The role of natriuretic peptide receptor-A signaling in unilateral lung ischemia-reperfusion injury in the intact mouse

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Submitted 8 May 2007; accepted in final form 20 January 2008

Dodd-o JM, Hristopoulos ML, Kibler K, Gutkowska J, Mukaddam-Daher S, Gonzalez A, Welsh-Servinsky LE, Pearse DB. The role of natriuretic peptide receptor-A signaling in unilateral lung ischemia-reperfusion injury in the intact mouse. Am J Physiol Lung Cell Mol Physiol 294: L714–L723, 2008. First published January 25, 2008; doi:10.1152/ajplung.00185.2007. —Ischemia-reperfusion (IR) causes human lung injury in association with the release of atrial and brain natriuretic peptides (ANP and BNP), but the role of ANP/BNP in IR lung injury is unknown. ANP and BNP bind to natriuretic peptide receptor-A (NPR-A) generating cGMP and to NPR-C, a clearance receptor that can decrease intracellular cAMP. To determine the role of NPR-A signaling in IR lung injury, we administered the NPR-A blocker anantin in an in vivo SWR mouse preparation of unilateral lung IR. With uninterrupted ventilation, the left pulmonary artery was occluded for 30 min and then reperfused for 60 or 150 min. Anantin administration decreased IR-induced Evans blue dye extravasation and wet weight in the reperfused left lung, suggesting an injurious role for NPR-A signaling in lung IR. In isolated mouse lungs, exogenous ANP (2.5 nM) added to the perfusate significantly increased the filtration coefficient sevenfold only if lungs were subjected to IR. This effect of ANP was also blocked by anantin. Unilateral in vivo IR increased endogenous plasma ANP, lung cGMP concentration, and lung protein kinase G (PKG) activation. Anantin enhanced plasma ANP concentrations and attenuated the increase in cGMP and PKG activation but had no effect on lung cAMP. These data suggest that lung IR triggered ANP release and altered endothelial signaling so that NPR-A activation caused increased pulmonary endothelial permeability.

anantin; atrial natriuretic peptide; permeability; guanosine 3’,5’-cyclic monophosphate; adenosine 3’,5’-cyclic monophosphate

ISCHEMIA-REPERFUSION (IR) lung injury complicates cardiopulmonary bypass surgery (7), lung transplantation (1), pulmonary artery thromboendarterectomy (26), and thrombosis of acute pulmonary thromboembolism (55). IR lung injury is characterized by endothelial dysfunction leading to increased pulmonary vascular permeability, edema, and increased resistance to blood flow (37). One potential consequence of a lung IR is the secretion of atrial and brain natriuretic peptides (ANP and BNP) from the right heart into the pulmonary circulation. In fact, markedly elevated plasma levels of ANP or BNP have been found in association with cardiopulmonary bypass surgery (2, 8), chronic thromboembolic pulmonary hypertension (16), and acute pulmonary thromboembolism (24). The effect of these natriuretic peptides on lung IR-induced endothelial barrier dysfunction is unknown.

ANP and BNP are synthesized primarily in the cardiac atria and ventricles, respectively, and are released into the blood stream by atrial and ventricular stretch (41). In contrast, C-type natriuretic peptide (CNP) is found in the brain and vascular endothelium. CNP acts locally to affect vascular function. The receptor targets of the natriuretic peptides include NPR-A, NPR-B, and NPR-C. These receptors are expressed on many cells, including pulmonary vascular endothelium (39). NPR-A, the receptor for ANP and BNP, and NPR-B, the CNP target, contain an intracellular particulate guanylate cyclase (pGC). pGC catalyzes the formation of cGMP, the downstream second messenger involved in most ANP/BNP signaling (39, 41). cGMP has three intracellular signaling targets: PKG, cGMP-sensitive phosphodiesterase (PDE), and cyclic nucleotide-gated cation channels (18). All three are present in pulmonary microvascular endothelium (36, 47, 48).

NPR-C lack pGC activity and are thought to function primarily as clearance receptors (41). NPR-C may also play a role in endothelial signaling, however, because NPR-C binding has been shown to inhibit adenylate cyclase activity in rat lung membranes (42) and decrease intracellular cAMP concentration in aortic endothelial cells (14). In rats, the lung contains the largest tissue concentration of NPR, with 90% being the NPR-C type (39). An NPR-C-mediated decrease in endothelial cAMP could result in an increase in pulmonary endothelial permeability because of the critical dependence of basal endothelial paracellular permeability on intracellular cAMP concentration (49).

The effect of ANP on pulmonary endothelial barrier function is controversial, with studies showing both increased (44, 59) and decreased (21, 25, 28, 30, 52, 57) lung endothelial permeability. We could not find published data regarding the effect of ANP or BNP on lung IR injury.

