (Sabrane *et al.*, 2005). The physiological role of BNP in this setting is unclear. As already mentioned, BNP-deficient mice do not have hypervolaemic hypertension (Tamura *et al.*, 2000 and pers. comm.) indicating that BNP is less critical than ANP for the maintenance of intravascular volume and blood pressure homeostasis.

The intracellular pathways that link the activation of endothelial GC-A/cGMP to the (mild) stimulation of albumin permeability in the systemic microcirculation remain unclear. In general the transport of plasma proteins and solutes across the endothelium involves two different pathways: one paracellular, through interendothelial junctions, and the other transcellular, via caveolae-mediated vesicular transport (Mehta and Malik, 2006; Komarova and Malik, 2010). In addition, an intact glycocalyx is part of the primary barrier that retains plasma proteins in the vascular space (reviewed by Curry and Adamson, 2010). All three determinants of endothelial barrier functions are specifically regulated by extracellular stimuli and intracellular mediators. In cultured human fetal, umbilical vein endothelial cells (HUVEC), ANP/GC-A-mediated cGMP production stimulates PDE 2 and thereby lowers intracellular cAMP levels (Surapisitchat *et al.*, 2007). Because cAMP regulates the stability of intercellular junctions via the Epac/Rap/Rac/Pak-1 pathway (Adamson *et al.*, 2008), a decrease in cAMP by ANP could raise *para*cellular endothelial permeability. However, it is important to remark that the role of PDE2 in ANP-stimulated permeability up-to-now was only demonstrated in HUVEC (Surapisitchat *et al.*, 2007), cells that probably share very few properties with microvascular endothelia *in situ* (Curry and Adamson, 2010). Nevertheless, a role for cGMP/cAMP crosstalk in the hyperpermeability actions of ANP *in vivo* was suggested by two recent elegant pharmacological studies in mice. Here, inhibition of PDE 4 with rolipram to increase endothelial cAMP and stabilize the endothelial barrier attenuated acute ANP-induced extravasation of iodinated albumin and plasma volume loss (Lin *et al.*, 2011, 2012). Another possible action of cGMP is through its ability to stimulate cGMP-dependent protein kinase I (cGKI) and subsequently the phosphorylation of vasodilator stimulated phosphoprotein (VASP), a protein associated with focal adhesion sites and adherens junctions (Smolenski *et al.*, 2000). However, again the functional relevance of this pathway in the microvascular systemic endothelium *in vivo* has not been demonstrated. In fact, our recent observations in the microcirculation of the mouse cremaster muscle indicate that the *trans*endothelial caveolae-mediated vesicular pathway participates in ANP-stimulated albumin extravasation (Chen *et al.*, 2012). We are presently investigating the postreceptor pathways mediating

increased endothelial albumin transcytosis after ANP/GC-A stimulation. Finally, in an isolated heart preparation, intracoronary infusion of ANP induced marked shedding of the capillary endothelial glycocalyx, as demonstrated by electron microscopy and by quantification of syndecan-1 in the coronary effluent (Bruegger *et al.*, 2005). ANP-induced degradation of the glycocalyx was accompanied by increased coronary fluid extravasation. Together, these observations *in vitro*/*ex vivo*/*in vivo* suggest that ANP modulates different components of the endothelial barrier.

Beyond blood pressure regulation: ANP attenuates pathological lung endothelial hyperpermeability

