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Research Article

Natriuretic peptides differentially attenuate thrombin-induced barrier dysfunction in pulmonary microvascular endothelial cells

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Abstract

Previous studies have described a protective effect of atrial natriuretic peptide (ANP) against agonist-induced permeability in endothelial cells derived from various vascular beds. In the current study, we assessed the effects of the three natriuretic peptides on thrombin-induced barrier dysfunction in rat lung microvascular endothelial cells (LMVEC). Both ANP and brain natriuretic peptide (BNP) attenuated the effect of thrombin on increased endothelial monolayer permeability and significantly enhanced the rate of barrier restoration. C-type natriuretic peptide (CNP) had no effect on the degree of thrombin-induced monolayer permeability, but did enhance the restoration of the endothelial barrier, similar to ANP and BNP. In contrast, the non-guanylyl cyclase-linked natriuretic peptide receptor specific ligand, cyclic-atrial natriuretic factor (c-ANF), delayed the rate of barrier restoration following exposure to thrombin. All three natriuretic peptides promoted cGMP production in the endothelial cells; however, 8-bromo-cGMP alone did not significantly affect thrombin modulation of endothelial barrier function. ANP and BNP, but not CNP or c-ANF, blunted thrombin-induced RhoA GTPase activation. We conclude that ANP and BNP protect against thrombin-induced barrier dysfunction in the pulmonary microcirculation by a cGMP-independent mechanism, possibly by attenuation of RhoA activation.

Keywords: Natriuretic peptides; Endothelium; RhoA GTPase; Signal transduction; Barrier function; Pulmonary edema

Introduction

The endothelial cell lining of the pulmonary microvasculature forms a semi-permeable barrier that separates blood from the pulmonary interstitium and regulates the flux of fluids, proteins, and cells between the capillary lumen and alveolar space. The integrity of this endothelial cell barrier may be disrupted by various inflammatory and thrombogenic mediators, as well as mechanical stress. Endothelial barrier dysfunction is a key feature of inflammation and central to the pathophysiology of the vascular leak and alveolar flooding associated with acute lung injury and sepsis.

The natriuretic peptides are a family of small proteins characterized by a 17 amino acid loop that plays important roles in vascular tone and intravascular volume homeostasis [1]. Atrial and brain natriuretic peptide (ANP and BNP) are synthesized primarily in the heart, kidney, and central nervous system; whereas, the C-type natriuretic peptide (CNP) is found primarily in the central nervous system and vascular endothelium. Each of the natriuretic peptides has been detected circulating in the plasma. The natriuretic peptides function by binding the particulate guanylate cyclase-linked natriuretic peptide A- or B-receptors (NPR-A, NPR-B), with subsequent cGMP production and activation of protein kinase G (PKG). Binding of the natriuretic peptides to a third receptor, natriuretic peptide receptor-C (NPR-C), results in internalization and degradation of the natriuretic peptides and thus, clearance from the

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circulation. Natriuretic peptide binding to NPR-C has also been shown to activate phospholipase C signaling cascade through diminished adenylate cyclase-mediated production of cAMP [2]. NPR-A has considerably greater affinity for ANP and BNP than for CNP; whereas NPR-B has greater binding affinity for CNP [3,4]. All three peptides bind to NPR-C with fairly similar affinities [3,4].

While the actions of the natriuretic peptides on cardiovascular, renal, and nervous system functions have been well described (reviewed in [1]), recent studies have begun to investigate the role of the natriuretic peptides in endothelium function. For example, low concentrations (picomoles) of ANP promoted proliferation and both chemotactic and random migration [5,6]; whereas, high concentrations (nanomoles) of ANP resulted in endothelial cell apoptosis [7]. Numerous studies have demonstrated a modulatory role of ANP on vascular permeability both in vitro and in vivo [8–19], although results have been conflicting depending upon the doses used, the animal species studied, and the experimental conditions. The majority of the studies describe an inhibitory effect of ANP on permeability in most vascular beds, but some investigators have found that ANP worsens vascular leak [20]. However, few of the studies of ANP on monolayer permeability have utilized lung-derived microvascular endothelial cells (LMVEC), making it difficult to extrapolate previous findings to the pulmonary microcirculation where pulmonary edema occurs. Furthermore, little is known about the roles of BNP and CNP in pulmonary vascular endothelial function. Circulating levels of all three peptides are elevated during acute lung injury [21–23]. Understanding how the natriuretic peptides modulate pulmonary capillary endothelial function and determining if there are differential effects between natriuretic peptides may lead to therapeutic approaches to combat diseases associated with pulmonary endothelial dysregulation, such as acute lung injury and pulmonary edema.

The current study was undertaken to characterize the effects of the natriuretic peptides on pulmonary microvascular endothelial barrier function. We provide data demonstrating differential effects of ANP, BNP, and CNP on thrombin-induced increases in LVMEC monolayer permeability. ANP and BNP both significantly blunted the extent of thrombin-induced permeability and enhanced the rate of barrier restoration following thrombin exposure. On the other hand, CNP only enhanced the rate of barrier restoration. Interestingly, 8-bromo-cGMP did not alter thrombin-induced barrier dysfunction or the rate of barrier restoration suggesting that ANP and BNP modulate thrombin-induced barrier dysfunction and restoration through signaling mechanisms independent of cGMP production. Finally, we provide data that ANP and BNP, but not CNP, inhibit thrombin-induced RhoA GTPase activation, suggesting an alternative mechanism by which ANP and BNP may inhibit pulmonary vascular leak.

