Atrial natriuretic peptide attenuates LPS-induced lung vascular leak: role of PAK1

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Birukova AA, Xing J, Fu P, Yakubov B, Dubrovskyi O, Fortune JA, Klibanov AM, Birukov KG. Atrial natriuretic peptide attenuates LPS-induced vascular leak: role of PAK1. Am J Physiol Lung Cell Mol Physiol 299: L652–L663, 2010. First published August 20, 2010; doi:10.1152/ajplung.00202.2009.—Increased levels of atrial natriuretic peptide (ANP) in the models of sepsis, pulmonary edema, and acute respiratory distress syndrome (ARDS) suggest its potential role in the modulation of acute lung injury. We have recently described ANP-protective effects against thrombin-induced barrier dysfunction in pulmonary endothelial cells (EC). The current study examined involvement of the Rac effector p21-activated kinase (PAK1) in ANP-protective effects in the model of lung vascular permeability induced by bacterial wall LPS. C57BL/6J mice or ANP knockout mice (Nppa−/−) were treated with LPS (0.63 mg/kg intratracheal) with or without ANP (2 μg/kg iv). Lung injury was monitored by measurements of bronchoalveolar lavage protein content, cell count, Evans blue extravasation, and lung histology. Endothelial barrier properties were assessed by morphological analysis and measurements of transendothelial electrical resistance. ANP treatment stimulated Rac-dependent PAK1 phosphorylation, attenuated endothelial permeability caused by LPS, TNF-α, and IL-6, decreased LPS-induced cell and protein accumulation in bronchoalveolar lavage fluid, and suppressed Evans blue extravasation in the murine model of acute lung injury. More severe LPS-induced lung injury and vascular leak were observed in ANP knockout mice. In rescue experiments, ANP injection significantly reduced lung injury in Nppa−/− mice caused by LPS. Molecular inhibition of PAK1 suppressed the protective effects of ANP treatment against LPS-induced lung injury and endothelial barrier dysfunction. This study shows that the protective effects of ANP against LPS-induced vascular leak are mediated at least in part by PAK1-dependent signaling leading to EC barrier enhancement. Our data suggest a direct role for ANP in endothelial barrier regulation via modulation of small GTPase signaling, pulmonary endothelium; permeability; acute lung injury; cytoskeleton; small interfering RNA in vivo

ACUTE LUNG INJURY (ALI) and its more severe form, acute respiratory distress syndrome (ARDS), are associated with high morbidity and mortality in patients. During the progression of ALI, the endothelial cell (EC) barrier of the pulmonary vasculature becomes compromised, leading to pulmonary edema, a characteristic feature of ALI. It is well-established that EC barrier dysfunction is initiated by agonist-induced cytoskeletal remodeling, which leads to disruption of cell–cell contacts and formation of paracellular gaps, allowing penetration of protein-rich fluid and inflammatory cells (14, 33). However, far less is known about the processes that determine EC barrier enhancement or protection in the injured lung.

Natriuretic peptides (NP) are a family of three peptide hormones: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). Although the major source of ANP is mammalian atrial myocytes, NP are found in other tissues, including aorta, lung, kidney, and brain (2). NP regulate a variety of physiological functions by interacting with receptors at the plasma membrane either to alter levels of second messengers such as cGMP and cAMP or to affect ion channels (see Ref. 1 for review). Previous studies of ANP action in the cardiovascular system have concentrated mainly on the diuretic, natriuretic, and vasodilatory actions of ANP (3). However, it is becoming increasingly recognized that ANP possesses a much broader range of biological activities, including effects on endothelial function and inflammation. It was shown that in endothelial cultures, ANP attenuated EC barrier dysfunction induced by lysophosphatidylcholine (37). In cultured pulmonary micro- and macrovascular endothelium, ANP inhibited hypoxia- and TNF-α-stimulated albumin permeability via suppression of NF-κB activity and p38 MAPK (24, 27). Recent reports also show that ANP attenuated thrombin-triggered decrease in EC resistance via modulation of Rho-dependent signaling (11, 28). These findings suggest an important role for ANP in lung function regulation in the settings of ALI.

Activation of the small GTPase Rac has been previously described as a major mechanism of EC barrier protection in response to various stimuli, including OX-PAPC, sphingosine 1-phosphate, hepatocyte growth factor, and prostacyclin (4, 6, 12, 21, 31). Our own (11) and other studies (28) delineated the key role of Rac-dependent mechanisms in EC barrier protection and downregulation of Rho-mediated EC hyperpermeability by ANP. Several cytoskeletal Rac effectors, such as the Arp2/3 complex, p21Arc, p21-activated kinase (PAK1), and cortactin, are intimately involved in cortical actin rearrangement and regulation of actin polymerization (16, 52). PAK1 is a serine/threonine protein kinase that activates the Arp2/3 complex and initiates peripheral actin polymerization (13).

