Dopamine inhibits pulmonary edema through the VEGF-VEGFR2 axis in a murine model of acute lung injury

Pawan K. Vohra,1 Luke H. Hoeppner,1 Gunisha Sagar,1 Shamit K. Dutta,1 Sanjay Misra,3 Rolf D. Hubmayr,2 and Debabrata Mukhopadhyay1

Departments of 1Biochemistry and Molecular Biology, and 3Radiology, 2Division of Pulmonary and Critical Care Medicine, Mayo Clinic College of Medicine, Rochester, Minnesota

Submitted 16 August 2010; accepted in final form 13 October 2011

Vohra PK, Hoeppner LH, Sagar G, Dutta SK, Misra S, Hubmayr RD, Mukhopadhyay D. Dopamine inhibits pulmonary edema through the VEGF-VEGFR2 axis in a murine model of acute lung injury. Am J Physiol Lung Cell Mol Physiol 302: L185–L192, 2012. First published October 14, 2011; doi:10.1152/ajplung.00274.2010.—The neurotransmitter dopamine and its dopamine receptor D2 (D2DR) agonists are known to inhibit vascular permeability factor/vascular endothelial growth factor (VEGF)-mediated angiogenesis and vascular permeability. Lung injury is a clinical syndrome associated with increased microvascular permeability. However, the effects of dopamine on pulmonary edema, a phenomenon critical to the pathophysiology of both acute and chronic lung injuries, have yet to be established. Therefore, we sought to determine the potential therapeutic effects of dopamine in a murine model of lipopolysaccharide (LPS)-induced acute lung injury (ALI). Compared with sham-treated controls, pretreatment with dopamine (50 mg/kg body wt) ameliorated LPS-mediated edema formation and lowered myeloperoxidase activity, a measure of neutrophil infiltration. Moreover, dopamine significantly increased survival rates of LPS-treated mice, from 0–75%. Mechanistically, we found that dopamine acts through the VEGF-VEGFR2 axis to reduce pulmonary edema, as dopamine pretreatment in LPS-treated mice resulted in decreased serum VEGF, VEGF2 phosphorylation, and endothelial nitric oxide synthase phosphorylation. We used D2DR knockout mice to confirm that dopamine acts through D2DR to block vascular permeability in our lung injury model. As expected, a D2DR agonist failed to reduce pulmonary edema in D2DR−/− mice. Taken together, our results suggest that dopamine acts through D2DR to inhibit pulmonary edema-associated vascular permeability, which is mediated through VEGF-VEGFR2 signaling and conveys protective effects in an ALI model.

dopamine receptor D2; vascular endothelial growth factor

ACUTE LUNG INJURY (ALI) and its severe form, the acute respiratory distress syndrome (ARDS), are prevalent causes of morbidity and mortality (51). One of the hallmarks of ALI is the accumulation of protein-rich alveolar edema fluid resulting from impaired vascular barrier properties (29, 33, 50, 53). Not only are vascular filtration coefficient and protein permeability increased, injured lungs are also defective in alveolar fluid clearance (35, 52). Although β-receptor agonists such as albuterol and isoproterenol reliably increase alveolar fluid clearance in experimental animals with injured lungs, their use in patients (42) has met limited success (32). Dopamine, a neurotransmitter, acts via two receptor isoforms (dopamine receptor D1 and D2, D1DR and D2DR). D1DR has been shown to increase transcapillary fluid flux through the trafficking of NaKATPase to the basolateral membrane of type II alveolar epithelial cells (5). Moreover, activation of D2DR induces NaKATPase gene expression (17). However, the actions of dopamine on pulmonary vascular barrier properties may not be limited to effects on transcapillary sodium transport. Activation of D2DR has also been implicated in the regulation of vascular endothelial growth factor (VEGF)-induced vascular permeability as well as tumor angiogenesis (2, 9, 46). Many experimental and human studies support the hypothesis that VEGF plays a critical role in shaping the vascular barrier function in ALI (3, 6, 15, 18, 25). In normal human lungs, the VEGF concentration in alveolar lining fluid is greater than that in plasma (43). Epithelial injury leads to decompartmentalization of VEGF, permitting its migration across tight junctions from the alveolar space to the vascular compartment (16, 25, 27, 49), and is therefore thought to play a pathogenic role in noncardiogenic pulmonary edema (26, 38).