Material and methods

Cell lines and reagents

Rat LVMEC, purchased from Vec Technologies, Inc. (Rensselaer, NY), were obtained from the periphery of the lung, consisting mostly of pulmonary microvascular and capillary endothelial cells. The LMVEC were propagated in MCDB-131 media and used between passages 6 and 13. Thrombin and the following natriuretic peptides were all purchased from Sigma Chemicals (St. Louis, MO); rat BNP 1–32, rat CNP 1–22, and rat C-ANF 3–23. Rat ANP 1–28, was purchased from American Peptides Co. (Sunnyvale, CA) and 8-bromo-cGMP was obtained from Biology Life Sciences Institute (Bremen, Germany). Antibodies directed against RhoA and Rac-1 were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). cGMP enzyme immunoassay kit was purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA).

The pGST-C21 and pGST-PBD constructs were gifts from Dr. J.G. Collard, The Netherlands Cancer Institute [24] and Dr. R.A. Cerione, Cornell University [25], respectively.

Endothelial monolayer permeability assay

Endothelial monolayer permeability was assessed by direct measurements of transendothelial electrical resistance (TER) using the electrical impedence system (ECIS) (Applied Biophysics, Troy, NY) as previously described [26,27]. Equivalent numbers of rat LMVEC were seeded on collagen coated gold electrode (8W10E) arrays, grown to confluence and permitted to adhere overnight. The endothelial cells were allowed to equilibrate and, with the exception of data presented in Figs. 1a and 5a, the vehicle, 1 μM ANP, 1 μM BNP, 1 μM CNP, 1 μM c-ANF, or 1mM 8-bromo-cGMP was added to the cells (grey arrow indicates point of addition). After 30 min, an additional bolus of vehicle, natriuretic peptide, c-ANF, or 8-bromo-cGMP was added in the presence or absence of (1 U ml^{-1}) thrombin (black arrow indicates point of addition) and the effects on monolayer permeability were measured over time.

RhoA and Rac-1 GTPase activation assays

A full description of this procedure has been described previously [26–28]. The endothelial cells were grown to confluence and treated with vehicle, 1 μM ANP, 1 μM BNP, or 1 μM CNP for 30 min and then exposed to 1 U ml^{-1} thrombin in the presence of vehicle or natriuretic peptide for the indicated times. Endothelial cells were washed once with PBS and lysed. Equivalent volumes of cleared lysates were incubated with 50 μg of bacterially produced GST-C21 for RhoA-GTP pull-down or GST-PBD for Rac-1-GTP pull-down bound to glutathione agarose beads for 1 h at 4°C. The beads were washed three times and suspended in