Confronting seemingly different findings from the literature suggests that ANP acts differently and even in opposing ways on microvascular endothelial permeability in the lung versus organs of the systemic circulation. Oedema of the lungs is one of the most serious complications of cardiac and renal insufficiency. Also, acute hypoxia or inflammatory agents increase vascular permeability and contribute to forms of noncardiogenic pulmonary oedema such as high-altitude pulmonary oedema (HAPE), acute respiratory distress syndrome (ARDS) or oedema provoked by infections of the lung or sepsis. Remarkably, *exogenous* synthetic ANP has been shown to protect from lung injury and endothelial barrier dysfunction in all these experimental noxious conditions. For instance, ANP attenuated pulmonary oedema induced by congestive heart failure in dogs (Riegger *et al.*, 1990) or by lung ischaemia-reperfusion injury in rodents (Dodd-o *et al.*, 2008). In mice, ANP pretreatment protected against lung injury, inflammation and endothelial barrier dysfunction induced by gram-negative bacterial wall LPS or *Staphylococcus Aureus* infection (Birukova *et al.*, 2010; Xing *et al.*, 2011). Clinical studies support the therapeutical relevance of these experimental observations: intravenous ANP infusion improved pulmonary gas exchange and the lung injury score in patients with acute lung injury during mechanical ventilation with positive end-expiratory pressure (Mitaka *et al.*, 1998) and diminished pulmonary oedema and pulmonary vascular permeability in intensive care patients without heart disease (Sakamoto *et al.*, 2010).

Of course in these complex *in vivo* experiments it was impossible to distinguish which cell types were targeted by ANP. In cardiac failure the anti-oedematic actions of ANP could be derived from systemic natriuretic actions and improved cardiac function due to left ventricular unloading. In oedema provoked by infections of the lung or hypoxia, the protective ANP effects could be mediated by the GC-A receptor on inflammatory cells such as macrophages, mast cells or neutrophils (Opgenorth *et al.*, 1990; Wiedermann *et al.*, 1992; Vollmar *et al.*, 1997; Kiemer and Vollmar, 2001; Tsukagoshi *et al.*, 2001). Indicating a direct action on the pulmonary vascular bed, various studies have shown that ANP has a direct anti-oedematic action in isolated perfused lung models subjected to increased capillary pressure, hypoxia or inflammatory stimuli such as detergents or prostaglandins (Inomata *et al.*, 1987; Imamura *et al.*, 1988). Yet, these studies could not distinguish whether the attenuation of interstitial oedema was due to the ANP-provoked vasodilatation, decreasing pulmonary capillary hydrostatic pressure, or to direct endothelial barrier-enhancing (stabilizing) effects. Suggesting the participation of a direct endothelial protective effect, ANP reduced hypoxia, TNF-α, thrombin, or bacterial endotoxin (LPS) – induced paracellular hyperpermeability of pulmonary microvascular and macrovascular endothelial cells cultured on permeable supports (Lofton *et al.*, 1991; Irwin *et al.*, 2005a; Klinger *et al.*, 2006; Scott *et al.*, 2010). Taken together, these numerous studies *in vitro*/*in vivo* indicate that ANP, at least when given as *exogenous* pharmacological agent, exerts endothelial barrier-protecting actions in the pulmonary circulation.

Interestingly, the lung itself might be a site of ANP synthesis. Immunoreactive ANP has been localized in peripheral lung tissue, respiratory epithelium and pulmonary veins (Toshimori *et al.*, 1988). Once more genetic mouse models can help to unravel whether *endogenous* cardiac and/or locally formed ANP or BNP (see following discussion) enhance the barrier properties of the pulmonary microcirculation under (patho)physiological conditions. Mice with targeted disruption of the gene encoding the ANP-degrading enzyme neutral endopeptidase (NEP24.11) showed a greater rise in plasma ANP levels, attenuated pulmonary vascular pressure and less pulmonary vascular albumin and fluid leak during high altitude exposure (Irwin *et al.*, 2005b). On the opposite, high altitude pulmonary oedema was exacerbated in mice with targeted deletion of the peptide ($ANP^{-/-}$), or in rats injected with an antibody against ANP (Irwin *et al.*, 2001). More severe LPS-induced lung injury and vascular leak were observed in $ANP^{-/-}$ mice (Birukova *et al.*, 2010). Again, these experiments could not dissect the cell types mediating the protective ANP effects. The EC GC-A KO mice could provide a valuable experimental tool to dissect *in vivo* whether endothelial actions of endogenous ANP help to stabilize the pulmonary vascular barrier under pathological conditions.