When cells are activated by a variety of agents, PAK1 is targeted and activated at focal adhesion complexes and membrane ruffles at the cell leading edge (15). PAK1 binds and becomes activated by the Rac1, Cdc42, and other members of Rac GTPase family, but not by Rho A-G Ras superfamilly members. PAK1 activation by Rac1 and Cdc42 leads to PAK1 autophosphorylation at Thr286, essential for its full catalytic function toward exogenous substrates (15). Activated PAK1...
phosphorylates LIM kinase (LIMK) and induces LIMK-dependent phosphorylation of the cofillin/actin-depolymerizing factor (ADF) family proteins leading to stimulation of actin polymerization (15). In addition, the association of activated PAK1 with focal adhesion complexes may further stimulate Rac signaling critical for EC barrier enhancement (5).

This work studied the barrier-protective effects of ANP in a model of LPS-induced lung injury and, using a small interfering RNA (siRNA) approach, tested a role for PAK1 in the mediation of the ANP-protective effects both in vitro and in vivo.

MATERIALS AND METHODS

Reagents and cell culture. Human pulmonary macrovascular (HPAEC) and microvascular (HLMVEC) EC were obtained from Lonza (Allendale, NJ). ANP was purchased from AnaSpec (San Jose, CA). TNF-α, IL-6, and IL-6 soluble receptor (SR) were obtained from R&D Systems (Minneapolis, MN). Primary antibodies to phosphorylated (p) PAK1, PAK1, and horseradish peroxidase (HRP)-linked anti-rabbit IgG were obtained from Cell Signaling Technology (Beverly, MA). VE-cadherin antibodies were obtained from BD Transduction Laboratories (San Diego, CA). All reagents used for immunofluorescence were purchased from Molecular Probes (Eugene, OR). Unless specified, all biochemical reagents were obtained from Sigma (St. Louis, MO).

Measurement of transendothelial electrical resistance across confluent HPAEC monolayers was performed using an electrical cell-substrate impedance sensing system (Applied BioPhysics, Troy, NY) as previously described (4, 8).

Immunofluorescence labeling. After agonist treatment, EC grown on glass coverslips were subjected to double immunofluorescence staining for VE-cadherin and F-actin as previously described (4, 7, 8).

Western blot analysis. Protein extracts from mice lungs or EC were separated by SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes, which were then incubated with p-PAK1-specific antibodies. Equal protein loading was verified by reprobing membranes with β-tubulin antibody. Immunoreactive proteins were detected using the enhanced chemiluminescent detection system (ECL) according to the manufacturer’s protocol (Amersham, Little Chalfont, United Kingdom).

Depletion of endogenous PAK1. To deplete endogenous PAK1, HPAEC were treated with gene-specific siRNA duplexes. For in vitro experiments, predesigned siRNA for human PAK1 has been previously described and was ordered from Ambion (Austin, TX) in purified, desalted, deprotected, and annealed double-strand form. In the in vitro transfection with siRNA was performed as previously described (4, 10). For in vivo experiments, predesigned standard purity Stealth PAK1-specific mouse siRNA sets were purchased from Invitrogen (Carlsbad, CA). Polymer-based administration of nonspecific or PAK1-specific siRNA conjugated with polycation polyethyleneimine (PEI-22) was shown to promote lung-specific DNA and siRNA delivery (48, 49) and was used as described elsewhere (45). The optimal concentration of siRNA was determined in the following series of preliminary experiments. Liposome-siRNA polypelexes were formed at a ratio of 1:10 (1 µg siRNA/10 µg lipid), and PAK1-specific siRNA (sipAK1) was tested in the 0.1–5.0 mg/kg dose range. siRNA at 1–4 mg/kg showed the most significant inhibition of the target gene after 72 h of transfection, as determined by RT-PCR analysis of lung tissues or by Western blot analysis. Treated mice showed no signs of nonspecific siRNA-induced inflammation. Nonspecific, nontargeting siRNA (Dharmacon, Lafayette, CO) was used as a control treatment for both in vitro and in vivo experiments.