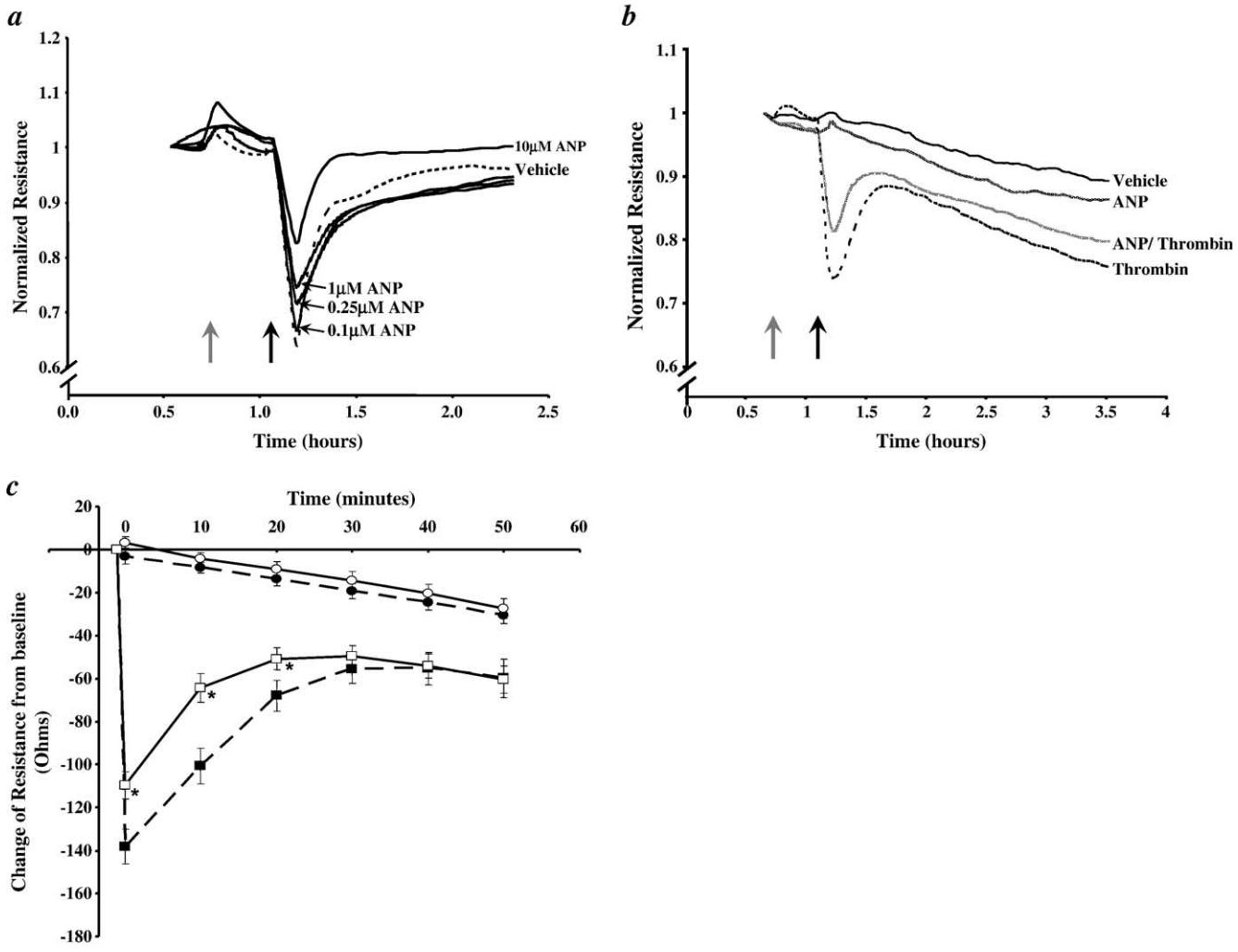

Fig. 1. The effects of ANP on thrombin-induced endothelial monolayer permeability. (a and b) Representative tracings of the resistance across the monolayers, normalized to the initial point of reading, are shown. Grey arrow indicates point of addition of vehicle and ANP. Black arrow indicates point of addition of the subsequent bolus of vehicle or ANP in the presence or absence of 1 U ml^{-1} thrombin. Concentrations of ANP used in panels b and c were 1 μM and in panel a are indicated. (c) The mean ± SE change of resistance from the baseline beginning at the point of vehicle (closed circle, dashed line), ANP (open circle, solid line), thrombin (closed square, dashed line), and ANP/thrombin (open square, solid line) addition is presented. $n = 9–10$ independent measurements. *$P <$ 0.0001 vs. thrombin alone.

Laemli buffer. Protein complexes bound to the beads were resolved on 15% SDS-PAGE. Parallel gels were run with corresponding crude cell lysates. All gels were transferred to Immobilon-P and immunoblotted for respective GTPase.

cGMP analyses

Equivalent numbers of endothelial cells were serum starved for 24 h. The cells were then preincubated with indicated natriuretic peptide for 30 min and then exposed to natriuretic peptide with or without thrombin. Following a 2, 10, 30, or 40 min exposure, the cells were collected by removing medium and lysed in 0.1 M HCl. Cell debris was subsequently removed by centrifugation at 600 × g for 10 min. The supernatant was dried and the amount of cGMP was determined using the commercially available enzyme immunoassay from BIOMOL Research Laboratories, Inc.

Statistical analysis

For three or more groups, differences among the means were tested for significance in all experiments, using ANOVA with Fisher's least significance difference test. Significance was reached when $P < 0.05$. All data are presented as mean + standard error. The number of experiments (n) is indicated for each set of data.

Results

Natriuretic peptides differentially modulate thrombin-induced barrier dysfunction

Thrombin induced an initial rapid increase in endothelial permeability with significant restoration of endothelial

barrier function within 30 min of exposure (Fig. 1). Preincubation with various concentrations of ANP dose dependently attenuated the degree of monolayer permeability induced by thrombin exposure (Fig. 1a). Furthermore, 1 μM ANP significantly enhanced the rate of endothelial barrier restoration following incubation with thrombin, as compared with the rate seen in monolayers incubated with thrombin alone (Figs. 1b and c). We next examined whether BNP and CNP modulated thrombin effects on endothelial monolayer permeability similarly as ANP. Like ANP, preincubation of the LMVEC with BNP significantly blunted the extent of thrombin-induced monolayer permeability and augmented the rate of barrier restoration (Fig. 2). Interestingly, while preincubation with CNP did not significantly attenuate the degree of barrier dysfunction induced by thrombin, CNP enhanced the rate of barrier restoration to a greater degree than that of ANP or BNP (Fig. 3). Barrier function returned to baseline levels within 20 min of thrombin exposure with CNP preincubation, as compared to a return to baseline after 40 min or more with the other natriuretic peptides.

To decipher the role of the natriuretic peptide receptors in modulating thrombin-induced endothelial monolayer permeability, we next examined the effects of the NPR-C agonist, c-ANF. c-ANF binds to all three natriuretic peptide receptors, but does not activate the particulate guanylase cyclase portion of the NPR-A or NPR-B. Preincubation of LMVEC with c-ANF did not blunt thrombin-induced monolayer dysfunction, nor did it enhance the rate of endothelial barrier restoration. Rather, restoration of endothelial barrier function was significantly delayed by c-ANF (Fig. 4). Thus, it is likely that the barrier enhancing effects of ANP, BNP, and CNP are occurring through the other natriuretic peptide receptors, NPR-A and/or NPR-B.