We have been interested in the effect of membrane-permeable cGMP analogs on pulmonary vascular permeability following IR in intact lungs (35) or reactive oxygen species (ROS) exposure in cultured monolayers (31, 36). Based on the cGMP-mediated protective effects observed in these studies, we anticipated that any endogenous ANP released by the heart during lung IR injury would also prove protective if endothelial cGMP increased.
The goal of the current study was to determine the role of endogenous ANP/BNP in an intact mouse model of unilateral left lung IR injury (10, 11). This preparation avoids direct cardiac manipulation, extracorporeal perfusion, and prolonged anesthesia. Each of these could independently affect natriuretic peptide secretion (4, 5, 17). We utilized the competitive NPR-A antagonist anantin (56) to block the effects of any ANP or BNP-mediated effects on the IR injury in our model. Anantin has no agonist activity and does not inhibit NPR-C binding or activity (53). SWR mice were chosen for study because this strain exhibited the greatest increase in left lung Evans blue dye (EBD) extravasation in our IR model (11).

Based on the previously observed cGMP-mediated protection in IR/ROS injury (31, 35, 36, 38), we hypothesized that anantin would exacerbate IR lung injury through blockade of NPR-A activation and inhibition of endothelial cGMP generation in the reperfused lung. To our surprise, we found that anantin significantly attenuated the IR-induced endothelial barrier dysfunction in the reperfused left lung of the intact mouse but had no effect in the non-IR-exposed right lung. These findings suggest an injurious role for ANP signaling in this form of lung injury. We confirmed these results in an isolated, perfused mouse lung IR model. In this model, exogenous ANP added to the perfusate increased the filtration coefficient ($K_f$) only in lungs subjected to IR. The effect of ANP was inhibited by anantin in this isolated lung IR model as well. In intact mice, unilateral IR increased circulating ANP. Anantin pretreatment further enhanced this IR-induced rise in circulating ANP. Anantin blocked IR-induced increases in plasma and left lung cGMP concentrations. Anantin also prevented IR-induced lung PKG$_I$ activation, confirming blockade of NPR-A function. Lung PDE2 expression was detected, but neither IR nor anantin altered lung or plasma cAMP concentrations. Overall, our results suggest that unilateral lung IR in the intact mouse triggered ANP release and increased endothelial permeability in the reperfused lung. This occurred in part through NPR-A signaling independent of measurable changes in cAMP.

**METHODS**

**In Vivo Mouse Lung IR**

The protocols in this study were approved by The Johns Hopkins Institutions Animal Care and Use Committee. Left lung ischemia (30 min) and reperfusion (150 min) were achieved as previously reported (10, 11). Briefly, SWR male mice (20–24 g, 6–10 wk old; The Jackson Laboratories, Bar Harbor, ME) were anesthetized (2 mg/ml etomidate-5 µg/ml fentanyl, 10 µg/g ip), intubated, and mechanically ventilated. The left pulmonary artery was isolated from the left thoracotomy and occluded with a slipknot (8–0 Prolene suture). One end of the slipknot was exteriorized for later release. The thoracotomy was closed (5–0 silk) after verification of lung reexpansion. The mouse was extubated, and the pulmonary artery tie was released after 30 min of vessel occlusion.

**EBD Extravasation**

EBD (30 mg/kg; Sigma, St. Louis, MO) was administered intravenously during the period of left pulmonary artery ischemia as previously described (10). After 150 min of reperfusion, the mice were killed by exsanguination as a saline flush cleared intravascular EBD. The left lower lobe and right lungs were separately weighed and incubated for 24 h at 37°C in formamide (1 ml of formamide per 100 mg of lung; Sigma). After incubation for 24 h, aliquots (200 µl) of the formamide supernatant were placed in 96-well plates for colorimetric evaluation using a spectrophotometer (620 nm). We previously showed that EBD extravasation in this preparation occurred only with IR and correlated with a radiolabeled albumin escape index when left lung ischemic times were varied (10).