Using a mouse ovarian tumor model, our group previously reported that the endothelium expresses the ligand dopamine along with its cognate D2DR, thereby regulating vascular permeability and endothelial cell (EC) barrier integrity (2, 7). Furthermore, we demonstrated that dopamine, through a D2DR-dependent mechanism, causes the down-regulation of VEGFR2 phosphorylation (47). We now provide evidence that the same mechanisms operate in murine endotoxin-induced ALI and can be targeted to improve morbidity and mortality in this preclinical model. Sepsis is the most common cause of ALI in humans (50, 51). Administration of the gram-negative bacterial endotoxin lipopolysaccharide (LPS) has been widely used as an animal model of sepsis-related lung injury. LPS is known to cause over-expression of VEGF (11, 12, 22, 54), which in turn upregulates nitric oxide (NO) production through phosphorylation of VEGFR2 and endothelial NO synthase (eNOS) (13, 14, 40). As postulated, our results suggest that dopamine acts through D2DR to inhibit pulmonary edema-associated vascular permeability, which is mediated through VEGF-VEGFR2 signaling, and conveys protective effects in an ALI model.

MATERIALS AND METHODS

Animals

Pathogen-free male Balb/C mice purchased from the National Cancer Institute (Frederick, MD) and D2DR knockout C57Bl/6 mice from Jackson Laboratory (Bar Harbor, ME) were used in LPS-induced
ALI. The animals were housed in separate cages in a temperature-controlled room with alternating 12-h/12-h light/dark cycles and were allowed 1 wk to acclimate to their surroundings. The animals were also fed a standard diet.

Reagents

LPS (Salmonella enterica), FITC-albumin, hexadecyl-trimethyl-ammonium bromide, hydrogen peroxide, quinpirole, eticlopride, and O-dianisidine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). The antibodies used were phospho-eNOS (S1177) and total eNOS (BD Biosciences, Franklin Lakes, NJ), VEGFR-2 (SC-504, Santa Cruz Biotechnology, Santa Cruz, CA), and phosphotyrosine 4G10 clone (Millipore, Billerica, MA).

Dopamine Preparation

A pharmaceutical-grade dopamine solution (40 mg/ml) was purchased from the Mayo Clinic Pharmacy. Dopamine (50 mg/kg body wt), quinpirole (10 mg/kg body wt), and eticlopride (10 mg/kg body wt) doses were selected on the basis of prior studies demonstrating their regulatory effects on vascular permeability (2).

Experimental Procedure

The wild-type animals were divided into six treatment groups as control (sham treated, n = 20), dopamine alone (n = 20), LPS alone (n = 20), dopamine + LPS (n = 20), quinpirole (D2DR specific agonist) + LPS (n = 20), and dopamine + LPS + eticlopride (D2DR antagonist, n = 20). In D2DR knockout study, animals were divided into six treatment groups as control (sham treated, n = 14), LPS alone (n = 14), quinpirole + LPS (10 mg/kg body wt) was injected intraperitoneally (IP) to induce lung edema (54). The control and treated animals were euthanized by spinal break. One group of animals was treated 30 min before the onset of LPS administration with an IP injection of dopamine (50 mg/kg body wt). One dose of dopamine was given the day of LPS treatment, and an additional dose was given the following day. The same procedure was repeated with quinpirole (10 mg/kg body wt).