Animal studies. All animal care and treatment procedures were approved by the University of Chicago Institutional Animal Care and Use Committee. Animals were handled according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. C57BL/6J mice and mice with a targeted disruption of the ANP gene (strain B6.129P2-Nppa<sup>−/−</sup>/Unc/I) were purchased from The Jackson Laboratories (Bar Harbor, ME). Male 8- to 10-wk-old homozygous Nppa<sup>−/−</sup> or matched control animals with an average weight of 20–25 g were anesthetized by intraperitoneal injection of ketamine (75 mg/kg) and acepromazine (1.5 mg/kg). Bacterial LPS (0.63 mg/kg body wt; Escherichia coli O55:B5) or sterile water was injected intratracheally in a small volume (20–30 µl) using a 20-gauge catheter (Penn-Century, Philadelphia, PA). In the first set of experiments, C57BL/6J mice were randomized to concurrently receive sterile saline solution or ANP (2 µg/kg) by intravenous injection in the external jugular vein to yield the following experimental groups: control, LPS only, ANP (2 µg/kg) only, and LPS and ANP (2 µg/kg). In experiments with knockout animals, Nppa<sup>−/−</sup> or wild-type mice were injected with LPS with or without ANP administration. In experiments with siRNA, PAK1-specific or nonspecific siRNA (final concentration 2 mg/kg) were intravenously injected using the PEI delivery protocol (45, 49) described above, 72 h before LPS administration with or without ANP. After 16 h, animals were killed by exsanguination under anesthesia. Bronchoalveolar lavage (BAL) was performed using 1 ml of sterile HBSS, and measurements of cell count, protein concentration, and myeloperoxidase activity were conducted as previously described (9, 19). Evans blue dye (30 ml/kg) was injected into the external jugular vein 2 h before termination of the experiment. Measurement of Evans blue accumulation in the lung tissue was performed by spectrofluorometric analysis of lung tissue lysates according to the protocol described previously (35, 38). For histological assessment of lung injury, the lungs were harvested without lavage collection and fixed in 10% formaldehyde. After fixation, the lungs were embedded in paraffin, cut into 5–6 µm sections, and stained with hematoxylin and eosin. Sections were evaluated at ×40 or ×10 magnification. In a separate set of experiments, mice were treated with LPS for various durations of time, and lungs were harvested without BAL collection. Expression of ANP mRNA was determined by quantitative (q) RT-PCR analysis of lung samples, and phosphorylation of PAK1 in lung tissue was analyzed by Western blot.

qRT-PCR. For the comparison of gene expression levels, total mRNA was isolated from mouse lung samples using MELT Total Nucleic Acids Isolation System (Ambion) according to the manufacturer’s protocol. Reverse transcription into cDNA was performed with 1 µg of total RNA using the SuperScript II polymerase (Invitrogen). Each cDNA sample was diluted to 2.6 µg/ml in sterile double-distilled water (ddH<sub>2</sub>O), and 5 µl of this dilution was used as template for qRT-PCR. Relative quantification was performed using mouse ANP gene-specific primers (forward 5′-GCC ATA TTG GAG CAA ATC CT-3′, reverse 5′-CAT CTT CTC CTC CAG GTG GT-3′) on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA), using QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA) to monitor double-stranded DNA (dsDNA) synthesis. Gene expression fold changes were calculated according to the ∆∆C<sub>T</sub> method (41). For normalization, commercially available mouse β-actin primers were used.

Statistical analysis. Results are expressed as means ± SD of three to eight independent experiments. Stimulated samples were compared with controls by using unpaired Student’s t-test. For multiple-group comparisons, a one-way ANOVA and post hoc multiple comparisons tests were used, and results with P < 0.05 were considered statistically significant.

RESULTS

ANP exhibits protective effects against EC barrier dysfunction induced by inflammatory mediators. The current study evaluated ANP effects in models of EC barrier dysfunction induced by inflammatory agents using HPAEC and HLMVEC.
EC monolayers were treated with LPS, TNF-α, or IL-6 with or without ANP pretreatment followed by measurements of trans-endothelial permeability. In the pulmonary macrovascular cells (HPAEC), LPS significantly increased permeability, which was attenuated by ANP pretreatment (Fig. 1A). Similar results were obtained in experiments conducted on lung microvascular endothelial cells (HLMVEC; Fig. 1B). ANP also protected pulmonary EC against hyperpermeability induced by TNF-α, a mediator of the LPS-triggered inflammatory cascade (Fig. 1C). Intratracheal LPS administration markedly activates IL-6 production in the lungs (39). In vitro, a combination of IL-6 and its soluble coreceptor, IL-6+SR (100 ng/ml; D), and transendothelial electrical resistance was monitored over time. Data are expressed as means ± SD of 3–10 independent experiments; n = 3 per condition for each experiment. HPAEC, human pulmonary macrovascular EC; HLMVEC, human lung microvascular EC.