**Effect of ANP on IR in Isolated Perfused Mouse Lungs**

SWR mice (25–30 g) were anesthetized with sodium pentobarbital (60 mg/kg) administered intraperitoneally. The trachea was cannulated, and the mice were mechanically ventilated as described above. A median sternotomy was performed, and the pulmonary artery and left atrium were cannulated via the right and left ventricles, respectively, utilizing a water-jacketed (37°C) chamber designed for the isolated perfused mouse lung preparation (Hugo Sachs Elektronik, March-Hugstetten, Germany). The inspired gas tensions were changed to 5% CO$_2$, and 21% O$_2$, and positive end-expiratory pressure was adjusted to 3 mmHg. In all experiments, perfusion was immediately initiated following pulmonary artery cannulation for a 10-min stabilization period with a constant recirculating flow (2 ml/min) of Brinster’s BMOC-3 media (11126034, Invitrogen) and 3% Bovine Serum Albumin Fraction V (Sigma) warmed to 37°C. In IR experiments, perfusion was then stopped with continued ventilation for 30 or 60 min before beginning reperfusion to mimic the ischemic time in the intact mouse protocol. Left atrial pressure was set at 6 mmHg in all experiments to achieve zone 3 conditions by adjusting the vertical distance between the lungs and the perfusion reservoir. Pulmonary arterial (Ppa), left atrial, and tracheal pressures were continuously monitored and digitally recorded as was reservoir weight, which served as the negative index of lung weight gain (W). The rate of fluid filtration into the lung (W) during the perfusion period was calculated at 20-min intervals by dividing the average W for each 20-min period by the final lung dry weight. After 60 min of perfusion, $K_f$ was measured by rapidly sealing and pressurizing the reservoir after occluding the left atrial cannula to allow controlled increases in static pulmonary vascular pressure to 10 and 15 mmHg at 15-min intervals as previously described (11). The W over the final 5 min at each pressure was used as an estimate of lung fluid filtration. The rate of fluid filtration at each vascular pressure was plotted against vascular pressure, and the slope of this relationship was used to estimate the $K_f$.

**Plasma and Tissue Measurement of ANP, cGMP, and cAMP**

ANP (Phoenix Pharmaceuticals, Mountain View, CA), cGMP (PerkinElmer Life Sciences, Wellesley, MA), and cAMP (Amersham, Piscataway, NJ) were measured using commercially available radioimmunoassay kits specific for each. Whole blood was collected from the left ventricle by direct ventricular puncture. Blood was collected into a chilled tube containing 100 µl of HBSS containing aprotinin (100–500 U/ml blood) and 0.5 M EDTA (pH 8, 30:1 ratio). Blood samples were immediately centrifuged (3,000 rpm for 5 min), and the supernatant frozen (−80°C) until use. Lung samples, flushed free of blood using normal saline, were boiled in 3 ml of 0.1 M acetic acid (left and right lungs separately, 5 min each) to inactivate intrinsic proteolytic activity. The acid was removed, and the tissue homogenized in 1:100 EDTA:HBSS and centrifuged (16,000 rpm for 20 min), and the supernatant frozen (−80°C).

**Gel Electrophoresis and Immunoblot Analysis**

To look for evidence of lung parenchymal PKGI activation, we performed immunoblots for the endothelial cytoskeletal protein vasoactive intestinal peptide (VIP)/vasoactive intestinal peptide (VIP) receptor (V1, V2, V3) and for monophasic phosphorylation of murine VIP on Ser$_{235}$ (Pser$_{235}$, VASP). PKGI preferentially phosphorylates murine VIP on Ser$_{235}$, which corresponds to Ser$_{239}$ in human VASP (27). Quantification of this specific VIP phosphorylation site correlates with PKGI activity in endothelial cells (31) and the monoclonal antibody directed against Pser$_{239}$-VASP in human cells.
( Alexis ) also detects Pser235-VASP in murine cells ( 27 ). PKG can also phosphorylate murine VASP on Ser53 ( 27 ), although this site, analogous to Ser157 in human VASP, is the preferred site for PKA ( 6 ), the downstream kinase for cAMP. Pser153-VASP is quantified by a shift in the apparent molecular mass in SDS-PAGE from 46 to 50 kDa ( 13 ).

Frozen lungs were homogenized in PBS ( 200 μl per 10 mg lung ) containing protease inhibitors as previously described ( 11 ). The samples were centrifuged ( 4,000 rpm for 5 min ), and the supernatant subjected to PAGE on an 11% gel. The separated proteins were transferred to nitrocellulose membrane ( 0.2-μm BA-S 83 ), and the membrane was probed with primary antibodies ( 1:200 dilution ). The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin ( Bio-Rad ) used at 1:2,000 dilution. In addition to VASP, lungs were also probed with antibodies directed against PDE2A ( FabGennix ), a PDE isozyme that is capable of cGMP-stimulated cAMP degradation, and PDE4 ( FabGennix ), another cAMP-degrading PDE. Bands were visualized using the enhanced chemiluminescence ( Amershams ) reagents and by exposing the blot to X-ray film ( BioMax MR, Kodak ). Densitometric quantification was performed using UN-SCAN-IT Gel Automated Digitizing System software version 5.1 ( Silk Scientific, Orem, UT ).