Thrombin-induced effects on endothelial barrier are not mediated through cGMP

To determine if the inhibitory effects of ANP and BNP on thrombin-induced barrier dysfunction were mediated by increased cGMP production via the NPR-A, we next assessed the effects of a non-degradable cGMP analogue, 8-bromo-cGMP, on the thrombin-induced effects on endothelial barrier function. Preincubation with 1nM, 1 μM, or 1mM 8-bromo-cGMP had no significant effect on thrombin-induced endothelial barrier dysfunction (Fig. 5a). There was a slight enhancement in the rate of barrier restoration with the 1mM 8-bromo-cGMP co-incubation; however, this effect was of shorter duration than that seen with any of the natriuretic peptides (Fig. 5b). Elevation of endogenous cGMP levels with the phosphodiesterase V inhibitor, sildenifil, also had no significant effect on thrombin-induced LMVEC monolayer permeability (data not shown).

Intracellular cGMP response to the natriuretic peptides and c-ANF in LMVEC was measured in order to demonstrate the presence of functional NPR-A and NPR-B receptors and the selectivity of c-ANF for the non-guanylate cyclase-linked receptor, NPR-C. Endothelial cell cGMP levels were assayed after exposure to natriuretic peptides in the presence or absence of thrombin. A significant enhancement in the production of cGMP was seen within 2 min exposure to ANP, BNP, and CNP (Fig. 5c). In contrast, there was no induction of cGMP in cells

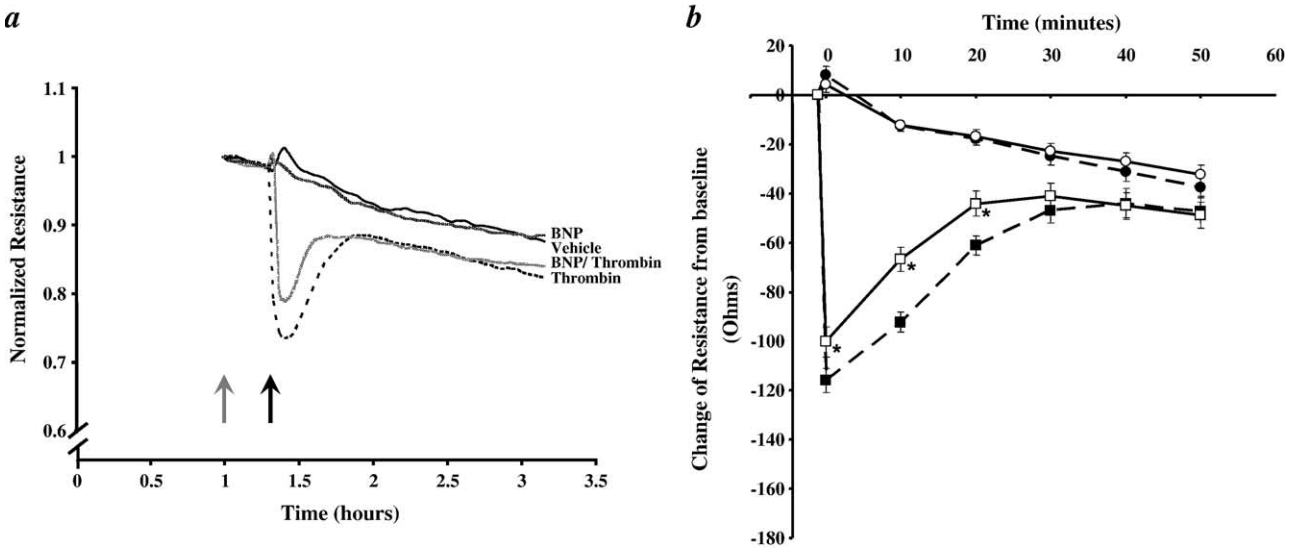

Fig. 2. BNP attenuates thrombin-induced endothelial monolayer permeability. (a) A representative tracing of the resistance across the monolayers, normalized to the initial point of reading, is shown. Grey arrow indicates point of addition of vehicle and 1 μM BNP. Black arrow indicates point of addition of the subsequent bolus of vehicle or BNP in the presence or absence of 1 U ml^{-1} thrombin. (b) The mean ± SE change of resistance from the baseline beginning at the point of vehicle (closed circle, dashed line), BNP (open circle, solid line), thrombin (closed square, dashed line), and BNP/thrombin (open square, solid line) addition is presented. n = 11–12 independent measurements. *$P < 0.0001$ vs. thrombin alone.

