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Opposite effects of ANP receptors in attenuation of LPS-induced endothelial permeability and lung injury

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Abstract

Atrial natriuretic peptide (ANP) has been recently identified as a modulator of acute lung injury (ALI) induced by pro-inflammatory agonists. While previous studies tested effects of exogenous ANP administration, the role of endogenous ANP in the course of ALI remains unexplored. This study examined regulation of ANP and its receptors NPR-A, NPR-B and NPR-C by LPS and involvement of ANP receptors in the modulation of LPS-induced lung injury. Primary cultures of human pulmonary endothelial cells (EC) were used in the *in vitro* tests. Expression of ANP and its receptors was determined by quantitative RT-PCR analysis. Agonist-induced cytoskeletal remodeling was evaluated by immunofluorescence staining, and EC barrier function was characterized by measurements of transendothelial electrical resistance. In the murine model of ALI, LPS-induced lung injury was assessed by measurements of protein concentration and cell count in bronchoalveolar lavage fluid (BAL). LPS stimulation significantly increased mRNA expression levels of ANP and NPR-A in pulmonary EC. Pharmacological inhibition of NPR-A augmented LPS-induced EC permeability and blocked barrier protective effects of exogenous ANP on LPS-induced intercellular gap formation. In contrast, pharmacological inhibition of ANP clearance receptor NPR-C significantly attenuated LPS-induced barrier disruptive effects. Administration of NPR-A inhibitor *in vivo* exacerbated LPS-induced lung injury, whereas inhibition of NPR-C suppressed LPS-induced increases in BAL cell count and protein content. These results demonstrate for the first time opposite effects of NPR-A and NPR-C in the modulation of ALI and suggest a compensatory protective mechanism of endogenous ANP in the maintenance of lung vascular permeability in ALI.

Keywords

natriuretic peptide receptor; cytoskeleton; pulmonary endothelium; vascular leak

INTRODUCTION

Tissue inflammation and increased vascular leak are cardinal features of acute lung injury (ALI), a severe illness associated with a mortality of 30–50% (Rubenfeld et al., 2005; Ware and Matthay, 2000). Despite recent advances in low tidal volume mechanical ventilation and

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a better understanding of the underlying inflammatory pathophysiology of ALI, there remain few effective treatments for this devastating illness.

Changes in the levels of circulating atrial natriuretic peptide (ANP) occur under pathologic conditions and play the homeostatic role to adjust physiologic pressure-load ratios. For example, cardiac pressure overload triggers ANP secretion by cardiomyocytes leading to fluid excretion by kidneys and vasodilation (Baxter, 2004; Curry, 2005). However, activation of ANP signaling may also accompany inflammatory reactions. Increased circulating levels of ANP have been detected in sepsis, pulmonary edema, ARDS and ALI (Eison et al., 1988; Imamura et al., 1988; Mitaka et al., 1992).

ANP belongs to a family of three cardiovascular peptide hormones named as atrial (ANP), brain (BNP), and C-type (CNP) natriuretic peptides and regulates a variety of physiological functions by interacting with receptors at the plasma membrane (Ahluwalia et al., 2004). Two of three ANP receptors identified so far, NPR-A and NPR-B, are also termed as particulate guanylate cyclase-linked natriuretic peptide A- or B-receptors, and catalyze cGMP production and activation of protein kinase G (PKG). ANP binding to natriuretic peptide receptor-C (NPR-C) results in internalization, degradation and clearance of natriuretic peptides from the circulation. Few reports suggest direct activation of intracellular signaling by NPR-C which involves activation of heterotrimeric protein Gi, phospholipase C, phosphoinositide turnover and protein kinase C (see (Anand-Srivastava, 2005) and (Potter et al.) for review).

Our experimental data combined with others show that ANP treatment exerts anti-inflammatory effects on hematopoietic and endothelial cells and may inhibit barrier disruptive effects of proinflammatory agents LPS and thrombin both in pulmonary endothelial cultures and animal models of ALI (Furst et al., 2005; Irwin et al., 2005; Klinger et al., 2006; Louzier et al., 2001; Mitaka et al., 1998; Sakurai et al., 2005; Xing and Birukova, 2010).

Signaling pathways of ANP-mediated barrier protection in pulmonary EC involve activation of Epac-Rap1 GTPase cascade, stimulation of Rac GTPase and downregulation of Rho signaling (Birukova et al., 2008b; Klinger et al., 2006; Xing and Birukova, 2010). Other study demonstrates that attenuation of VEGF-induced EC permeability by ANP occurred via ANP-induced inhibition of Src and PI3K-Akt signaling leading to attenuation of VEGF-induced serine/threonine phosphorylation of tight junction proteins ZO-1 and occludin (Pedram et al., 2002).