The intracellular pathways that link the activation of endothelial GC-A/cGMP signalling to the attenuation of pathological lung hyperpermeability have been addressed in many *in vitro* studies with cultured macro- and microvascular lung endothelial cells. In general, three cGMP-modulated proteins (as third messengers) are expressed in pulmonary endothelia: cGMP-stimulated PDE 2, a dual substrate esterase, which appears to hydrolyse cGMP under resting conditions but targets cAMP in the presence of stimulation; cGMP-inhibited PDE 3; and cGKI. Unfortunately, the expression levels of these third messengers of cGMP are highly variable depending on the species, source (macro- vs. microvascular, venous vs. arterial), passage, confluency and cell culture conditions of endothelial cells (recently reviewed by Surapisitchat and Beavo, 2011). Even more, their expression levels are modulated by inflammatory mediators (see the following discussion). Therefore this review will emphasize only those studies that combined *in vitro* studies with studies of permeability in isolated perfused lungs or intact animals.

PDE 2

The second messenger cAMP has, in general, endothelial barrier-enhancing effects. Inflammatory agents such as TNF-α and thrombin have been shown to induce the endothelial expression of PDE 2 (Seybold *et al.*, 2005). Subsequent

decreases in endothelial cAMP levels are concomitant to increases in permeability. Accordingly, in isolated mice lungs, PDE 2 inhibition was effective in preventing thrombin-induced lung oedema, indicating an important signalling role of this cGMP target in lung endothelium (Seybold *et al.*, 2005). However, because ANP via GC-A/cGMP would stimulate (not inhibit) PDE2 activity and thereby lower endothelial cAMP levels (Surapisitchat *et al.*, 2007), this pathway cannot mediate barrier-protecting actions of the peptide.

PDE 3

In cultured human macrovascular pulmonary artery endothelial cells (HPAEC), ANP treatment induced time-dependent increases in intracellular cAMP concentrations, indicating inhibition of PDE 3 (Birukova *et al.*, 2008). ANP/cAMP, via PKA-dependent inhibition of Rho and via PKA-independent Epac/Rap1/Rac/PAK1 mediated signalling cascades, prevented thrombin-induced stress fibre formation and detachment of adherens junctions (Birukova *et al.*, 2008). The cytoskeletal Rac effector PAK1 (p21-activated kinase) is intimately involved in cortical actin rearrangement and regulation of actin polymerization. PAK1 is a serine/threonine protein kinase that activates the Arp2/3 complex and initiates peripheral actin polymerization. In addition, the association of activated Epac/PAK1 with focal adhesion complexes may further stimulate Rac signalling critical for ANP-dependent endothelial barrier enhancement (Xing and Birukova, 2010). Of note in mice, molecular inhibition of PAK1 with siRNA supressed the protective effects of ANP treatment against LPS-induced lung injury and endothelial barrier dysfunction (Birukova *et al.*, 2010).

cGK I

Several studies indicate that the cGMP/cGKI pathway might participate in the protecting effects of ANP/GC-A on lung endothelium. In cultured murine lung microvascular endothelial cells ANP, via GC-A/cGMP/cGK I, attenuated H_2O_2 cytotoxity by increasing the expression of catalase and glutathione peroxidase 1, thereby reducing endothelial cytotoxity from reactive oxygen species (ROS) (De Vito *et al.*, 2010). The inhibitory effect of ANP on ROS production has also been shown in isolated perfused lungs (Fürst *et al.*, 2005). However, the exact link between cGKI and the activation of these downstream cascades is unknown.