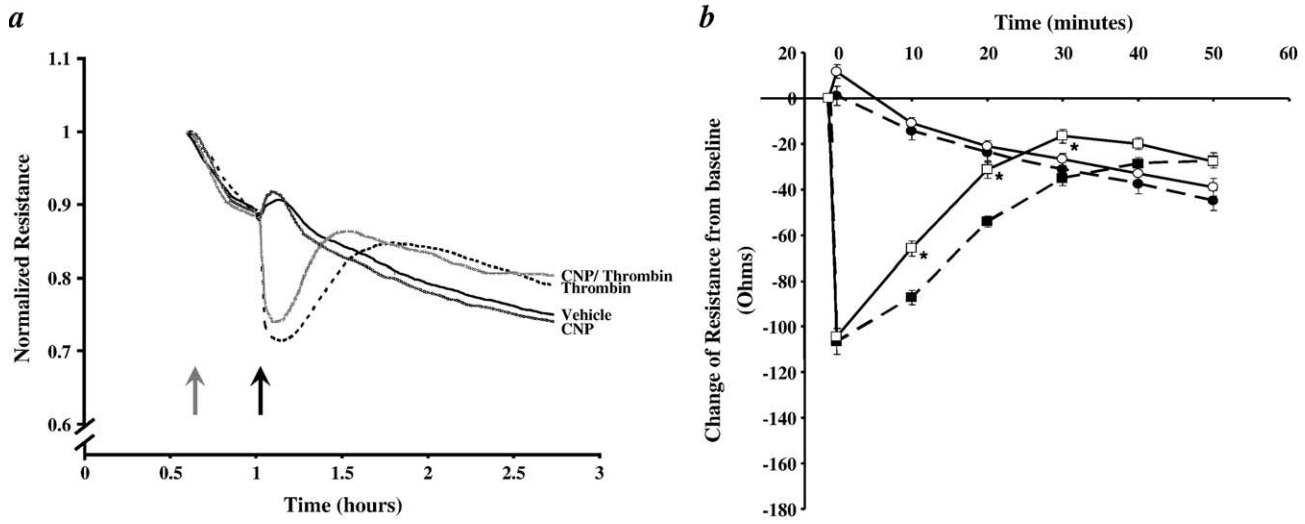

Fig. 3. CNP augments endothelial barrier restoration following thrombin exposure. (a) A representative tracing of the resistance across the monolayers, normalized to the initial point of reading, is shown. Grey arrow indicates point of addition of vehicle and 1 μM CNP. Black arrow indicates point of addition of the subsequent bolus of vehicle or CNP in the presence or absence of 1 U ml^{-1} thrombin. (b) The mean ± SE change of resistance from the baseline beginning at the point of vehicle (closed circle, dashed line), CNP (open circle, solid line), thrombin (closed square, dashed line), and CNP/thrombin (open square, solid line) addition is presented. $n = 10–12$ independent measurements. *$P < 0.0001$ vs. thrombin alone.

treated with c-ANF or in cells given thrombin alone. In addition, no significant differences were seen in the level of cGMP produced in endothelial cells exposed to ANP and thrombin, as compared with endothelial cells treated with ANP alone (Fig. 5d).

ANP attenuated thrombin-induced RhoA activation

Thrombin is thought to be involved in the pathogenesis of acute lung injury by disrupting cytoskeletal structure and directly increasing endothelial permeability [29–31]. More recently, thrombin has been shown to induce a rapid activation of RhoA in pulmonary endothelial cells [27,32,33]. Our results confirm that this peak activation of RhoA occurs within 1 min of exposure to thrombin and is then followed by a rapid decline to baseline levels. The addition of either ANP or BNP to monolayers of LMVEC significantly reduced the degree of thrombin-induced RhoA activation (Fig. 6a). Significant attenuation of thrombin-induced RhoA activation by ANP was noted at 1 min after treatment with ANP (Fig.

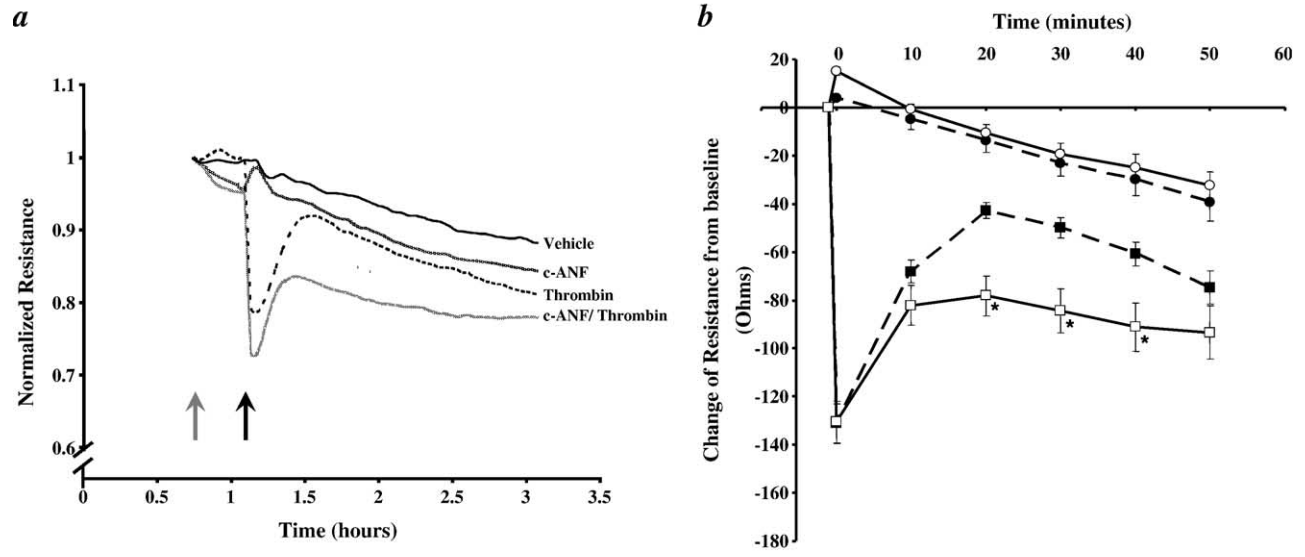

Fig. 4. NPR-C activation diminishes endothelial barrier restoration following thrombin exposure. (a) A representative tracing of the resistance across the monolayers, normalized to the initial point of reading, is shown. Grey arrow indicates point of addition of vehicle and 1 μM c-ANF. Black arrow indicates point of addition of the subsequent bolus of vehicle or c-ANF in the presence or absence of 1 U ml^{-1} thrombin. (b) The mean ± SE change of resistance from the baseline beginning at the point of vehicle (closed circle, dashed line), c-ANF (open circle, solid line), thrombin (closed square, dashed line), and c-ANF/ thrombin (open square, solid line) addition is presented. $n = 7–21$ independent measurements. *$P < 0.0001$ vs. thrombin alone.