Expression of all three ANP receptor types has been previously reported in lung tissue (Potter, 2005), but their presence in pulmonary EC exposed to ALI-related inflammatory agents has not been investivgated. Given the importance of ANP in modulation of lung vascular permeability and control of lung barrier under inflammatory conditions, in this work we characterized LPS effects on ANP and NPR expression by lung EC, studied the involvement of ANP receptors in the modulation of LPS-induced lung injury, and examined effects of NPR-A and NPR-C specific inhibitors on the LPS-induced EC permeability and lung barrier dysfunction.

MATERIALS AND METHODS

Cell culture and reagents

Human lung microvascular endothelial cells (HLMVEC), human pulmonary artery endothelial cells (HPAEC) and cell culture basal medium with growth supplements were obtained from Lonza (Allendale, NJ). Cells were cultured according to the manufacturer’s

protocol, and used at passages 5–8. ANP was purchased from AnaSpec (San Jose, CA). TNFα was obtained from R&D Systems (Minneapolis, MN). Potent selective antagonists of natriuretic peptide receptor-A (A71915, designated as NPR-Ai) and natriuretic peptide receptor-C (cANF, designated as NPR-Ci) characterized previously (Delporte et al., 1992; Moro et al., 2004; Pandey et al., 2000; Potter et al., 2006) were purchased from Bachem Bioscience (King of Prussia, PA). All reagents for immunofluorescence were purchased from Molecular Probes (Eugene, OR). Unless specified, biochemical reagents were obtained from Sigma (St. Louis, MO).

Permeability measurements

The cellular barrier properties were analyzed by measuring transendothelial electrical resistance (TER) across confluent endothelial cell monolayers using an electrical cell-substrate impedance sensing system (Applied Biophysics, Troy, NY) as previously described (Xing et al., 2011).

Endothelial cell imaging

EC monolayers plated on glass cover slips were treated with the agonist of interest, fixed in 3.7% formaldehyde solution in PBS for 10 min at 4°C, washed three times with PBS, permeabilized with 0.1% Triton X-100 in PBS-Tween (PBST) for 30 min at room temperature, and blocked with 2% BSA in PBST for 30 min. Incubation with VE-cadherin antibodies were performed in blocking solution (2% BSA in PBST) for 1 hr at room temperature followed by staining of actin filaments with Texas Red-conjugated phalloidin. After immunostaining, slides were analyzed using a Nikon video imaging system (Nikon Instech Co., Tokyo, Japan) as described elsewhere (Birukov et al., 2004; Birukova et al., 2006; Birukova et al., 2008a).

Quantitative RT-PCR (qRT-PCR) analysis

Reverse transcription (RT) was performed with 1 μg of total RNA isolated from control and LPS treated human lung micro- or macrovascular EC. RT-PCR reactions were performed as previously decribed (Birukova et al., 2010). Primer sequences are presented in Table-1. Primers for the qPCR reactions were designed for QRT with the length of the amplicons 100 bp. β2-Microglobulin is expressed in lung tissue well above the average calculated for 79 human cell types and tissues (Schmittgen and Zakrajsek, 2000; Su et al., 2004) and was used as an internal standard in this study.

Animal studies

All protocols involving the use of animals were approved by the University of Chicago Institutional Animal Care & Use Committee for the humane treatment of experimental animals. Adult male C57BL/6J mice, 8–10 week old, with average weight 20–25 grams (Jackson Laboratories, Bar Harbor, ME) were anesthetized with an intraperitoneal injection of ketamine (75 mg/kg) and acepromazine (1.5 mg/kg) according to institutional regulations. LPS (0.2 mg/kg body weight, Escherichia coli O55:B5) or sterile water was injected intratracheally in a small volume (20 μl) using a 20 gauge catheter Penn-Century Inc., (Philadelphia, PA). Mice were randomized to concurrently receive sterile saline solution or ANP (2 μg/kg) by intravenous injection (i/v) in the external jugular vein to yield the following experimental groups: control, LPS only, ANP (2 μg/kg) only, and LPS + ANP (2 μg/kg). In experiments with ANP receptor inhibitors, NPR-Ai (10 μg/kg, i/v) or NPR-Ci (10 μg/kg, i/v) was given concurrently with LPS instillation.

Bronchoalveolar lavage (BAL) analysis

After 18 hr, animals were sacrificed by exsanguination under anesthesia. BAL was performed using 1 ml of sterile Hanks Balanced Salt Buffer. The collected lavage fluid was centrifuged at 2500 rpm for 20 min at 4°C, the supernatant was removed and frozen at −80°C for subsequent protein study. The cell pellet was then resuspended in 1 ml of red blood cell lysis buffer (ACK Lysing Buffer, BioSource International) for 5 min and then re-pelleted by centrifugation at 2500 rpm for 20 min at 4°C. The cell pellet was again resuspended in 200 μl of PBS, and 20 μl of cell suspension were used for cell counting by a standard hemocytometer technique. The BAL protein concentration was determined by a modified Lowry colorimetric assay using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis

Results are expressed as means ± SD. Stimulated samples were compared to controls by unpaired Student's t-test. For multiple-group comparisons, a one-way analysis of variance (ANOVA), followed by the post hoc Tukey test, were used. $P<0.05$ was considered statistically significant.