Pathological lung hyperpermeability is often related to the activation of hormones acting through Gαq-protein coupled receptors, such as thrombin, endothelin-1 (ET-1) or histamine. All these hormones signal via increasing intracellular Ca^{2+}, for instance through phospholipase C/DAG-mediated activation of transient receptor potential (TRP) channels (Kini *et al.*, 2010). An increase in $[Ca^{2+}]_i$ in endothelial cells leads to activation of myosin light chain kinase and endothelial contraction, resulting in decreased barrier function. Importantly, members of both the canonical (TRPC6) and vanilloid (TRPV4) subfamilies of TRP channels have been shown to be negatively regulated by ANP/cGMP and by NO-derived cGMP via cGMP/cGKI-dependent inhibitory phosphorylation (Takahashi *et al.*, 2008; Yin *et al.*, 2008). Even more, endothelial TRP channels are in close proximity to endothelial NO synthase, GC-A and cGKI due to their colocalization in caveolar microdomains (Hong *et al.*, 2008; Klaiber *et al.*, 2011). These observations lead to the hypothesis that inhibition of TRP channel activity and of TRP-induced rises in intracellular Ca^{2+} levels, could provide another possible mechanism how ANP, via cGMP production and cGKI activation, prevents destabilization of the microvascular endothelial lung barrier by inflammatory mediators.

Does ANP exert anti-inflammatory endothelial actions also in the systemic circulation?

Increased endothelial permeability is also characteristic of many systemic diseases and pathological conditions, including atherosclerosis, tumour growth, oedema and sepsis. As mentioned earlier, the physiological stimulatory effect of ANP on endothelial permeability in the skin and skeletal muscle is very mild and different to the actions of inflammatory mediators. Hence, ANP is unlikely to provoke or to contribute to peripheral inflammation. Even more, beyond the direct physiological mild permeability-increasing (volume-regulating) effects on *quiescent* endothelial cells, ANP seems to exert the opposite, namely barrier-enhancing effects on an *inflammation-activated* endothelium in the systemic circulation. In cultured HUVEC ANP prevented barrier disruption by inflammatory agents acting through very different types of receptors and cellular pathways, such as VEGF (Pedram *et al.*, 2002), TNF-α (Kiemer *et al.*, 2002) or histamine (Fürst *et al.*, 2008). At the molecular level, ANP pretreatment attenuated the TNF-α-induced expression of adhesion molecules and monocyte chemoattractant protein-1 by inhibiting NF-kB activation and p38 mitogen-activated protein kinase signalling (Kiemer *et al.*, 2002). It also attenuated histamine-evoked disruption of adherens junctions and stress fibre formation as well as VE-cadherin and myosin light chain (MLC2) phosphorylation (Fürst *et al.*, 2008). However, all these studies were conducted in HUVEC as model system, macrovascular cells of *fetal* origin, which down-regulate specific ANP-modulated signalling pathways (such as cGKI) or up-regulate others (such as the NP clearance receptor, NPR-C) (Smolenski *et al.*, 2000). Also, the expression of critical pathways involved in endothelial barrier functions, such as the glycocalyx and caveolae, is remarkably lower in cultured cells when compared with native microvascular endothelia (Chappell *et al.*, 2009; Curry and Adamson, 2010). Therefore it was crucial to study the relevance of these *in vitro* observations *in vivo*. Fürst *et al.* (2008) showed that intra-arterial application of ANP attenuates the histamine-induced leak of FITC-dextran from the mouse cremaster microcirculation and prevented histamine-provoked systemic fluid extravasation in rats. One potential limitation of this study is that the authors did not study whether this high systemic dose of ANP provoked hypotension and volume contraction, unwarranted effects that could lead to reactive constriction of precapillary arterioles and thereby interfere with actions of histamine in an endothelium-independent way. Taking into account this potential limitation, these *in vivo* studies suggest that synthetic ANP might serve as potential therapeutic option for the prevention of vascular leakage. However, these studies do not unravel whether *endogenous*

ANP and/or BNP act as anti-inflammatory hormones under pathophysiological conditions. Against this possibility, our intravital microscopy studies showed that the acute stimulatory effects of histamine on FITC-albumin or FITC-dextran extravasation from postcapillary venules of the skin or cremaster muscle were not different in mice with global or endothelial GC-A deletion, as compared with respective control mice (Schreier *et al.*, 2008; Chen *et al.*, 2012). Of course these observations do not rule out a protective role for endogenous ANP or BNP in (sub)chronic inflammation, a situation that may enhance the local or systemic expression levels of the peptides (see following discussion) or modulate specific endothelial (post)receptor (postGC-A) signalling pathways, such as the relative PDE2/PDE3 expression levels (Surapisitchat *et al.*, 2007; Surapisitchat and Beavo, 2011).