Lung Membrane Binding Studies

Lung microsomal membranes were prepared as previously described ( 33 ). Optimal binding conditions were established in the membrane preparations using freshly prepared 125I-ANP, which was iodinated by the lactoperoxidase method and purified by high-performance liquid chromatography ( 33 ). Membrane preparations in 20-μg aliquots were incubated for 2.5 h at 22°C with ~20,000 cpm 125I-ANP with and without increasing concentrations ( 1×10−12 to 1×10−6 M ) of ANP in a total volume of 0.2 ml. The reaction was stopped by adding 3 ml of cold 50 mM Tris-HCl buffer, pH 7.4, followed by filtration on Whatman GF/C filters ( Millipore ) presoaked in 1% normal data were log-converted before using the appropriate parametric test. Values presented in the text are means ± SE. Differences were considered significant when P ≤ 0.05.

RESULTS

Lung EBD Extravasation Following IR

As shown in Fig. 1, 30 min of left lung ischemia followed by 150 min of perfusion in diluent-treated mice resulted in a twofold increase in left lung EBD extravasation compared with the contralateral right lung value. High-dose ( but not low-dose ) anantin treatment significantly attenuated this increase in barrier dysfunction by 55%. The addition of l-NMA to anantin treatment to inhibit nitric oxide production from nitric oxide synthase, another potential source of endothelial cGMP production, resulted in protection that was not different from anantin treatment alone. The inset graph in Fig. 1 shows the mean values of the right and left wet lung weights from the high-dose anantin-treated mice subjected to 30 min of left lung ischemia and 150 min of perfusion each normalized to the average right and left wet weights, respectively, from diluent-treated mice subjected to the same IR protocol. The diluent ( 0.170 ± 0.059 g ) and anantin-treated right lungs ( 0.110 ± 0.009 g ) had similar mean wet weights ( average ratio of 1.01 ± 0.06 ) indicating that anantin treatment did not affect fluid balance in the right non-IR lung. In contrast, the left lung wet weight from anantin-treated mice ( 0.045 ± 0.003 g ) was significantly less ( P < 0.05 ) than diluent-treated mice following IR ( 0.141 ± 0.052 g ) by an average of 66% as shown by an anantin-to-diluent left lung wet weight ratio of 0.34 ± 0.02 ( P < 0.005 ).
Effect of Exogenous ANP on $K_f$ in Mouse Isolated Lung IR

As shown in Fig. 2A, exogenous ANP administration at the time of reperfusion resulted in a significant sevenfold increase in $K_f$ in lungs exposed to IR ($P < 0.0001$). By contrast, ANP had no effect on $K_f$ in lungs perfused for 60 min without a preceding ischemic time. This IR-dependent ANP effect on $K_f$ was completely prevented by anantin pretreatment.

Of note, the IR groups in Fig. 2 consisted of a mixture of consecutive lungs subjected to either 30 ($n = 12$) or 60 ($n = 13$) min of ischemia and 60 min of reperfusion. This is because a shift in the response of the lungs to IR injury occurred over the time these experiments were performed. To allow the combination of all of the data for the analysis in Fig. 2, we adjusted for the shift in injury by normalizing the $K_f$ in each experiment to the contemporaneous mean $K_f$ value obtained in the group subjected to perfusion without IR or ANP. There were no associated systematic differences in $P_{pa}$ or tracheal pressure, so these data sets were combined without normalization.

The administration of exogenous ANP (2.5 nM) to the perfusion reservoir had no effect on the time course of $P_{pa}$ or, therefore, pulmonary vascular resistance (PVR) in the presence or absence of a 30-min period of ischemia (Fig. 2B). There was a significant interaction between IR condition and time ($P < 0.0001$) independent of ANP or diluent treatment indicating that $P_{pa}$ (and PVR) in IR lungs started higher and then decreased over time early in reperfusion compared with non-IR lungs. Anantin pretreatment increased $P_{pa}$ at all time points indicating an increased PVR ($P < 0.0001$).

Figure 2C shows that there was no difference in $W$ in IR lungs whether ANP was present or absent from the perfusate. In non-IR lungs, however, ANP treatment resulted in a con-
sistent decrease in W over the last 40 min of perfusion compared with diluent-treated lungs (P < 0.01). For the reasons described above, W values for each experiment were normalized to the contemporaneous mean 20-min value of W in lungs subjected to 60 min of perfusion without ischemia or ANP. The significant early increase in Ppa and PVR in the IR lungs compared with non-IR lungs was associated with a significant early IR-induced increase in W (P < 0.0001) independent of ANP vs. diluent treatment. This early IR-induced increase in W was significantly exacerbated by anantin pretreatment (P < 0.0001). There were no differences between groups or over time in peak tracheal pressure, which averaged 6.7 ± 0.07 mmHg (data not shown).