Lung Injury Analysis

Lung injury is a broad term that can be applied to conditions ranging from mild interstitial edema without cellularity to massive and fatal destruction of the lung. Here, we used physical and biochemical methods to investigate the effect of dopamine on LPS-induced lung injury. The LPS-challenged animals become very sick and started dying within 2–3 h at this stage. We selected 24 h as the time point in all groups for histological comparison except for bronchoalveolar lavage (BAL) analysis, which was done at 6 h as well as 24 h after LPS challenge.

Lung Water Content

After completion of the animal experiment, mice were euthanized by spinal break. The left lung was harvested for the wet-to-dry weight ratio. The dry weight was determined after incubating the lungs at 80°C for 72 h, and the wet-to-dry weight ratio was calculated (56).

Histology

The right lung was inflated with 10% (vol/vol) formaldehyde embedded in paraffin, and cut into 5-μm-thick sections. Sections were stained with hematoxylin and eosin, and images were taken with a Nikon Eclipse E800 microscope with a ×20 objective. The extent of lung injury was determined by a blinded observer using a semiquantitative score based on congestion, interstitial edema, neutrophil infiltration, and air space hemorrhage, as follows: 0 = no change; 1+ = focal, mild, subtle change, 2+ = multifocal mild changes; 3+ = multifocal prominent changes; and 4+ = extensive prominent changes (1, 48). Histological comparison was done at 24 h in all groups.

Survival

A survival study was carried out separately with initial intervention. Animals (n = 8, each group) were observed daily and survival calculated 158 h after the initial intervention. D2DR knockout animals (n = 8, each group) were monitored up to 32 h.

Biochemical Assays

Pulmonary microvascular permeability. FITC-labeled albumin, a macromolecular marker, is widely used to evaluate pulmonary microvascular permeability (56). Two hours before euthanasia, FITC-labeled albumin (5 mg/kg body wt) was administered via a tail-vein injection at 6 h (n = 3) and at 24 h (n = 3). Immediately after euthanasia, the lungs were lavaged three times with phosphate-buffered saline (0.5 ml per lavage) and the samples combined. Fluid recovery was roughly 95%. The BAL samples were centrifuged at 3,000 revolution/min for 10 min. FITC fluorescence in the BAL fluid was measured using a fluorescence spectrophotometer with excitation at 484 nm and emission at 510 nm (56).

Myeloperoxidase assay. Tissue myeloperoxidase (MPO) activity is a quantitative marker for the presence of neutrophils in the lungs (55). MPO activity was measured in tissue homogenates at 24 h after LPS injection in placebo and dopamine treated animals (n = 6). The MPO activity was expressed as absorbance per gram of tissue (39).

VEGF-A Levels and VEGFR-2 Phosphorylation

Serum was collected from the different experimental groups at 24 h after the LPS challenge. Mouse VEGF-164 levels in serum were measured by an ELISA assay (R&D Systems, Minneapolis, MN) with a specific mouse VEGF primary antibody that has no cross reactivity with human VEGF. Phosphorylation of VEGFR2 was assessed by Western blot analysis. Frozen whole-lung tissues were homogenized in 400 μl of RIPA lysis buffer supplemented with protease and phosphatase inhibitors. The homogenates were centrifuged at 14,000 revolution/min for 15 min at 4°C, and the supernatants were collected. VEGFR2 was immunoprecipitated with a VEGFR2 antibody and resolved on a 10% SDS-PAGE. The samples were analyzed for VEGFR2 phosphorylation using the antiphosphotyrosine clone 4G10. A mouse anti-IgG-horseradish peroxidase conjugate was used to detect the band with the enhanced chemiluminescence reagent (GE Healthcare Life Sciences, Piscataway, NJ). The intensities of the bands were analyzed using Image J from the National Institutes of Health.