RESULTS

Expression of ANP receptors by pulmonary endothelial cells

Expression of ANP receptors NPR-A, NPR-B and NPR-C was examined by qRT-PCR analysis of total RNA preparations from HPAEC and HLMVEC cultures using gene specific primers described in Materials section. The results showed that all three ANP receptors are expressed at comparable levels in cultured HPAEC (Figure 1A) and HLMVEC (Figure 1B).

Effect of LPS on the expression of ANP and ANP receptors in pulmonary EC

To test potential effects of LPS treatment on expression levels of ANP or its receptors, human pulmonary macrovascular and microvascular EC were treated with LPS for 1 hour or 18 hours. Levels of ANP, NPR-A, NPR-B, and NPR-C mRNA expression were determined by qRT-PCR as described in the Methods. One-hour LPS treatment induced 3.5-fold increase in ANP mRNA expression in pulmonary macrovascular and 3.9-fold increase in microvascular EC (Figure 2). The ANP mRNA levels in HPAEC remained significantly elevated after 18 hrs of LPS treatment (Figure 2A), but returned to basal levels in lung microvascular EC (Figure 2B). The 2.2-fold increase in expression of NPR-A after 1 hr and 18 hrs of LPS treatment was detected in HPAEC culture, while expression changes in HLMVEC cultures did not reach statistically significant levels. LPS did not affect NPR-B and NPR-C expression levels in both EC types.

Effects of ANP receptor inhibition on LPS-induced EC permeability

The results described above demonstrate the activation of ANP and NPR-A expression in pulmonary EC challenged with pro-inflammatory agonists and suggest the activation of ANP-dependent mechanisms by LPS. The following experiments tested involvement of ANP acting receptor NPR-A and clearance receptor NPR-C in endothelial barrier dysfunction induced by LPS and TNFα. Previous studies showed that LPS significantly decreased transendothelial electrical resistance (TER) in human pulmonary EC, reflecting endothelial barrier compromise, with maximal response at 6 hrs (Birukova et al., 2007). In the current experiments cells were pretreated with NPR-A pharmacological inhibitor A71915 or NPR-C peptide inhibitor cANF for 30 min followed by LPS challenge. When LPS concentration was 300 ng/ml, which induces maximal barrier-disruptive response, EC permeability was neither affected by NPR-A inhibitor, nor by NPR-C inhibitor (Figure 3A,

left bar graphs). However, when LPS was used at sub-maximal concentrations (50 ng/ml), inhibition of NPR-A increased LPS-mediated permeability, whereas inhibition of NPR-C suppressed LPS-induced EC disruption and returned TER values to basal levels (Figure 3). These data strongly suggest a role for intrinsic ANP signaling as a compensatory physiological mechanism of EC barrier protection against EC barrier compromise induced by pro-inflammatory agents.

Effects of ANP receptor inhibition on LPS- or TNFα-induced EC cytoskeletal remodeling

HPAEC pretreated with NPR-A and NPR-C inhibitors (A71915 and cANF, respectively) or vehicle were challenged with sub-maximal concentration of LPS (50 ng/ml) or TNFα (5 ng/ml) for 6 hours, and effects of ANP on LPS- and TNFα-induced cytoskeletal remodeling and gap formation in EC monolayers were examined by immunofluorescence staining of F-actin. Both LPS and TNFα induced formation of actin stress fibers and paracellular gaps reflecting EC barrier compromise (Figure 4A, **upper panels**). Despite the effects on EC permeability described above, pretreatment with NPR-A inhibitor did not significantly alter the overall pattern of LPS- or TNFα–induced EC gap formation and EC cytoskeletal remodeling (data not shown). Apparent discrepancy between microscopy data and results of permeability studies depicted in Figure 3 may be explained by limitations of morphometric analysis, which did not allow for detection of subtle changes in cell morphology of LPS- or TNFα–challenged EC monolayers upon treatment with NPR-A inhibitor. In contrast to NPR-A, inhibition of ANP clearance receptor NPR-C markedly decreased stress fibers and paracellular gap formation in both LPS- and TNFα-treated cells (Figure 4B).

We further analyzed potential role of NPR-A receptor in the mediation of protective effects by exogenous ANP. EC pretreatment with ANP prevented EC monolayer disruption induced by LPS and TNFα (Figure 4C, **upper panels**). However, inhibition of NPR-A abolished ANP-activated protective mechanisms (Figure 4C, **lower panels**).