**Plasma and Lung ANP, cGMP, and VASP Phosphorylation Following IR in Intact Mouse Lungs**

There was a significant increase in plasma ANP concentration during the 30-min period of left lung ischemia that persisted at 15 min of reperfusion in diluent-treated animals (P < 0.03). Anantin treatment did not affect the increase in plasma ANP concentration during left lung ischemia but significantly increased plasma ANP at 15 min of reperfusion (P < 0.04) compared with diluent-treated mice (Fig. 3A). There were no detectable effects of either IR or anantin on lung tissue ANP concentrations (Fig. 3B).

To determine if the changes in plasma ANP resulted in NPR-A signaling via the generation of cGMP, we measured the time course of plasma and lung cGMP concentrations in diluent and anantin-treated mice. Plasma cGMP averaged 4.7 ± 0.78 pmol/ml in untreated baseline mice and was not altered by IR in diluent-treated mice (Fig. 4A). In contrast, anantin treatment caused a significant decrease in plasma cGMP concentration at 60 min compared with both the baseline concentration in untreated mice (P < 0.001) and diluent-treated mice at the same time point by a significant (P < 0.005) ANOVA interaction.

The cGMP concentration in the reperfused left lung from diluent-treated mice significantly increased at 15 and 60 min of reperfusion compared with the unchanged right lung cGMP time course (P < 0.02). Anantin treatment resulted in a significant decrease in left lung cGMP concentration at 60 min based on a significant three-factor (treatment, time, lung) ANOVA interaction (P < 0.04).

The changes in plasma and lung cGMP concentrations were followed by a significant increase in lung Psr235-VASP at 150 min (Fig. 5) that occurred in both lungs equally despite the increased cGMP concentration observed in the reperfused left lung compared with the right (Fig. 4B). Anantin significantly attenuated Psr235-VASP formation. IR did not alter Psr153-VASP phosphorylation in either lung over time.

**Effect of Anantin on Lung ANP Binding Kinetics**

ANP-induced cGMP generation through binding of NPR-A has been shown to downregulate the density of NPR-C in pulmonary endothelial cells (22), suggesting that successful blockade of NPR-A with anantin could increase lung membrane NPR-C density. Because NPR-C represent 95% of expressed natriuretic peptide receptors on pulmonary endothelial cells (22), this effect would be expected to increase total ANP binding capacity on isolated lung membranes without altering ANP binding kinetics. As shown in Fig. 6, anantin treatment did increase the number of specific ANP binding sites as evidenced by the significant increase in Bmax compared with diluent pretreatment (427 ± 40 vs. 325 ± 30 fmol/mg protein; P < 0.05). Anantin had no effect on Kd.

**Lung PDE2 Expression and cAMP Concentration Following IR**

To determine if ANP-induced changes in lung cGMP concentration could affect endothelial barrier function through a PDE-dependent effect on lung cAMP concentration, we measured PDE2 protein expression at baseline and following IR. We also measured the cAMP-specific PDE4 but found no detectable expression (data not shown). PDE2 expression was detectable in uninjured mouse lung homogenates (n = 2), but there were not any statistically significant changes in expression as a function of IR (data not shown, n = 7). There was an unexplained difference between left and right lungs in PDE2
expression such that the left lungs had consistently greater PDE2 expression compared with the corresponding right lungs both at baseline and following IR. There were also no detectable differences in plasma or lung cAMP concentration as a function of IR or anantin treatment (data not shown, n/110057–17).

DISCUSSION

In this study, we investigated the effect of the NPR-A blocker anantin on IR lung injury in the intact mouse. Anantin attenuated the IR-induced pulmonary vascular protein extravasation and lung wet weight, suggesting an injurious role for endogenously released ANP in IR lung injury. This finding was supported by experiments performed in isolated perfused mouse lungs. In these experiments, administration of a physiological concentration of ANP exacerbated the $k_f$ in lungs subjected to IR. This effect was also blocked by anantin pretreatment and did not occur in lungs undergoing control perfusion without ischemia.

Limitations of our findings include the use of a single mouse strain. We chose this strain because it demonstrated an increased sensitivity to IR lung injury (11). Thus our results are not necessarily applicable to other inbred mouse strains.

Fig. 4. Time course of plasma (A) and lung (B) cGMP concentrations from mice treated with diluent (n = 8–10) or anantin (n = 9–10) before undergoing 30 min of left lung ischemia followed by 0, 15, or 60 min of reperfusion. Plasma cGMP at −30 min shown in A was obtained from right ventricular blood (closed diamond) from untreated, anesthetized mice subjected to sham surgery (n = 12). Basal lung cGMP concentrations from right (open diamond) and left (closed diamond) lungs from these untreated control mice (n = 12) are shown in B. Values are means ± SE. *P < 0.0005 vs. corresponding diluent value by two-factor (drug treatment, time) ANOVA interaction. †P < 0.001 vs. basal plasma cGMP concentration by one-factor ANOVA. ‡P < 0.02 vs. corresponding right lung value by two-factor (time, lung) ANOVA interaction. #P < 0.04 by three-factor (drug treatment, time, lung) ANOVA interaction. LL, left lung; RL, right lung.