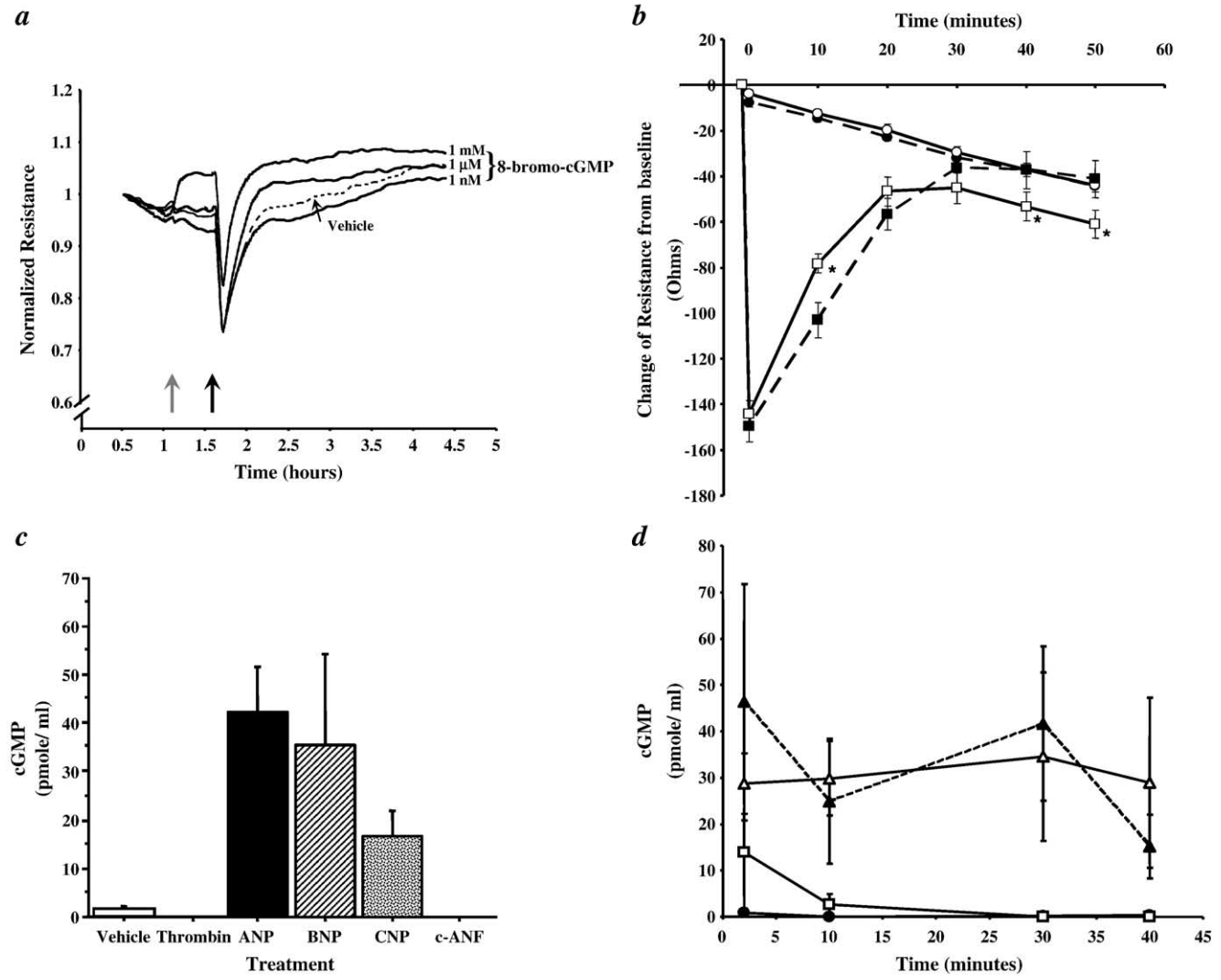

Fig. 5. ANP modulates thrombin-induced endothelial barrier function independently of cGMP. (a) Representative tracing of the resistance across the monolayers, normalized to the initial point of reading, is shown. Grey arrow indicates point of addition of vehicle and 8-bromo-cGMP. Black arrow indicates point of addition of the subsequent bolus of vehicle or 8-bromo-cGMP in the presence or absence of 1 U ml^{-1} thrombin. Concentration of 8-bromo-cGMP used in panel b was 1 μM and in panel a is indicated. (b) The mean ± SE change of resistance from the baseline beginning at the point of vehicle (closed circle, dashed line), 8-bromo-cGMP (open circle, solid line), thrombin (closed square, dashed line), and 8-bromo-cGMP/thrombin (open square, solid line) addition is presented. $n = 16$ independent measurements. *$P < 0.05$ vs. thrombin alone. (c and d) Intracellular cGMP levels were assessed in endothelial cells exposed to the natriuretic peptides in the presence or absence of thrombin. The cells were incubated with vehicle, 1 μM ANP, 1 μM BNP, 1 μM CNP, or 1 μM c-ANF for 30 min, then exposed to an additional bolus of the natriuetic peptides or the agonist in the presence or absence of 1 U ml^{-1} thrombin for the indicated times. Cells were then lysed and the amount of cGMP produced was measured using an enzyme immunoassay. (c) Cells were collected after 2 min and cGMP levels were determined. (d) Vehicle (closed circle); ANP (open triangle); thrombin (open square); and ANP/thrombin (closed triangle, dashed line). $n = 3–6$; P = not significant.