Our (11) previous study has shown a Rac-dependent mechanism of ANP barrier-protective effects against thrombin-induced barrier dysfunction in HPAEC. In the current study, we investigated a role for Rac effector PAK1 in the protective effects of ANP in a model of LPS-induced EC hyperpermeability. We monitored PAK1 phosphorylation as a reflection of its activation (15) in the ANP-treated micro- and macrovascular EC. In both cell types, ANP triggered sustained PAK1 phosphorylation (Fig. 2). Because PAK1-phosphospecific antibodies also cross-react with autophosphorylated PAK2 and PAK3, the impact of these isoforms in the overall phosphosignal cannot be completely excluded.

Fig. 1. Effects of atrial natriuretic peptide (ANP) on endothelial cell (EC) barrier dysfunction induced by inflammatory agonists. EC were plated on gold microelectrodes and grown to confluence. At the time point indicated by the 1st arrow, cells were pretreated with ANP (100 nM, 20 min). At the time point indicated by the 2nd arrow, cells were stimulated with LPS (200 ng/ml; A and B), TNF-α (20 ng/ml; C), or a combination of IL-6 (25 ng/ml) and its soluble coreceptor, IL-6+SR (100 ng/ml; D), and transendothelial electrical resistance was monitored over time. Data are expressed as means ± SD of 3–10 independent experiments; n = 3 per condition for each experiment. HPAEC, human pulmonary macrovascular EC; HLMVEC, human lung microvascular EC.

Fig. 2. Analysis of p21-activated kinase (PAK1) phosphorylation (p-) in response to ANP. HPAEC (top) or HLMVEC (bottom) were treated with either vehicle or ANP (100 nM) for 0.5, 3, 6, or 18 h. Phosphorylation of PAK was detected by Western blot with corresponding phosphospecific antibodies. Equal protein loading was confirmed by determination of PAK1 and β-tubulin content in total cell lysates. Results are representative of 4 independent experiments.
ANP protects against LPS-induced inflammation and vascular leak in vivo. The following experiments examined ANP effects in the murine model of LPS-induced lung injury. One group was challenged with LPS for 16 h, whereas another LPS-stimulated group received concurrent ANP administration. Control mice were treated with vehicle (saline solution) or ANP alone. At the end of the experiment, lung injury was evaluated by measurements of BAL cell count, protein concentration, myeloperoxidase activity, histological analysis of lung sections, and measurements of Evans blue accumulation in the lung tissue. LPS instillation caused pronounced lung inflammation with a 15-fold increase in BAL cell counts (1.74 ± 0.81 × 10⁶ cells/ml vs. 0.11 ± 0.06 × 10⁶ cells/ml in controls; *P < 0.001). A single intravenous injection of ANP at the beginning of the experiment significantly attenuated LPS-induced leukocyte accumulation in BAL (1.07 ± 0.36 × 10⁶ cells/ml vs. 1.74 ± 0.81 × 10⁶ cells/ml for LPS samples; *P < 0.001; Fig. 3A). LPS also induced activation of myeloperoxidase in BAL, which is a marker of neutrophil activation and tissue oxidative stress (11.73 ± 3.83 U/ml vs. 0.89 ± 0.15 U/ml in controls; *P < 0.002). Myeloperoxidase activity was suppressed by ANP treatment (8.10 ± 0.92 U/ml vs. 11.73 ± 3.83 U/ml in LPS samples; *P < 0.05; Fig. 3B). Analysis of BAL protein content was used as a parameter of lung vascular permeability. LPS also significantly increased the protein concentration in BAL fluid (0.70 ± 0.05 mg/ml vs. 0.23 ± 0.03 mg/ml in controls; *P < 0.001), which indicates increased vascular permeability to macromolecules. LPS-induced increases in BAL protein content were significantly attenuated by intravenous ANP administration (0.44 ± 0.09 U/ml vs. 0.70 ± 0.05 mg/ml for LPS samples; *P < 0.001; Fig. 3C).

Histological analysis of lung tissue showed alveolar wall thickening, increased leukocyte infiltration into the lung interstitium and alveolar space, and areas of local alveolar hemorrhage indicative of vascular disruption. These effects were attenuated in ANP-treated mice (Fig. 3D). ANP alone showed no effects on lung histology or BAL parameters.