Involvement of ANP receptors in the development of LPS-induced lung injury *in vivo*

We have previously found increased ANP expression in the mouse lungs after intratracheal LPS injection (Birukova et al., 2010). Specific role of ANP acting and clearance receptors in the development of LPS-induced lung injury was further examined in animal models.

Pharmacologic inhibitors of NPR-A or NPR-C were intravenously administrated in mice simultaneously with intratracheal LPS injection. Treatment of LPS-challenged mice with exogenous ANP in the absence of NPR inhibitors was used as a positive control. After 18 hr BAL was performed, and cell count and protein concentration were determined as described in the Methods. At high LPS dose (0.7 mg/kg), exogenous ANP exhibited protective effect against LPS-induced increased cell counts and protein accumulation in BAL, however effects of ANP receptor inhibitors were negligible (data not shown).

When LPS was administered at lower dose (0.2 mg/kg), which induces moderate but significant elevation of BAL protein and cell count, inhibition of ANP acting receptor NPR-A further promoted LPS-induced increase in cell count as compared to animals treated with LPS alone (Figure 5A), while effects of NPR-A inhibitor on LPS-induced increase in BAL protein content did not reach statistical significance (Figure 5B).

Experiments with inhibition of ANP clearance receptor NPR-C showed that NPR-C inhibitor significantly decreased cell count and protein concentration in BAL samples from LPS-challenged mice even without addition of exogenous ANP (Figure 5). Taken together, these data suggest a compensatory protective role of ANP in the regulation of lung vascular permeability.

DISCUSSION

Natriuretic peptide levels have important prognostic value in multiple clinic settings. Elevated plasma natriuretic peptide levels are associated with higher disease severity and mortality (Doust et al., 2005; Karmpaliotis et al., 2007; Rana et al., 2006; Ueda et al., 2006), however causal relationships between these events remain to be clarified. For example, administration of ANP reduced mortality rate and exhibited beneficial effects in patients with ALI (Mitaka et al., 1998). These observations led to suggestion that increase in plasma ANP levels may serve as physiological adaptive mechanism to acute injury (Mitaka et al., 1998; Sakurai et al., 2005).

This study shows constitutive expression of ANP and three ANP receptors in human lung macrovascular and microvascular endothelial cells. The results also demonstrate that exposure to LPS upregulated ANP in both EC types and increased NPR-A expression in human pulmonary macrovascular EC. These data are supported by other studies, which showed that in septic conditions, LPS caused three- to six-fold increases in ANP mRNA expression by circulating macrophages (Vollmar and Schulz, 1995). Our results strongly suggest that induction of ANP and NPR-A expression in LPS-challenged pulmonary EC may have functional consequence. Inhibition of NPR-A function attenuated protective barrier protective effects of exogenous ANP, while inhibition of ANP clearance receptor prevented increases in EC permeability and cytoskeletal perturbations in response to LPS and TNFα. Thus, the current data demonstrate for the first time the opposing roles of NPR-A and NPR-C receptors in control of ANP-mediated protection against LPS-induced endothelial permeability and lung barrier dysfunction induced by inflammatory agonists. These results strongly suggest a role of ANP clearance receptor in modulation of barrier protective effects of endogenously upregulated ANP.

Our results do not show effects of LPS on NPR-C mRNA expression levels. On the other hand, decreased number of ANP clearance receptors has been reported in the injured lungs and suggested to retain ANP in circulation (Hollister et al., 1989; Onuoha et al., 2000). These results may illustrate increased NPR-C shedding or degradation under pro-inflammatory conditions without affecting NPR-C mRNA levels. Further studies are required to elucidate these potential mechanisms.

Experiments using NPR-A specific inhibitor demonstrate that activation of NPR-A mediates ANP protective effect against LPS-induced permeability. Analysis of signaling mechanisms involved in ANP-induced barrier protection was not in the focus of this study. However, previous studies by our and other groups showed critical role of Epac-Rap1-Rac signaling in ANP protection against thrombin-induced barrier dysfunction (Birukova et al., 2008b; Klinger et al., 2006; Xing and Birukova, 2010). Additional mechanisms of ANP protective action may involve induction of mitogen-activated protein kinase phosphatase-1 (MKP-1) dependent on Rac and Nox2 activities (Birukova et al., 2008b; Klinger et al., 2006; Xing and Birukova, 2010). In turn, ANP-induced MKP-1 activation inhibits TNFα-induced p38 activation, actin stress fiber formation and endothelial permeability (Kiemer et al., 2002). Interestingly, bypassing NPR-A guanylate cyclase activation by direct introduction of stable cGMP analog 8Br-cGMP mimicked ANP effect on ROS production and upregulation of MKP-1 expression by endothelial cells. However, 8Br-cGMP is not sufficient to activate Epac-Rap1-Rac cascade of pulmonary EC barrier enhancement (A. Birukova, unpublished studies). These data strongly suggest that ANP triggers other receptor-mediated signaling mechanisms in addition to cGMP, which contribute to its protective effects in various pathological settings.