Fig. 5. Time course of lung expression of monophosphorylated vasodilator-stimulated phosphoprotein Ser235 (Pser235-VASP) and Pser153-VASP from diluent-treated mice (triangles) subjected to 30 min of left lung ischemia followed by 60 (n = 3) or 150 (n = 4) min of reperfusion. An additional group of mice (n = 5) was treated with anantin (circles) and studied after 30 min of ischemia and 150 min of reperfusion for Pser235-VASP expression. Values at −30 min were obtained from untreated, anesthetized mice (n = 2). Pser235-VASP blots, normalized to GAPDH expression, and Pser153-VASP blots, normalized to total VASP, are expressed as fraction of measurements obtained in the untreated, control mice. Values are means ± SE of phosphorylated VASP determined from densitometry of immunoblots. Representative immunoblots for Pser235-VASP from single diluent-treated mice from each time point are shown above the mean data. *P < 0.03 vs. Pser153-VASP two-factor (phosphorylation site, time) ANOVA interaction. †P < 0.05 vs. 60 min.

Fig. 6. Effect of pretreatment with anantin (n = 10 mice) or diluent (n = 15 mice) on competitive binding of $^{125}$I-ANP to in vitro mouse lung membranes. Binding is represented as % bound/total radioactivity used. Values are means ± SE. *P < 0.05 vs. diluent. $K_d$, equilibrium dissociation constant; $B_{max}$, maximum binding capacity.

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second potential limitation is that we produced pulmonary artery ischemia in both our intact and isolated mouse lung preparations without airway occlusion. This approach differed from many lung IR studies (12, 40). Thus our results may differ from other animal models in which pulmonary artery IR was accompanied by lack of ventilation and hypoxia-reoxygenation.

The effect of ANP and NPR-A activation on pulmonary endothelial barrier function is controversial. In uninjured animals, exogenous ANP administration increased protein flux across the systemic and pulmonary vascular endothelium (44). Interestingly, the injurious effect of ANP on the lung was less pronounced than that observed in systemic organs in these studies (44, 62). In contrast, administration of exogenous ANP attenuated the acute lung injury in intact lungs caused by acid aspiration (52), oxidant generation (28), or oleic acid administration (30).

Consistent with these data, inhibition of endogenous ANP activity with anti-ANP antibody or NPR-A blockers worsened lung injury from sepsis (50) and acid aspiration (54). Exogenous ANP administration also attenuated pulmonary microvascular endothelial barrier dysfunction in vitro (21, 25). By contrast, both inhibition (21, 57) and exacerbation (59) of endothelial barrier dysfunction in pulmonary artery conduit endothelium have been reported.

We could find no published studies of ANP in lung IR injury. Lung IR has been shown to cause decreased lung parenchymal cGMP concentration (35, 38, 40). Moreover, stimulation of soluble GC (sGC) with NO (38) or pretreatment with membrane-permeant cGMP analogs (35, 38, 40) attenuated IR-induced increases in endothelial permeability, suggesting a protective role for endothelial cGMP. It may not be possible to extrapolate the consequences of an increase in endogenous ANP release from these studies, however, because exogenous administration of cGMP analogs may have different effects from endogenous ANP signaling. In addition, NPR-C signaling may dominate in pulmonary endothelium. Finally, the effect of cGMP on endothelial barrier function may depend on the state of endothelial activation (19) and the endothelial compartment in which cGMP is generated (43).

In the current study, anantin selectively attenuated the increase in EBD and wet weight in the reperfused left lung in a dose-response manner (Fig. 1) without altering right lung values. This suggests that IR-induced NPR-A activation preferentially enhanced injury only in IR-exposed endothelium. This result supports the hypothesis that the activation state of the endothelium may be an important determinant of the response to ANP stimulation. For example, Holschermann et al. (19) found that exogenous ANP decreased basal endothelial permeability in unstimulated aortic endothelial monolayers but further increased permeability if administered after intracellular calcium concentration ([Ca<sup>2+</sup>]) was increased. Reperfusion lung injury has been shown to likely require increased pulmonary endothelial [Ca<sup>2+</sup>]; (23, 58). It is unknown whether changes in [Ca<sup>2+</sup>], alter the effects of ANP/cGMP signaling on pulmonary endothelial barrier function.