Fig. 3. Effects of ANP on pulmonary vascular leak and inflammation in vivo. Mice were treated with LPS [0.63 mg/kg intratracheal (it)] or vehicle with or without concurrent ANP injection (2 μg/kg iv) for 16 h. Cell counts (A), myeloperoxidase (MPO) activity (B), and protein concentration (C) were determined in bronchoalveolar lavage (BAL) fluid taken from control (Con) and experimental animals (n = 6 per group); *P < 0.001. D: whole lungs were fixed in 10% formaldehyde, embedded in paraffin, and used for histological evaluation by hematoxylin and eosin staining as described in MATERIALS AND METHODS (magnification: ×40; n = 3–4 per group, ≥20 fields per slide were inspected).
The protective effects of ANP against LPS-induced lung vascular dysfunction were further assessed by measurement of Evans blue leakage into the lung tissue. LPS induced noticeable Evans blue accumulation in the lung parenchyma, which was significantly decreased by ANP (Fig. 4A). These results were further confirmed by quantitative analysis of Evans blue-labeled albumin extravasation in the lung preparations (Fig. 4B). LPS dramatically increased Evans blue accumulation in the lungs (31.85 ± 4.95 vs. 14.28 ± 3.40 μg/g wet lung weight in nontreated controls; \( P < 0.001 \)), which was significantly decreased in ANP-treated mice (19.50 ± 5.35 vs. 31.85 ± 4.95 μg/g wet lung weight for LPS alone; \( P < 0.01 \)). Collectively, these data show significant protective effects of ANP in the mouse model of LPS-induced ALI.

To test our hypothesis about physiological compensatory role of ANP, we performed PCR analysis of lung tissue samples from mice treated with LPS for various time points. LPS administration induced a rapid increase in the expression of ANP mRNA (Fig. 5A). Importantly, ANP elevation was accompanied by increased PAK1 phosphorylation/activity determined in lung tissue samples from LPS-treated animals (Fig. 5B). These results support a physiological role for ANP as a compensatory mechanism activated during development of lung injury.

**ANP knockout mice develop more severe LPS-induced lung injury.** To assess a role for endogenous ANP production in control of LPS-induced lung injury, we used ANP knockout mice (Nppa−/−). In the absence of LPS, no significant difference was observed in BAL cell count, protein concentration, and Evans blue extravasation between wild-type controls and ANP knockout animals. In contrast to wild-type controls, Nppa−/− mice developed more prominent lung inflammation and vascular leak in response to LPS challenge characterized by a nearly 2-fold increase in BAL cell counts (3.45 ± 1.11 × 10⁶ cells/ml vs. 1.82 ± 0.22 × 10⁶ cells/ml in wild-type controls; \( P < 0.05 \)) and BAL protein content (1.36 ± 0.51 mg/ml vs. 0.72 ± 0.06 mg/ml in wild-type controls; \( P < 0.05 \); Fig. 6A). In addition, LPS induced more pronounced Evans blue leakage from the vascular space into the lung parenchyma in Nppa−/− mice compared with wild-type controls (85.9 ± 26.85 vs. 41.23 ± 9.76 μg/g wet lung weight, respectively; \( P < 0.05 \); Fig. 6B). Similarly, LPS treatment of Nppa−/− mice caused more prominent neutrophil accumulation and increased areas of alveolar hemorrhage compared with controls (Fig. 6C). Importantly, analysis of lung tissue samples from LPS-treated mice revealed that PAK phosphorylation was dramatically decreased in Nppa−/− mice compared with wild-type controls (Fig. 6D).

The potential protective role of ANP in the development of ALI was further tested in rescue experiments. Nppa−/− mice were intravenously injected with ANP concurrent with LPS intratracheal administration for 16 h, and parameters of lung barrier function and injury were compared between ANP knockout mice treated with ANP or vehicle. A single ANP injection decreased parameters of LPS-induced lung injury, as detected by BAL cell counts (2.99 ± 0.97 × 10⁶ cells/ml vs. 3.45 ± 1.11 × 10⁶ cells/ml in Nppa−/− mice treated with vehicle; \( P < 0.05 \); Fig. 6D).
Similar abolishment of ANP-protective effects by PAK1 knockdown was observed in the model of LPS-induced EC barrier disruption (Fig. 7B). Analysis of EC permeability using transendothelial electrical resistance measurements showed that ANP attenuated LPS-induced hyperpermeability (Fig. 7C, left). However, siRNA-induced PAK1 knockdown abolished the protective effect of ANP on HPAEC barrier after LPS challenge (Fig. 5C, right). Taken together, these data strongly suggest an important role for PAK1 in the mediation of ANP-protective effects in the pulmonary EC.