Measurement of eNOS

Histology slides were stained for eNOS using a blood vessel staining kit (ECM590, Millipore) per the manufacturer’s instructions. Immunohistological staining of slides from all treatment groups was performed with phospho-eNOS (Serine 1177) and total eNOS antibodies (BD Biosciences). A quantitative analysis of immunostained slides was done with Metamorph software (greater than three slides per group).

Statistics

The data in the figures are represented as means and SD. The error bar was based on SD values. Differences between the treated groups vs. the injured group (LPS/saline) were assessed using the unpaired Student’s t-test.
Student’s t-tests for unpaired observation, and ANOVA and/or Dunnett’s test. Statistical significance was assigned when the P value was ≤0.05.

RESULTS

Given the well-established role of dopamine in preventing vascular permeability, we sought to determine the potential therapeutic effects of dopamine in a murine model of ALI. To this end, we challenged mice with LPS to induce pathophysiological features of pulmonary edema such as alveolar flooding and collapse, tissue infiltration with inflammatory cells, and pulmonary hemorrhage.

Dopamine Modulates the Pathophysiological Features of LPS-Induced ALI

The intraperitoneal administration of LPS was associated with alveolar flooding, airspace collapse, tissue infiltration with inflammatory cells, and perivascular hemorrhage (n = 6, Fig. 1A). These histopathological features of ALI were much less pronounced in animals pretreated with dopamine as reflected in the statistically significant difference in histological lung injury scores (P = 0.013 vs. sham, n = 6, Fig. 1B). These results are consistent with the hypothesis that dopamine affords pulmonary vascular barrier protection against proinflammatory insults.

Dopamine Ameliorates the LPS-Induced Increase in Lung Water and Microvascular Protein Permeability

The intraperitoneal administration of LPS was associated with a statistically significant increase in lung water as reflected in the greater wet-to-dry weight ratio compared with sham-treated controls (P < 0.05 vs. sham, n = 3, Fig. 2A). Consistent with the histopathological findings, the pretreatment of LPS challenged mice with dopamine prevented the increase in lung water, resulting in a wet-to-dry weight ratio similar to that measured in sham-treated controls. The administration of the D2DR-specific agonist quinpirole before LPS challenge was equally effective in preventing pulmonary edema, suggesting that the mechanism of dopamine-mediated barrier protection to proinflammatory insults involves D2DR activation.

The effects of dopamine on pulmonary microvascular protein permeability was inferred from FITC-albumin concentrations in BAL fluid obtained at 6 h (P = 0.007 vs. sham, n = 3, Fig. 2B) and 24 h (P < 0.0003 vs. sham, n = 3, Fig. 2B) after an LPS challenge. FITC-albumin concentrations of LPS-treated mice were greater than those of sham-treated controls at both time points. In contrast, pretreatment with dopamine largely prevented the translocation of FITC-albumin into the alveolar space, indicating preservation/restoration of microvascular protein permeability. A similar response was observed in LPS-challenged animals pretreated with quinpirole (P = 0.005 vs. sham, n = 3, Fig. 2B at 6 h and P < 0.0004 vs. sham, n = 3, Fig. 2B at 24 h) in support of the role of D2DR in dopamine-mediated barrier protection.

Dopamine Prevents LPS-Mediated Neutrophil Recruitment

Neutrophil recruitment in response to injury was inferred from MPO activity in lung homogenates of LPS- and placebo-treated mice. As anticipated from the histopathological findings and numerous reports in the literature, LPS induced neutrophil recruitment and therefore raised MPO activity of lung tissue homogenates. Pretreatment with dopamine and quinpirole substantially reduced MPO activity (P < 0.05 vs. sham, n = 6, Fig. 2C) in lungs of LPS-treated mice, indicating that D2DR agonists inhibit neutrophil recruitment to proinflammatory insults.