Protective effect of NPR-A and NPR C inhibitors on LPS-induced EC barrier dysfunction *in vitro* was registered in this study only at low LPS concentrations. These data suggest the limited potential for ANP produced by stimulated pulmonary EC cultures to counteract barrier disruptive effects of LPS and TNFα. However, significant improvement of BAL parameters of lung barrier dysfunction in LPS-challenged mice treated with NPR-A inhibitor supports the utility of endogenous ANP in the negative regulation of LPS-induced ALI. Supportive studies using NPR-C knockout mice showed almost two-fold elongation of half-life time of circulating ANP (Matsukawa et al., 1999).

In conclusion, the results of this study suggest a novel compensatory mechanism of the maintenance of lung vascular permeability in pathologic inflammatory settings via upregulation of ANP signaling. We demonstrate for the first time the role of NPR-C clearance receptor in mediating anti-inflammatory and barrier protective effects of endogenous ANP in ALI models. Thus, beneficial effects of *in vivo* administration of NPR-C clearance receptor inhibitor on BAL parameters of lung injury described in this study may be considered as a novel therapeutic modality in treatment of ALI.

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Highlights

- This study examined the role of ANP and its receptors the modulation of LPS-induced lung injury.
- Inhibition of NPR-A augmented LPS-induced EC permeability and blocked protective effects of exogenous ANP.
- Inhibition of ANP clearance receptor NPR-C attenuated LPS-induced barrier disruptive effects.
- *In vivo* NPR-A inhibitor exacerbated LPS-induced lung injury, whereas inhibition of NPR-C suppressed LPS-induced lung injury.