PAK1 is a key regulator of ANP-protective effects in vivo. A role for PAK1 in the ANP-mediated lung protection against LPS-induced injury was further examined in animal models. Using PEI-based siRNA delivery protocol (49), we performed in vivo knockdown of PAK1. Control injections were performed with nonspecific RNA duplexes. Targeted PAK1 depletion in the lungs was confirmed by Western blot analysis of PAK1 content in various tissue samples (Fig. 8A), and inhibition of PAK1 mRNA expression after 72 h of transfection was further confirmed by RT-PCR analysis of lung tissues (Fig. 8B). Similar increases in cell counts and protein content induced by LPS were observed in BAL from both control and siPAK1-treated mice (Fig. 8C). However, PAK1 depletion impaired the protective effects of ANP against LPS-induced lung injury, as indicated by increased BAL cell counts (1.92 ± 0.61 × 10^6 cells/ml in siPAK1-treated mice vs. 1.25 ± 0.37 × 10^6 cells/ml in controls treated with nonspecific RNA; P < 0.003) and protein concentration (0.66 ± 0.19 mg/ml for siPAK1 vs. 0.36 ± 0.11 mg/ml for nonspecific RNA controls; P < 0.001) after combined LPS and ANP treatment (Fig. 8C). Histological analysis of lung tissue showed that the ANP-protective effect against LPS-induced neutrophil infiltration was diminished in PAK1-depleted mice compared with mice transfected with nonspecific RNA (Fig. 8D). No significant differences were found between nontransfected animals and mice transfected with nonspecific RNA.

APN-mediated protection against LPS-induced pulmonary vascular leak was further assessed by measurements of Evans blue leakage into the lung tissue. LPS treatment of nontransfected controls (Fig. 4) or mice transfected with nonspecific RNA (Fig. 8E) induced considerable Evans blue vascular leakage into surrounding lung tissue, which was attenuated by ANP pretreatment. Similar levels of Evans blue extravasation in response to LPS were observed in the lungs of siPAK1-transfected mice, however, this vascular leak was not attenuated by ANP administration (29.33 ± 4.04 μg/g wet lung weight in PAK1 knockdowns vs. 14.27 ± 6.81 μg/g wet lung weight in controls treated with nonspecific RNA; P < 0.02). These results confirm our in vitro data and strongly suggest a role for PAK1 in the mediation of the barrier-protective effects of exogenous ANP in the pulmonary vasculature.

DISCUSSION

Plasma levels of ANP and BNP in normal patients are ~10 fmol/ml and become elevated 10- to 30-fold in patients with septic shock or congestive heart or acute renal failure (29, 42, 50). Elevated plasma NP levels are associated with higher disease severity and mortality rates (18, 25, 43, 50). However, the precise causal relationship between ANP elevation and the severity of ALI remain unclear. For example, introduction of

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Fig. 5. Analysis of LPS-induced ANP expression and PAK phosphorylation in the lungs. Mice were treated with LPS (0.63 mg/kg it) or vehicle for various time points. A: expression of ANP mRNA was determined by quantitative (q) RT-PCR analysis of right and left lung tissue samples. The graph represents pooled data; n = 6 for each experimental group; *P < 0.05. B: PAK phosphorylation in lungs was analyzed by Western blot with phosphospecific antibodies. Equal protein loading was confirmed by probing of membranes with total PAK1 and β-tubulin antibodies. Results of densitometry are shown as means ± SD; n = 6 per group; *P < 0.05. RDU, relative density units.

5.08 ± 2.45 × 10^6 cells/ml for LPS-treated Nppa^−/− mice; P < 0.05), BAL protein content (0.83 ± 0.26 mg/ml vs. 1.23 ± 0.28 mg/ml for LPS-treated Nppa^−/− mice; P < 0.05; Fig. 6E), and measurements of Evans blue accumulation in the lung tissue (37.0 ± 14.23 vs. 67.07 ± 13.83 μg/g wet lung weight in LPS-treated Nppa^−/− mice; P < 0.05; Fig. 6F). These data strongly support a physiological role for ANP in the modulation of agonist-induced lung injury and vascular permeability.

PAK1 mediates ANP-induced protection of EC barrier. The current study investigated a role for Rac effector PAK1 in the protective effects of ANP in the model of EC hyperpermeability caused by inflammatory agonists.