Effects of ANP and BNP on endothelial regeneration and neovascularization

Regeneration of the endothelium after vascular damage in the adult organism involves different stages, which include the activation of pre-existing mature endothelial cells to proliferation and migration. In contrast to angiogenesis, vasculogenesis is based on the recruitment and incorporation of bone marrow-derived endothelial progenitor cells (EPCs) to sites of neovascularization. The role of the NP/GC-A/cGMP system in angiogenesis and vasculogenesis is even more controversial than the role in permeability. Both enhancing and preventing effects of NPs have been reported. In HUVEC and human coronary arterial endothelial cells, low ANP concentrations stimulated proliferation and migration, whereas high concentrations exerted inhibitory effects (Kook *et al.*, 2003). Proangiogenic actions of ANP and/or BNP were also observed in bovine aortic endothelia (BAEC) (Chen *et al.*, 2008) and microvascular endothelia from rat fat pad capillaries (RFPECs) (Kuhn *et al.*, 2009). Moreover, BNP stimulated the proliferation, adhesion and migration of EPCs (Shmilovich *et al.*, 2009). Intriguingly, other *in vitro* studies showed opposite effects. Here, ANP inhibited the proangiogenic effects of VEGF on cultured macrovascular (BAEC) and microvascular endothelia (human microdermal EC; human retinal EC) (Pedram *et al.*, 2001; Lara-Castillo *et al.*, 2009). *In vivo*, an increase of circulating BNP levels achieved by intraperitoneal injections (Shmilovich *et al.*, 2009) or by targeted overexpression of the *BNP* gene in the liver (Kook *et al.*, 2003) accelerated vascular regeneration in limb ischaemia experimentally generated in mice by femoral artery ligation. This was concomitant to increased number of circulating EPCs (Shmilovich *et al.*, 2009). Importantly, a clinical study showed the therapeutical potential of intravenously administered recombinant human ANP (carperitide^{R}) in patients with peripheral arterial diseases (Park *et al.*, 2008). On the other hand, other groups reported anti-vasculogenic actions of ANP. In rats, intravitreal injections of ANP significantly reduced the size of laser-injury – induced (VEGF-dependent) choroidal neovascularization (Lara-Castillo *et al.*, 2009). To study the role of the *endogenous* peptides in postischaemic angiogenesis and arteriogenesis, we recently compared functional recovery and angiogenesis after critical hindlimb ischaemia in mice with endothelial GC-A deletion and respective control mice (Kuhn *et al.*, 2009). Histology and MR angiography revealed that postischaemic angiogenesis and arteriogenesis were both significantly impaired in EC GC-A KO mice, demonstrating protective endothelial effects of endogenous NPs in this situation.

As mentioned at the beginning, whereas ANP is mainly produced in cardiac myocytes, BNP production has been demonstrated in many other cell types. In particular hypoxia, via stabilization of the hypoxia-inducible factor HIF-1α, induces (extra)cardiac BNP expression (Wilhide and Jones, 2006; Weidemann *et al.*, 2008). Intriguingly, our immunohistochemical studies together with RT-PCR analyses indicated that satellite cells, activated within the hindlimb early after ischaemia, express BNP (Kuhn *et al.*, 2009). Indeed, a recent clinical study in patients with peripheral arterial disease demonstrated increased local plasma levels of BNP (Jouni *et al.*, 2011). These experimental and clinical obervations suggest that BNP, produced by satellite cells proliferating in the ischaemic muscle, may stimulate the regeneration of neighbouring endothelia in a paracrine way. Together with other studies they corroborate the forementioned hypothesis that BNP, in contrast to ANP, might be a stress-responsive hormone that mainly acts as a local paracrine factor modulating endothelial proliferation and tissue remodelling within the heart as well as in other tissues. Of course these observations do not exclude a role for endogenous ANP to modulate angiogenesis or vasculogenesis after ischaemia or tissue damage.