Dopamine Increases Survival Rates of LPS-Challenged Mice

In preliminary observations, we found that animals became very sick and unable to move around 24 h (n = 8, Fig. 2D) and died around 24–27 h (Fig. 2D), whereas 75% of mice pretreated with dopamine were alive at 158 h (n = 6, Fig. 2D), suggesting that dopamine-mediated hemodynamic effects along with protection of endothelial barrier integrity may be contributing to the increased lifespan in this preclinical ALI model (41).
Dopamine Inhibits Pulmonary Edema by Decreasing Serum VEGF Levels and Phosphorylation of VEGFR2

We previously demonstrated in an ovarian tumor model that dopamine acts through D2DRs to induce endocytosis of VEGFR2, preventing VEGF binding and receptor activation, thereby preserving vascular barrier properties. Because serum VEGF levels are increased in noncardiogenic pulmonary edema, we tested whether dopamine altered the lung response to an LPS challenge by a similar mechanism. As postulated, we observed a significant reduction in serum VEGF levels ($P<0.001$ vs. sham, $n=6$, Fig. 3A) and VEGFR2 ($P=0.005$ vs. sham, $n=6$, Fig. 3, B and C) phosphorylation in dopamine-pretreated animals challenged with an LPS-induced lung injury compared with sham-treated groups. Our findings are consis-
tent with, but not definitive for, the effect of dopamine on systemic VEGF levels. Similar results were obtained using the D2DR-specific agonist quinpirole ($P = 0.003$ vs. sham, $n = 6$, Fig. 3A). Eticlopride, (an antagonist of D2DR) abrogated dopamine-mediated decrease in VEGF ($P = 0.1$ vs. sham, $n = 6$, Fig. 3A) levels. These results provided a further support that D2DR, but not D1DR, plays a role in preventing pulmonary edema by inhibiting VEGF-VEGFR2 signaling.

Dopamine Inhibits VEGFR2 Signaling

To further demonstrate that VEGFR-2 signaling plays a critical role in sepsis-induced lung injury, we used immunohistochemistry to evaluate eNOS levels, a downstream target of VEGFR-2 signaling. On the basis of our results illustrating effects of dopamine on the VEGF-VEGFR2 signaling axis, we expected decreased levels of phospho-eNOS (10, 30) in dopamine-treated mice challenged with LPS. However, there was no significant change in eNOS phosphorylation observed in sham-treated and dopamine-pretreated LPS animals ($P = 0.11$, $n = 6$, Fig. 4A), indicating that the reduction in VEGFR2 phosphorylation influenced eNOS activation ($P = 0.001$ vs. sham, $n = 6$, Fig. 4A and B). Although we did not observe significant change in protein expression levels ($P = 0.33$ vs. sham, $n = 5$, Supplemental Fig. S1, A and B; supplemental figures are available online at the *American Journal of Physiology Lung Cellular and Molecular Physiology* website), we observed a significant reduction in eNOS phosphorylation in these mice, compared with LPS-treated animals.

**Validation of Postulated Mechanisms in a D2DR Knockout Mouse Model**

D2DR (−/−), heterozygous (+/−) and wild-type mice (+/+) were challenged with LPS, and effects on histopathology, phospho-eNOS tissue labeling, and survival were examined. Pretreatment with dopamine or the D2DR agonist quinpirole prevented LPS-induced lung injury in wild-type as well as heterozygous D2DR (+/−) mice. These drugs had no lung protective effect in D2DR-null mice. ($P = 0.003$ vs. sham, $n = 6$, Fig. 5, A and B). In keeping with these results, the prophylactic administration of dopamine and/or quinpirole was associated with decreased phospho-eNOS labeling in wild-type and...
heterozygous D2DR (+/−) but failed to ameliorate LPS-induced activation of eNOS in D2DR-null mice (P = 0.54, n = 6, Fig. 6, A and B; P = 1.0, n = 5, Supplemental Fig. S2, A and B). Quinpirole significantly improved the survival rates (n = 8 each group) of LPS-treated wild-type and D2DR (+/−) (n = 6, Fig. 6C) mice but failed to improve the survival of in D2DR (−/−) animals at 32 h (Fig. 6C). Taken together, these results show that dopamine protects against LPS-mediated inflammation and barrier dysfunction via a D2DR activation-dependent mechanism and that a single allele of the D2DR gene appears sufficient to mediate this effect.