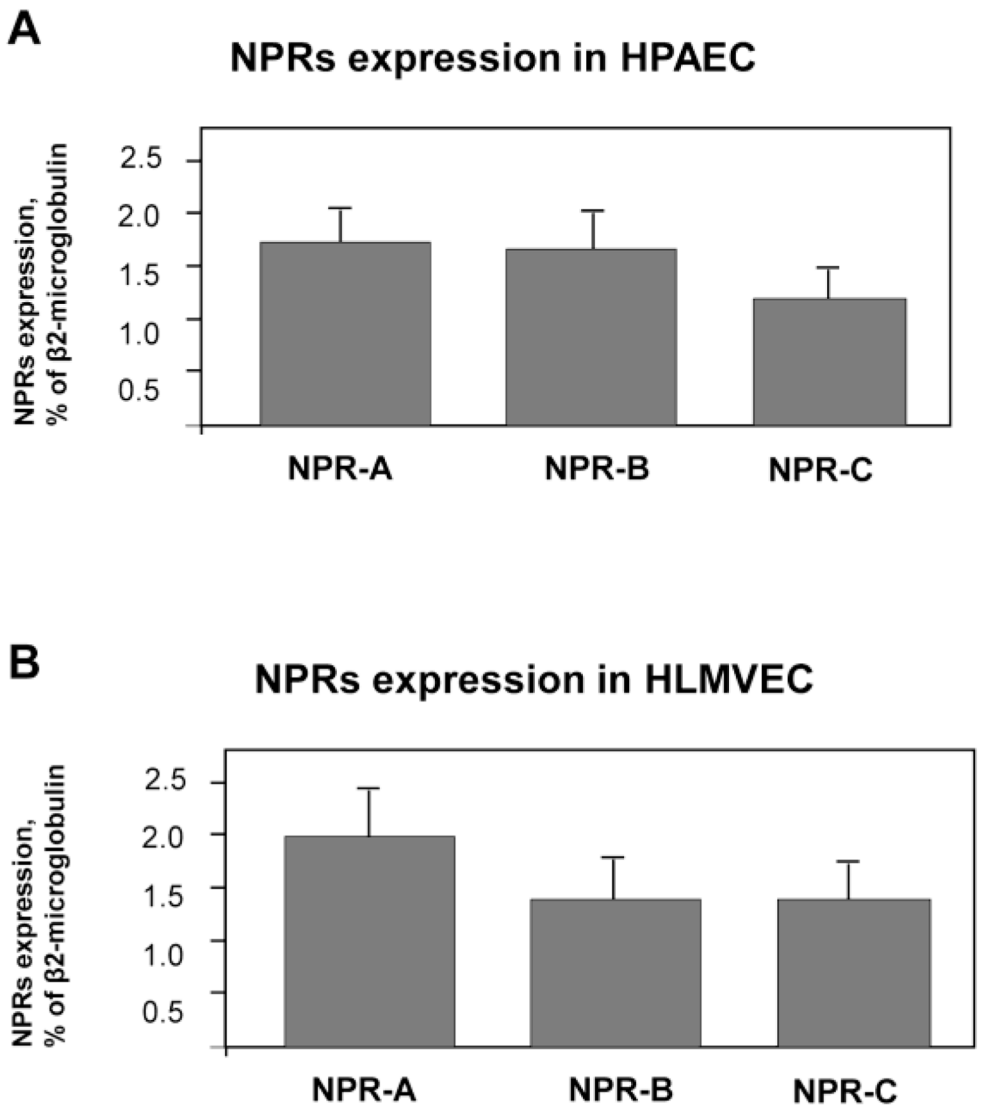

Figure 1. Expression of ANP receptors in pulmonary endothelium

HPAEC **(A)** or HLMVEC **(B)** were grown to confluence followed by isolation of mRNA. Quantative RT-PCR was performed as described in the Methods. Levels NPR-A, NPR-B, and NPR-C mRNA were normalized to the housekeeping gene β2-microglobulin. Results are representative of three independent experiments.

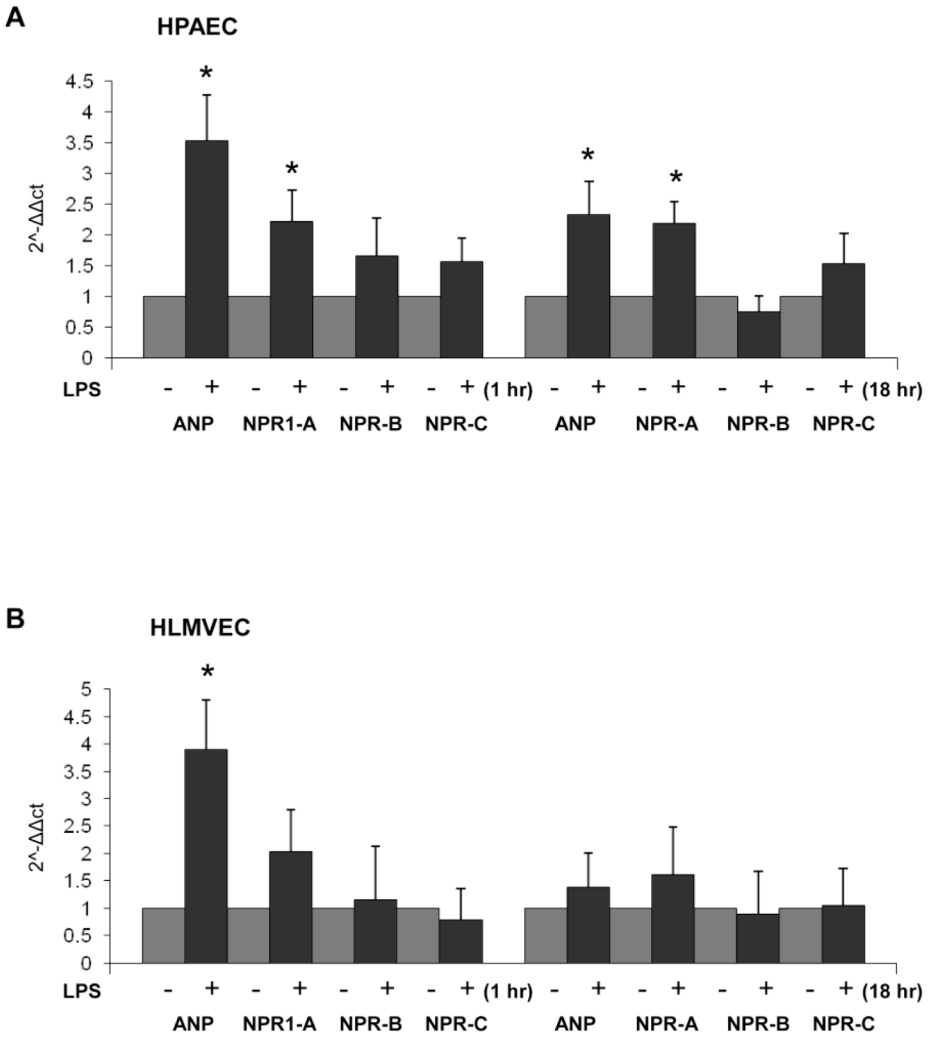

Figure 2. Effect of LPS on ANP and ANP receptor expression in the lung macrovascular (HPAEC) and microvascular (HLMVEC) endothelial cells

HPAEC (**A**) or HLMVEC (**B**) monolayers were pretreated with vehicle or LPS (300 ng/ml) for 1 hour or 18 hours. Levels of ANP, NPR-A, NPR-B, and NPR-C mRNA were determined by qRT-PCR analysis. The graph represents pooled data; n=6 for each experimental group; *$p<0.05$.