In the following experiments, the role of PAK1 was assessed using a siRNA approach. After 48 h of transfection with PAK1-specific or nonspecific siRNA, EC were preincubated with ANP and then stimulated with TNF-α (6 h). Stress fibers, paracellular gaps, and adherens junctions were visualized by double immunofluorescence staining for F-actin and VE-cadherin. ANP markedly attenuated TNF-α-induced stress fiber formation, reduced number of paracellular gaps, and prevented disruption of VE-cadherin-positive adherens junctions in both nontransfected EC and cells transfected with nonspecific RNA. In contrast, siRNA-induced knockdown of PAK1 abolished the protective effects of ANP against TNF-α-induced EC barrier disruption (Fig. 7A). Overlapping areas of adherens junctions and actin filaments appear in yellow in Fig. 7A, bottom.
Fig. 6. Analysis of LPS-induced lung injury in ANP knockout mice. ANP knockout mice (Nppa+/−) or matched controls were treated with LPS (0.63 mg/kg it) for 16 h. A: cell count and protein concentration were measured in BAL fluid as described in MATERIALS AND METHODS. B: LPS-induced vascular leak was analyzed by spectrometric determination of Evans blue-labeled albumin extravasation into the lung tissue. C: LPS-induced neutrophilic infiltration was evaluated by histological analysis of lung sections from control and Nppa+/− mice. D: PAK phosphorylation in lung samples from LPS-treated mice was analyzed after 1 h of LPS administration by Western blot with phosphospecific antibodies. Equal protein loading was confirmed by probing of membranes with total PAK1 and β-tubulin antibodies. Results of densitometry are shown as means ± SD; n = 6 per group; *P < 0.05. E and F: effects of single ANP injection (2 μg/kg iv) on LPS-induced lung injury in Nppa+/− mice were assessed by measurements of cell count and protein concentration in the BAL fluid (E) and by analysis of Evans blue accumulation in the lung tissue (F). n = 4–7 per group; *P < 0.05. WT, wild-type.
Fig. 7. Effects of PAK1 depletion on the ANP-mediated barrier protection in human pulmonary EC. EC grown on glass coverslips were transfected with nonspecific (ns) RNA or with PAK1-specific (siPAK1) small interfering RNA (siRNA). A: cells were treated with ANP (100 nM, 20 min) before TNF-α (20 ng/ml, 6 h) challenge. Analysis of cytoskeletal remodeling was performed by double immunofluorescence staining with VE-cadherin and Texas red-phalloidin. Paracellular gaps are marked by arrows. Bottom shows merged images of F-actin and VE-cadherin staining. B: in other experiments, cells were pretreated with ANP (100 nM, 20 min) followed by LPS (200 ng/ml, 6 h). Actin rearrangement was analyzed by immunofluorescence staining for F-actin. C: transendothelial resistance was monitored in control and PAK1-depleted EC over 10 h. Data are expressed as means ± SD of 3–8 independent experiments, n = 3 per experimental condition. Dotted lines mark the time points used for analysis of EC cytoskeletal remodeling depicted in A and B.
ANP reduced the mortality rate and had a beneficial effect in patients with ALI (34).

The main thrust of this work was testing exogenous ANP as a potential protective strategy in the model of LPS-induced ALI and analysis of the effects of endogenous ANP deficiency on LPS-induced lung barrier dysfunction. Our data show that ANP significantly attenuated pulmonary EC barrier dysfunction on treatment with LPS or inflammatory cytokines IL-6 and IL-1β.

Fig. 8. Role of PAK1 in ANP-mediated protection against LPS-induced vascular leak in vivo. Mice were transfected with nonspecific or PAK1-specific siRNA for 72 h. A: Western blot analysis of siRNA-mediated PAK1 knockdown was performed in the lung, heart, liver, and kidney tissue samples. Results of densitometry are shown as means ± SD; *P < 0.05. B: expression of PAK1 mRNA was determined by qRT-PCR analysis of lung tissue samples from control and siPAK1-treated mice (n = 4–6 per condition). C: animals were treated with LPS (0.63 mg/kg it, 16 h) with or without ANP treatment (2 μg/kg iv). After 16 h, cell count and protein concentration were determined in BAL fluid (n = 5–9 per group; *P < 0.001; **P < 0.003). D: whole lungs were used for histological evaluation by hematoxylin and eosin staining as described in MATERIALS AND METHODS (magnification: ×40; n = 4 per group, ≥20 fields per slide were inspected). E: effect of ANP on LPS-induced vascular leak was analyzed by Evans blue-labeled albumin extravasation into the lung tissue; n = 5–6 for each experimental group; *P < 0.02.
TNF-α and markedly reduced lung injury caused by intratracheal injection of LPS. These results strongly suggest a protective role of exogenous ANP against LPS-induced lung EC barrier dysfunction. Our data are also in good agreement with other experimental models, which showed that oxidant-induced pulmonary edema observed in perfused rabbit lungs was inhibited by ANP pretreatment (32). However, ANP-protective effects apparently depend on the type of injury. ANP- and NP receptor-A (NPR-A)-mediated elevation of cGMP observed in the ischemia-reperfusion model of lung injury increased lung vascular permeability (17). Interestingly, the same group showed that activation of PKG and cGMP decreased basal levels of lung EC permeability and attenuated pulmonary EC barrier dysfunction caused by hydrogen peroxide (36, 44).