6b). This time course correlates well with the natriuretic peptide associated changes in endothelial barrier dysfunction (Fig. 1). Consistent with the permeability studies, neither CNP (Fig. 6a) nor c-ANF (data not shown) blunted thrombin-induced RhoA activation significantly.

Similar to a previous study [34], we also noted an increase in Rac-1 activity following 1 min exposure to 1 μM ANP in LMVEC (data not shown). Thrombin stimulation has been shown to diminish Rac-1 activity levels with concomitant increase in endothelial barrier dysfunction [35,36]. Thus, it is possible that ANP activation of Rac-1 may also contribute to natriuretic peptide-mediated attenuation of thrombin-induced monolayer permeability in LMVEC.

Discussion

In the present study, we found that ANP and BNP limit thrombin-induced increases in permeability of pulmonary microvascular endothelial cells and that all three natriuretic peptides aid in the restoration of normal barrier function in these cells. These findings are consistent with those of recent reports by Irwin et al. [12] that ANP inhibited

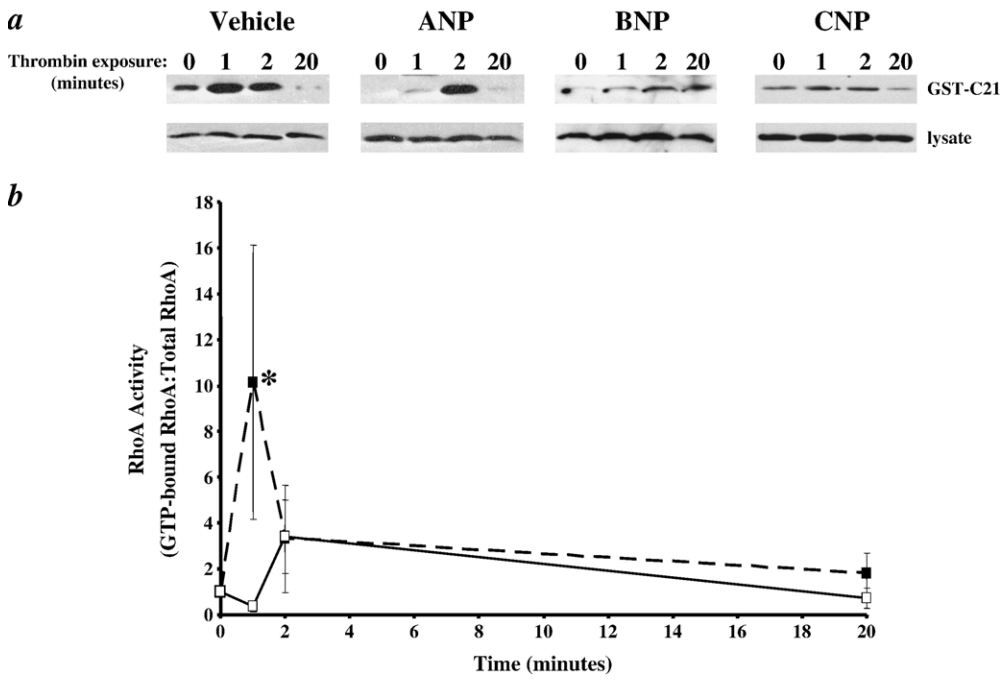

Fig. 6. ANP and BNP blunt thrombin-induced RhoA activation. The endothelial cells were allowed to equilibrate and the vehicle, 1 μM ANP, 1 μM BNP, or 1 μM CNP was added to the cells. After 30 min, an additional bolus of vehicle or natriuretic peptide was added in the presence or absence of (1 U ml^{-1}) thrombin and the effects on RhoA GTPase activity were determined following the indicated time of exposure by measuring the level of active RhoA, GTP-bound RhoA, relative to total RhoA present in crude lysate by immunoblot analysis. (a) Representative immunoblots are shown. (b) Data are presented as the mean ± SE of the ratio of GTP-bound RhoA to total RhoA. Vehicle/thrombin (closed square, dashed line); and ANP/thrombin (open square). $n = 3–4$; *$P < 0.01$ vs. time 0 min of respective treatment. P = no significance between time 0 and any ANP/thrombin exposure times.

hypoxia- and TNFα-induced increases in albumin flux across pulmonary endothelial cell monolayers, isolated from bovine small pulmonary arteries, approximately 200 μm in diameter. Their findings suggested that ANP has a direct inhibitory effect on permeability in precapillary pulmonary arteries. The findings of the present study extend these observations to the pulmonary microcirculation and add further support to the hypothesis that ANP and the natriuretic peptides in general play an important role in limiting the degree of pulmonary edema formation that occurs in acute lung injury.