**DISCUSSION**

Our results clearly demonstrate that, in a clinically relevant murine model of sepsis, pretreatment with dopamine and quinpirole significantly attenuates the characteristic features of ALI. Specifically, decrease in the wet-to-dry lung weight ratio, preserved aeration of alveoli as well as intravascular retention of albumin, consistent with barrier protection. These observations are consistent with our premise that the barrier-protective mechanism facilitated by dopamine or quinpirole is mediated by its interaction with VEGF signaling. Our hypothesis is substantiated by the observation that pretreatment with dopamine significantly decreases VEGF level as well as VEGFR2 phosphorylation. However, we do not exclude the possibility of an effect on the delivery of VEGF to alveolar endothelium via recruited monocyte/macrophages and neutrophils, both of which have VEGF in their granules, especially given the observed effect of dopamine on the inhibition of lung parenchymal inflammation. Although our results are indicative of the fact that dopamine mediated barrier protection, in our murine model sepsis is predominantly mediated by VEGF pathway. There are several alternative or complementary mechanisms through which dopamine may influence the formation and clearance of pulmonary edema.

It is a well-established fact that the clinical use of dopamine might significantly increase venous return and cardiac-filling pressure in patients with cardiac dysfunction, sepsis, and respiratory failure (20, 45). We did not measure left atrial or pulmonary venous pressure in our animal model, but we observed significant reduction in the levels of lung water content, as assessed by weight and histology, in dopamine-treated mice. These results are in accord with the well-estab-
lished effects of dopamine on alveolar fluid clearance in ALI (31, 34–37). Although we did not measure alveolar fluid clearance independent of lung water or protein permeability, we concluded that a significantly decreased concentration of FITC-albumin in the BAL fluid of dopamine- and LPS-treated mice would not be consistent with a clearance-dominated mechanism of action. In contrast, with capillary fluid egress, alveolar fluid clearance remains the most viable and active energy-dependent process, mediated by coordinated interactions between apical sodium channels and basolateral Na/K ATPase. Both type 1 and type 2 alveolar epithelial cells possess the machinery for transepithelial ion transport, though water simply moves along osmotic gradients (4). Typical alveolar fluid clearance-measurement methods infer water flux from changes in alveolar concentration relative to cell-impermeable molecules such as albumin. Therefore, we anticipated a higher rather than lower FITC-albumin concentration in the BAL fluid of dopamine-treated mice if enhanced clearance had been the dominant mechanism. The preponderance of our data supports the fact that observed dopamine-induced change in albumin extravasation likely arises from the changes in the hydraulic conductance. However, the conclusion that vasoconstriction prevents alveolar edema formation cannot be made on data obtained from albumin extravasation ratio alone.

The numerous therapeutic interventions include simvastatin, adenosine triphosphate (ATP), sphingosine 1-phosphate (S1P) and activated protein C (APC), currently (23) being proposed to protect and maintain endothelial barrier integrity in ALI. A previous report from our group clearly enunciated the role of D2DR in protection of endothelial barrier integrity and modulation of vascular permeability (47). The above-mentioned report became the premise for testing the role of a direct modulator of EC barrier function in our murine model of ALI (23). Excessive production of NO has also been implicated in pathophysiology of many diseases including sepsis. Previous reports have suggested that increased phosphorylation of eNOS upon lung injury plays a pivotal role in vascular permeability (8, 19, 21); however, a consensus has not been reached on the mechanism of action, as the signaling cues correlating VEGF-NOS pathways has not been ascertained until now (24).


References


DOPAMINE INHIBITS PULMONARY EDEMA IN LUNG INJURY