The intracellular pathways that link the activation of endothelial GC-A/cGMP signalling to stimulation or inhibition of endothelial proliferation and migration have been addressed in many *in vitro* studies with cultured macro- and microvascular endothelial cells. A recent elegant study by Koika *et al.* (2010) demonstrated *in vivo* (in chicken chorioallantoic membranes and rat cornea) that cGKI is involved as downstream signalling molecule of stimulatory, proangiogenic actions of cGMP. Concordantly, postischaemic angiogenic and vasculogenic responses were attenuated in global cGKI-knockout mice and promoted in mice with overexpression of the enzyme (Yamahara *et al.*, 2003; Aicher *et al.*, 2009). As already mentioned, one effect of endothelial NP/cGMP/cGKI signalling in macro- (HUVEC) and microvascular endothelial cells (RFPEC) is the phosphorylation of VASP, a protein associated with focal adhesion sites and adherens junctions (Smolenski *et al.*, 2000; Kuhn *et al.*, 2009). In HUVECs and BAECs, this pathway was shown to be important for reorganization of the actin cytoskeleton and endothelial tube formation (Chen *et al.*, 2008). Other studies in HUVECs showed that cGKI phosphorylates p21-activated kinase (pak)1, thereby stimulating Pak/VASP association, which also seems to be important for endothelial migration (Fryer *et al.*, 2006). Members of the MAPK family, including ERK1/2 and p38 MAPK, and the serine/threonine kinase Akt/PKB are also important mediators of endothelial growth and migration. In macrovascular EC, ANP and BNP increased the levels of phospho-Akt and phospho-ERK1/2 (Kook *et al.*, 2003). Taken together, these *in vitro* data suggest that activation of VASP, MAPK/ERK and Akt/PKB cooperate in the NP/GC-A/cGKI-dependent stimulation of processes required for angiogenesis, such as endothelial proliferation and migration. However, further complicating this issue, others found that ANP prevents the stimulatory effects of VEGF on MAPKs

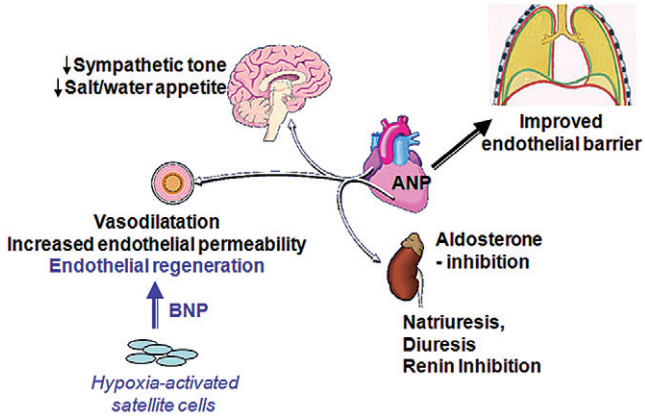

Figure 2

Scheme of the main GC-A-mediated actions of ANP and BNP. Cardiac ANP mildly stimulates endothelial permeability in the peripheral circulation to adjust systemic intravascular volume (endocrine actions). In contrast, in the lung, the peptide possibly stabilizes endothelial barrier functions. BNP is formed by different (extra)cardiac cells in response to hypoxia. In the skeletal muscle, BNP released by satellite cells improves postischaemic regeneration of neighbouring endothelia (paracrine actions).

(c-Jun N-terminal kinase, p38 MAPK) and cyclin D1 in cultured macrovascular BAEC (Pedram *et al.*, 2001). The reasons for these discrepant functional and molecular responses of endothelial cells to NPs remain unresolved. Many of these presumed differences may relate to the origin and differences in passage number of the cells being studied. However, it is also possible that the NP/GC-A signalling pathway exerts dual effects in endothelial cells, mildly stimulating basal permeability and regeneration but attenuating the inflammatory and proangiogenic properties of agonists such as VEGF.