We included a group of mice in which nitric oxide synthase inhibition was combined with anantin because we initially anticipated that NPR-A blockade would enhance lung IR injury. We therefore wanted to be able to determine if cGMP from endogenous NO-stimulated sGC played any role in modulating postreperfusion endothelial permeability. In addition, there are data suggesting a reciprocal relationship between pGC and sGC activities when one is inhibited (29). As shown in Fig. 1, this combined treatment group demonstrated the same amount of protection as anantin alone. This suggests that NO signaling played no role in the IR-induced permeability change. This result is consistent with previous data from intact rat lung IR injury showing markedly decreased lung surface NO production during reperfusion (40).

To confirm that the beneficial effects of anantin on pulmonary endothelial barrier function in the mouse reflected the interruption of ANP-stimulating NPR-A (rather than a nonspecific effect of anantin), we examined the effect of exogenous ANP administration in an isolated mouse lung model of IR. We reasoned that IR-induced changes in endogenous ANP would likely be attenuated in this model because the right heart was excluded from the circuit. We therefore added ANP to the perfusion reservoir to achieve a concentration that both mimicked our plasma measurements in the intact mice (Fig. 3) as well as previously published plasma measurements from intact mice (51).

Consistent with the effect of anantin in the intact unilateral IR model, exogenous ANP administration worsened endothelial barrier function only in IR-exposed lungs (Fig. 2A). Moreover, this IR-dependent effect was completely prevented by anantin pretreatment, further supporting an injurious role of NPR-A signaling in IR-exposed lungs. The ANP-induced increase in K<sub>i</sub> did not appear to be secondary to an increase in vascular surface area. That is, ANP had no effect on the time course of P<sub>pa</sub> (Fig. 2B), which, in a constant perfusion system, directly reflects changes in PVR. In contrast, ANP significantly decreased the rate of lung fluid filtration during perfusion in the non-IR perfused lungs (Fig. 2C). There was, however, no difference in mean K<sub>i</sub> between these groups. Not surprisingly, IR resulted in a significant increase in P<sub>pa</sub> and W early in reperfusion. This could occur if a significant component of the increase in PVR developed downstream from the major site of fluid filtration. Anantin pretreatment exacerbated this effect, suggesting that ANP possibly countered an increase in downstream resistance through NPR-A stimulation. This result also suggests that it was unlikely that the protective effect of anantin on the increased K<sub>i</sub> following ANP could be due to a decrease in vascular surface area.

To confirm that the anantin-induced effects in our intact lung IR model was due to NPR-A blockade, we made direct measurements of cyclic nucleotides and VASP phosphorylation. VASP is a well-characterized target of both activated PKG<sub>i</sub> and PKA (6). As shown in Fig. 3, we were able to detect a reperfusion-dependent increase in circulating ANP concentrations compared with baseline control values. This suggests that unilateral IR lung injury in the mouse likely stimulates atrial ANP secretion secondary to atrial distension or hypoxia (41). Interestingly, the anantin-treated mice had a significantly greater increase in plasma ANP compared with diluent early in reperfusion. This was either due to displacement of ANP from NPR-A by anantin or to a stimulatory effect of anantin on atrial myocyte ANP secretion. A stimulatory effect of anantin on atrial myocyte ANP secretion has been previously observed and was thought to be secondary to loss of an ANP-mediated feedback inhibitory effect (34). We observed a trend toward increased lung tissue ANP concentration in IR lungs, but there
were no differences detected as a function of anantin. This was not surprising given the large number of NPR-C compared with NPR-A in lung cellular membranes (39).

The IR-induced increased plasma ANP concentrations did not affect plasma cGMP concentration (Fig. 4A). IR did, however, significantly and selectively increase left lung cGMP concentration at 15 and 60 min of reperfusion compared with both baseline and right lung values by an ANOVA interaction. This suggests that IR either potentiated pGC activity or inhibited a PDE responsible for lung cGMP degradation. Anantin treatment significantly decreased both plasma and left lung cGMP concentrations at 60 min. This suggests that the increased cGMP in the left lung was, in fact, due to IR-induced ANP release. Given the poor membrane permeability of cGMP, we suspect that the anantin-induced decrease in plasma cGMP resulted from decreased endothelial cGMP generation and secretion, which has been shown to occur in endothelium by active secretion through an ATPase-dependent transporter (45).