Few studies have explored the effect of the other natriuretic peptides on vascular permeability. Lofton et al. [37] found that similar to ANP, BNP inhibited thrombin-induced permeability in bovine aortic endothelial cells. In the present study, we found that BNP and ANP similarly inhibited thrombin-induced permeability in LMVEC. This may be important considering the longer half-life of circulating BNP, as well as the greater availability of BNP for therapeutic use. Recent studies have shown a beneficial effect of BNP in the acute treatment of patients with decompensated congestive heart failure [38–40]. Although this effect was attributed to the diuretic and vasodilatory effects of BNP leading to a reduction in pulmonary venous pressure [38–40], our results suggest that BNP may also act to help restore pulmonary microvascluar endothelial barrier function and limit the degree of pulmonary edema formation. CNP, on the other hand, did not attenuate the level of permeability induced by thrombin, but it did enhance the rate of restoration of the endothelial barrier function. Interestingly, this effect appeared to be more pronounced and of greater duration than with the other natriuretic peptides, suggesting that ANP and BNP may play a greater role in limiting the degree of barrier dysfunction, whereas CNP exerts its effect primarily on restoring endothelial barrier integrity.

The mechanism(s) responsible for natriuretic peptide modulation of endothelial barrier function in the pulmonary microcirculation is unclear. Studies in aortic, coronary artery, and proximal pulmonary arterial endothelial cells [37] have demonstrated that the inhibitory effects of ANP on cell permeability can be reproduced by the addition of cGMP analogs. Recently, Kiemer et al. [13] found that 8-bromo-cGMP could partially mimic the effect of ANP on inhibiting TNFα-induced actin polymerization, a key feature of increased endothelial cell permeability. However, the degree of inhibition was less than 50% that of ANP. In the present study, we were unable to duplicate the inhibitory effects of the natriuretic peptides on thrombin-induced increases in permeability with 8-bromo-cGMP alone. In addition, CNP enhanced the rate of restoration of the endothelial barrier function to at least the same degree and for a longer period of time as ANP and BNP, despite a smaller rise in cGMP levels. These findings suggest that, in LMVEC, the inhibitory effect of natriuretic peptides on thrombin-induced increases in permeability is not mediated

primarily by changes in intracellular cGMP. We were also unable to demonstrate a role of NPR-C in mediating thrombin-induced endothelial permeability as has been described in rat coronary arterial endothelial cells [9]. In fact, targeted activation of NPR-C with c-ANF had no effect on the degree of thrombin-induced permeability and markedly impaired restoration of barrier function. Thus, our findings suggest that the protective effect of the natriuretic peptides on LMVEC barrier dysfunction is mediated through the NPR-A/NPR-B, but through a different, cGMP-independent mechanism. Alternatively, it is possible that PKG associates with NPR-A in such a way that increased cGMP is compartmentalized with NPR-A, resulting in inhibition of endothelial barrier dysfunction [41].

A large body of literature supports the role of Rho GTPases in the regulation of endothelial barrier dysfunction (reviewed in [42]). Specifically, RhoA activation has been shown to be associated with thrombin-induced barrier dysfunction [27,32,33,36,43,44]; whereas, Rac-1 activity has been associated with protecting against barrier dysfunction [45,46]. Our data demonstrated an activation of Rac-1 by ANP and an attenuation of thrombin-induced RhoA activation by preincubation with ANP and BNP, but not with CNP. Previous studies have suggested that RhoA is inactivated by protein kinase A (PKA) in lymphocytes [47] and cGMP-dependent protein kinase (cGK) in vascular smooth muscle cells [48], possibly through phosphorylation. Additionally, Rac-1 protective effects on endothelial barrier function were dependent upon cAMP generation through PKA [46]. Furthermore, ANP has been shown to protect against hepatocyte apoptosis induced by ischemia/reperfusion injury via a PKA-dependent pathway [49]. Thus, it is possible that ANP and BNP regulate the activities of Rho GTPases in lung microvascular endothelial cells via increasing cGMP and activating localized PKG and PKA, and thus limiting the degree of pulmonary vascular leak.

Several recent studies suggest that ANP plays a significant role in blunting pulmonary edema formation in animal models of sepsis. ANP has been shown to inhibit leukocyte adhesion to hypoxic endothelial cells [50] and neutralization of endogenous ANP with antibodies directed against ANP has been shown to exacerbate pulmonary edema in endotoxin-primed rats exposed to high altitude [11]. Finally, infusion of the NPR-A selective antagonist, HS-142-1 has been shown to increase lung lymph flow and pulmonary transvascular fluid flux in an ovine model of sepsis [12]. These studies strongly suggest that endogenous ANP plays an important role in mitigating the degree of pulmonary vascular leak associated with acute lung injury.

In summary, the natriuretic peptides differentially modulate lung microvascular endothelial barrier function in vitro. These effects are achieved primarily through NPR-A, but not via modulation of intracellular cGMP levels alone. Natriuretic peptide limitation of thrombin-induced RhoA activation may be an alternative explanation. We hypothesize that activation of NPR-A with ANP or BNP increases cGMP and activates localized PKG causing attenuation of RhoA activation that helps to protect from increased lung microvascular permeability in vivo.

Acknowledgments

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