Summary and conclusions

This review emphasizes the important role of the endothelial GC-A receptor in the physiological regulation of arterial blood pressure and intravascular volume by cardiac ANP. This involves the modulation of transvascular albumin and fluid exchange in the systemic microcirculation, especially in the skin and skeletal muscle (Figure 2). However, beyond this mild permeability-increasing (volume-regulating) effect on *quiescent* endothelial cells, GC-A/cGMP signalling possibly exerts the opposite, namely barrier-enhancing effects on an *inflammation-activated* endothelium of the pulmonary and maybe also systemic microcirculation (Figure 2). At difference to ANP, the systemic circulating levels of BNP normally are very low. However, the expression of this hormone is markedly induced in the heart and in extracardiac tissues by stressors such as pressure load (in cardiac myocytes) and hypoxia (in many cell types, for instance in muscular satellite cells). Paracrine actions of BNP on the GC-A receptor of neighbouring endothelial cells possibly participate in the coordination of tissue regeneration/hypertrophy and accompanying angiogenesis (Figure 2).

Endothelial dysfunction but also altered NP/GC-A/cGMP signalling are common features of patients with lower extremity peripheral artery disease (Park *et al.*, 2008) and patients with cardiac hypertrophy/heart failure (Hirooka *et al.*, 1990; Tsutamoto *et al.*, 1992; Kerem *et al.*, 2010; reviewed by Kuhn, 2003). Inactivating posttranslational modifications of the receptor, such as GC-A dephosphorylation, possibly contribute to the attenuation of the cardiovascular and renal effects of NPs (Bryan and Potter, 2002; Schröter *et al.*, 2010). Our studies of EC GC-A KO mice emphasize that an inhibition of endothelial NP/GC-A/cGMP signalling could accelerate the progression of these diseases and their complications (impaired angiogenesis, hypervolaemia, lung oedema) and support further work to assess the importance of the NP/GC-A system, particularly its endothelial alterations, in human (patho)physiology. The therapeutical potential of synthetic ANP (carperitide®), BNP (neseritide®) or chimeric peptides in the treatment of pulmonary oedema, congestive heart failure and arterial diseases is currently under intensive investigation (McKie *et al.*, 2010; Sakamoto *et al.*, 2010).

The activity of the NP/GC-A/cGMP signalling pathway does not only depend on ligand-receptor activation but also on the kinetics of cGMP degradation. In many cell types, PDE 5 is the principal enzyme responsible for cGMP hydrolysis, controlling both the magnitude, duration and intracellular compartimentalization of the NP/GC-A/cGMP signal. The role of PDE5 in endothelial cells has only been partly characterized. Cultured pulmonary microvascular endothelial cells apparently do not express PDE 5, at least under basal conditions (Zhu *et al.*, 2009). However the PDE 5 inhibitor sildenafil prevented neonatal hyperoxic pulmonary inflammation (de Visser *et al.*, 2009). Moreover, PDE 5 seems to control the angiogenic potential of endothelial cells in the peripheral circulation. Hence, the PDE 5 inhibitors, sildenafil and tadalafil, promoted vascular repair in multiple experimental models of tissue damage and wound repair (Zhu *et al.*, 2009). Sildenafil, based on its potent pulmonary vasodilatating effect, has been approved for the treatment of pulmonary arterial hypertension (Rubin *et al.*, 2011). Future studies will be required to study whether and how PDE5 inhibitors can influence endothelial (dys)functions in disease and endothelial responses to endogenous or exogenous (synthetic) NPs.

This paper aims to illustrate the complexity of NP/GC-A/cGMP signalling in the control of endothelial permeability as well as in the regulation of postnatal proliferation, migration and differentiation of endothelial cells and their progenitors. It emphasizes how difficult but critical it is to prove *in vivo* (in specific vascular beds and under specific (patho)physiological conditions) the relevance of observations on cultured endothelial cells.

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Conflicts of interest

The author declares that there is no conflict of interest.

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