Although we found a preferential increase in left lung cGMP early in reperfusion by 150 min, we found evidence of equivalent phosphorylation of the PKG$_{1}$-preferred Ser$^{235}$-VASP in right and left lungs. This suggests that there was either a cGMP-independent mechanism of VASP phosphorylation (20) or that the right lung cGMP measurements were insufficiently sensitive to pick up key compartmental changes in cGMP concentration. We suspect the latter possibility was correct because we confirmed that this P$_{ser}^{235}$-VASP signal originated from NPR-A/pGC activity by showing that this phosphorylation signal was significantly attenuated by anantin pretreatment (Fig. 5). We found no changes over time in P$_{ser}^{235}$-VASP in either lung suggesting that PKA activity was not increased. These data do not necessarily implicate PKG$_{1}$ or VASP in the excess endothelial barrier dysfunction caused by NPR-A stimulation in our experiments because cGMP has PKG$_{1}$-independent effects (41) that could be playing a role in this regard. Additional experiments with PKG$_{1}$ antagonists or PKG$_{1}$ knock-out mice will be necessary to address this question.

As mentioned above, the predominant NPR on pulmonary endothelium is NPR-C. This function both as a clearance receptor (42) and as an inhibitor of endothelial adenylate cyclase activity (14). Increasing pulmonary endothelial cGMP concentration by NPR-A stimulation was shown to downregulate NPR-C expression (22). We therefore hypothesized that the decreased lung cGMP concentration caused by anantin-induced inhibition of NPR-A should upregulate NPR-C expression. If NPR-C binding succeeded in decreasing endothelial adenylate cyclase activity, this effect could also serve to limit the observed barrier protection mediated by NPR-A blockade. Alternatively, increased NPR-C signaling could be part of the explanation for anantin-induced protection because specific NPR-C stimulation was recently shown to inhibit cardiac IR injury (15) and endothelial-neutrophil interactions (46). As shown in Fig. 6, we did find that anantin treatment increased maximal ANP binding to mouse lung membranes without affecting the $K_{d}$, suggesting increased NPR-C number assuming that the small number of NPR-A remained blocked. Additional experiments with specific NPR-C agonists will be necessary to further explore the role of NPR-C in the protective effect of anantin.

Finally, we considered whether the apparent injurious effects of NPR-A stimulation in lung IR injury could be mediated through a cGMP-stimulated cAMP-degrading PDE. The specific PDEs expressed in lung endothelium differ as a function of species and conduit vs. microvascular endothelium (61). PDE2, -4, and -5 appear to represent the most significant pulmonary endothelial PDEs (32, 60, 61). PDE5 hydrolyzes cGMP (3), whereas PDE4 degrades cAMP. PDE2 can hydrolyze both cGMP and cAMP and is stimulated by cGMP (3). Thus cGMP stimulation of PDE2 activity can alter barrier function by decreasing cAMP (47). Cytokines were recently shown to increase endothelial PDE2 expression (47) and are known to be released in lung IR injury (12). There were no measurable differences in lung PDE2 protein, and PDE4 expression was not detected (data not shown). In addition, there were no differences in either plasma or lung cAMP concentrations over time suggesting that cGMP-mediated PDE2 stimulation played no role in IR injury.

Of note, we found that both cGMP and cAMP concentrations were significantly greater in the left compared with the right lungs in the baseline control mice. There was an opposite pattern of PDE2 expression with significantly greater right lung expression in baseline control mice (data not shown). We speculate that the right-left lung cyclic nucleotide differences could be explained by the differential expression of PDE2, but we have no explanation for the difference in PDE expression between lungs. Regardless of the explanation of these right-left differences, they did not interfere with our ability to detect the differential changes in NPR-A signaling caused by both IR and anantin.

In summary, these data show that unilateral lung IR injury in the intact mouse is accentuated by the endogenous release of ANP into the circulation. As demonstrated through NPR-A blockade with anantin, the released ANP exacerbated IR-induced endothelial barrier function in the reperfused left lung of the intact mouse but did not affect endothelial permeability in the uninjured right lung. This detrimental effect of ANP on lung endothelial barrier function following unilateral IR was confirmed with measurements of $K_{p}$ in an isolated mouse model of bilateral IR. In the intact mouse, the IR-induced release of endogenous ANP resulted in an increase in lung cGMP concentration and PKG$_{1}$ activation as evidenced by the phosphorylation of VASP at the PKG$_{1}$-preferred serine site. The increased cGMP concentration in the reperfused lung did not result in changes in cAMP concentration or VASP phosphorylation at the PKA-preferred site, suggesting that ANP did not worsen barrier function through reciprocal changes in cAMP concentration. The mechanisms behind the IR-specific adverse effects of NPR-A signaling on lung endothelial barrier function will require additional study.

ACKNOWLEDGMENTS

We thank Dr. Naresh Punjabi for advice regarding the statistical analysis.

GRANTS

This work was supported by a Grant-In-Aid (J. M. Dodd-o) from the American Heart Association (AHA) with funds contributed in part by the AHA, Maryland Affiliate, and by National Heart, Lung, and Blood Institute Grants HL-67189 and HL-075236 (D. B. Pearse).

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