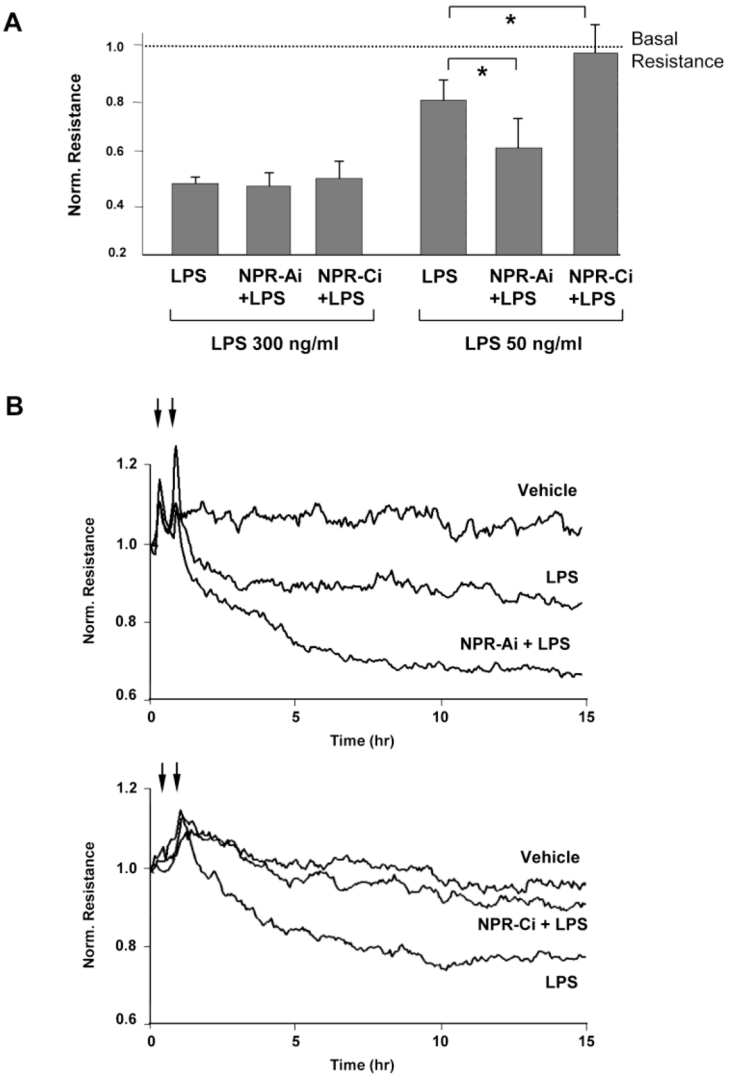

Figure 3. Involvement of ANP receptors in LPS-induced alteration of EC permeability

HPAEC plated on microelectrodes were pretreated with NPR-Ai (500 nM, 30 min) or NPR-Ci (500 nM, 30 min) followed by stimulation with LPS (300 ng/ml or 50 ng/ml). **A -** Bar graphs representing permeability measurements observed after 6 hours of agonist stimulation. Data are expressed as mean ± SD of five independent experiments; *p<0.05. **B -** EC were stimulated with 50 ng/ml LPS (second arrow) with or without ANP receptor inhibition (first arrow) and measurements of transendothelial electrical resistance were performed over 15 hours. Shown are representative graphs of five independent measurements.