Our results show that ANP knockout mice developed more severe lung injury in response to intratracheal LPS, which was alleviated by exogenous ANP. On the other hand, we found that LPS increased ANP mRNA levels in the lungs of wild-type mice. In addition to activation of ANP gene expression, lung injury conditions are associated with decreased number of ANP clearance receptors in the lung endothelium, which promotes retaining of ANP in circulation leading to elevated ANP levels (22, 40). LPS-induced activation of ANP is highly consistent with other studies showing a beneficial role of endogenous ANP in other models. These studies showed that passive neutralization of ANP in hydrochloric acid-treated animals further increased lung weight (51) and ANP blockade exacerbated high altitude pulmonary edema in endotoxin-primed rats (23). Our data also support a beneficial role for endogenous ANP in attenuation of ALI. Collectively, these results strongly suggest that an increase in plasma ANP levels may serve as an adaptive physiological mechanism to acute injury. The protective effects of ANP may also be associated in part with its anti-inflammatory activities. ANP inhibited vascular permeability, TNF-α secretion, NF-κB inflammatory cascade, and activation of p38 stress MAPK induced by LPS, hypoxia, or TNF-α stimulation of EC (20, 24, 27). ANP also suppressed NF-κB-mediated inflammatory signaling in vivo (30). However, these mechanisms were not the focus of this study.

ANP-induced Rac signaling leads to activation of Rac downstream cytoskeletal and signaling effectors, including serine/threonine kinase PAK1 (11, 15). PAK1 plays a crucial role in cell motility, cytoskeletal remodeling, cell contraction, and barrier regulation by serving both as a signaling kinase and an important adaptor protein (15). The results of this study show that ANP activates PAK1 in the pulmonary EC and in LPS-treated lungs, and siRNA-induced PAK1 knockdown attenuates protective effects of exogenous ANP in pulmonary EC culture and in the murine model of LPS-induced lung vascular leak. The role of PAK1 in control of lung vascular permeability was also addressed in other studies, which used PAK function blocking peptide (47). This peptide does not inhibit PAK kinase activity but impairs PAK binding to Nck and prevents PAK translocation to the sites of action. This peptide prevented PAK junctional translocation and increased permeability (47). Another blocking peptide preventing the interaction between PAK1 and PIX blocked VEGF-induced permeability in vitro and decreased LPS-induced lung vascular leakage in vivo (46), whereas expression of active PAK1 in EC significantly inhibited thrombin-induced EC barrier dysfunction (26). Taken together, published studies and our results emphasize the complexity of PAK1 functions in different cell types during ALI and demonstrate that inhibition of different PAK1 modalities (adaptor, targeting, or kinase functions) causes distinct effects on vascular permeability and the progression of ALI.

Our results show that LPS-induced lung injury and barrier dysfunction were more severe in ANP−/− mice and were accompanied by decreased PAK1 activation in the lungs but were attenuated by injection of exogenous ANP. ANP is known to stimulate Rac and Rac-dependent PAK1 activation in pulmonary EC (11). In turn, PAK1 knockdown attenuated protective effects of pharmacological ANP doses in mice challenged with LPS. However, PAK1 depletion did not exacerbate LPS-induced lung injury despite the finding that LPS induces endogenous ANP production. The clear explanation of these effects at the moment is not available and requires further investigation. It is possible that timing, rapid onset, duration, and magnitude of PAK1 activation caused by injection of exogenous ANP concomitant with LPS challenge, as opposed to effects of endogenous ANP induction secondary to LPS treatment, may be definitive factors in these differences. Thus we do not consider exacerbation of ALI in ANP knockout mice and lack of effects of PAK1 knockdown on the LPS-induced lung dysfunction as contradictory results. These results emphasize once again the complexity of physiological responses at the level of the whole organism.

In summary, this study led to three major conclusions: 1) exogenous ANP protects against lung barrier dysfunction and neutrophil accumulation induced by intratracheal LPS challenge; 2) protective effects of exogenous ANP against LPS-induced lung dysfunction are mediated by lung vascular endothelium and controlled by Rac GTPase effector PAK1; PAK1 molecular inhibition abolishes ANP-protective effects against vascular endothelial leak both in animal models and in EC culture; and 3) LPS stimulates endogenous ANP production in the lung, whereas LPS-induced lung dysfunction is more expressed in ANP knockout mice. We speculate that such ANP elevation my represent an intrinsic mechanism that negatively regulates endotoxin-induced inflammatory process and lung dysfunction but may also engage other (i.e., anti-inflammatory) mechanisms in addition to endothelial effects described in this study.

Protective effects of exogenous ANP described in this study suggest a new therapeutic option for the treatment of lung vascular leak associated with septic lung inflammation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


Stockton R, Reutershan J, Scott D, Sanders J, Ley K, Schwartz MA. Induction of vascular permeability: beta PIX and GIT1 scaffold the