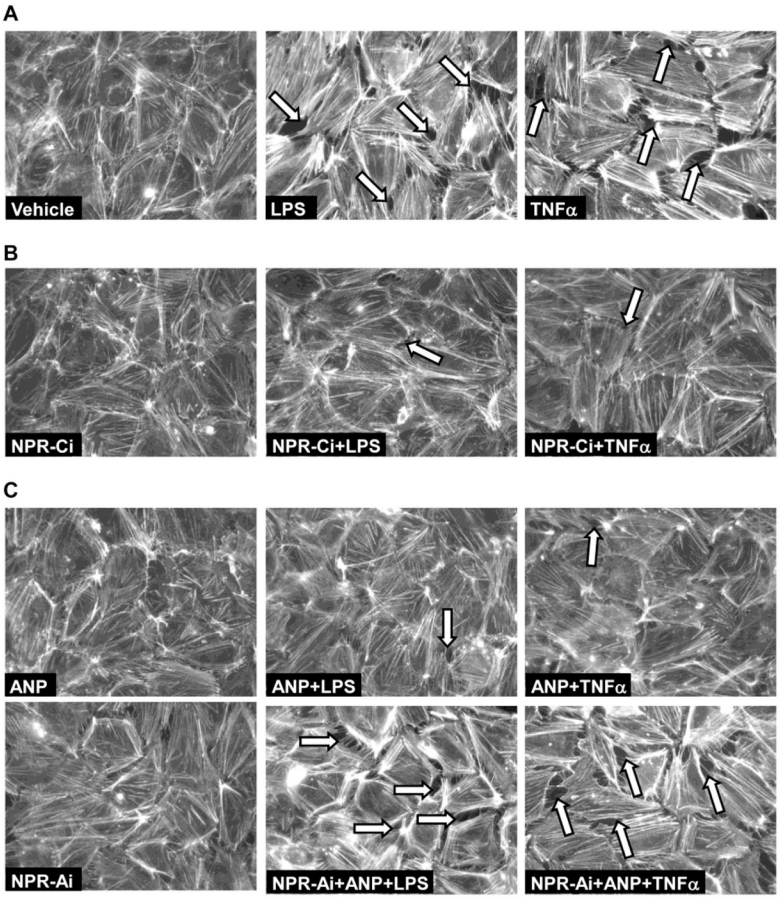

Figure 4. Effect of ANP receptor inhibition on LPS- or TNF -induced EC cytoskeletal remodeling

A - HPAEC monolayers were stimulated with LPS (50 ng/ml) or TNFα (5 ng/ml) for 6 hours; **B -** HPAEC monolayers stimulated with LPS (50 ng/ml) or TNFα (5 ng/ml) for 6 hours with NPR-Ci (500 nM, 30 min) pretreatment; **C -** Cells were pretreated with NPR-Ai (500 nM, 30 min) followed by addition of ANP (100 nM, 30 min) and stimulation with LPS (50 ng/ml) or TNFα (5 ng/ml) for 6 hours. Immunofluorescence staining with Texas-Red phalloidin was performed to visualize F-actin filaments. Paracellular gaps are shown by arrows. Shown are results of three independent experiments.

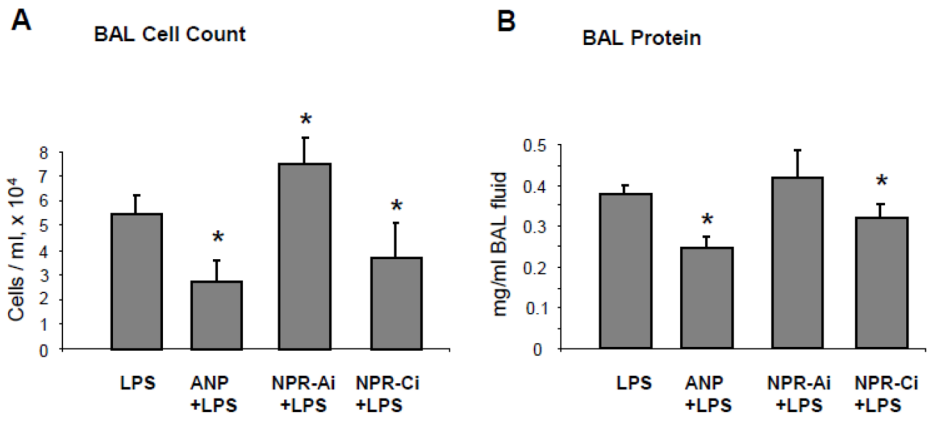

Figure 5. Involvement of ANP receptors in LPS-induced lung injury *in vivo*

Mice were treated with LPS (0.2 mg/kg, i/t) or vehicle with or without concurrent ANP (2 μg/kg, i/v), NPR-Ai (10 μg/kg, i/v), or NPR-Ci (10 μg/kg, i/v) injection for 18 hr. Cell count **(A)** and protein concentration **(B)** were determined in bronchoalveolar lavage fluid taken from control and experimental animals. Data are expressed as mean ± SD; n=6 per group; *$p<0.05$. Comparisons were made between groups marked by brackets.

Table 1

Primers used for qRT-PCR analysis of ANP and NPR expression in human lung endothelial cells.

ANP:	
forward	5′-GATAACAGCCAGGGAGGACAA-3′
reverse	5′ – CTGCAGCTTAGATGGGATGA – 3′
NPR1:	
forward	5′-TGGTGACCCT-GCTCAATGACCTGTACACT -3′
reverse	5′ – ACCACCATGTAGGCATCGCC-AATTGT – 3′
NPR2:	
forward	5′-TGAGCGGCCAGACTTTGGACA-GATTAA -3′
reverse	5′ – CTGTTCCATGCGCAGCAGGAGGTT – 3′
NPR3:	
forward	5′-ACAGGCATGGCATGACCAGTGGAGA -3′
reverse	5′ – TGTTTGTCTCCTTCTCTTCCATGAGCCATCT – 3′
β2-microglobulin:	
forward	5′-TCAGGTTTACTCACGTCATCCAGCAGAGAA -3′
reverse	5′ – CAGTAAGTCAACTTCAATGTCGGATGGATGAA – 3′