Airway vasculature after mycoplasma infection: chronic leakiness and selective hypersensitivity to substance P

Marilyn L. Kwan, Antonio D. Gómez, Peter Baluk, Hiroya Hashizume, and Donald M. McDonald
Cardiovascular Research Institute and Department of Anatomy, University of California, San Francisco, California 94143

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Kwan, Marilyn L., Antonio D. Gómez, Peter Baluk, Hiroya Hashizume, and Donald M. McDonald. Airway vasculature after mycoplasma infection: chronic leakiness and selective hypersensitivity to substance P. Am J Physiol Lung Cell Mol Physiol 280: L286–L297, 2001.—Angiogenesis and microvascular remodeling are features of chronic airway inflammation caused by *Mycoplasma pulmonis* infection in rats. As airway blood vessels undergo remodeling, they become unusually sensitive to substance P-induced plasma leakage. Here we determined whether the remodeled vessels are leaky under baseline conditions, whether their heightened sensitivity is specific to substance P, and whether the leakage is reversible. Four weeks after infection, the amount of baseline leakage of Evans blue in the tracheal mucosa was two to five times the normal level. Gaps <1 μm in diameter were located between endothelial cells in some remodeled vessels. Substance P, but not platelet-activating factor or 5-hydroxytryptamine, produced an exaggerated leakage response. Inhalation of the β2-adrenergic receptor agonist salmeterol reduced the leakage by <60%. We conclude that the blood vessel remodeling after *M. pulmonis* infection is associated with microvascular leakiness due, in part, to the formation of endothelial gaps. This leakage is accompanied by an abnormal sensitivity to substance P but not to platelet-activating factor or 5-hydroxytryptamine and can be reduced by β2-agonists.

angiogenesis; β2-adrenoceptor agonists; endothelial cells; Evans blue; inflammation; *Mycoplasma pulmonis*; salmeterol; vascular permeability; Wistar rats; Fischer 344 rats

remodeling (changes in existing vessels) occur in asthma (12, 31, 33). However, the leakiness of the airway microvasculature has been more difficult to assess. Changes in the number, caliber, and permeability of mucosal vessels are functionally important because even modest increases in wall thickness due to edema and engorged blood vessels could amplify decreases in airway conductance produced by bronchoconstriction (28).

*Mycoplasma pulmonis* infection, which causes chronic inflammatory airway disease in rats and mice (34, 35), has proven useful as a model for studying remodeling and permeability changes in the airway microvasculature (40). After *M. pulmonis* infection, microvascular remodeling and angiogenesis accompany the influx of inflammatory cells, epithelial thickening, mucous gland hypertrophy, and fibrosis (13, 34, 41, 58). One feature of the remodeled blood vessels is increased sensitivity to stimuli that evoke neurogenic inflammation, which is mediated by neuropeptides from sensory nerves (41). Substance P and the sensory nerve irritant capsaicin trigger an abnormally large amount of plasma leakage in the airways of infected rats (38, 41). This hyperreactivity is due to increased expression of substance P [neurokinin-1 (NK1)] receptors on endothelial cells of the remodeled vessels (5). It is unknown whether the remodeled vessels have a generalized sensitivity to inflammatory mediators or whether the heightened responsiveness is restricted to substance P and other NK1 receptor agonists.

Newly formed and remodeled blood vessels have been found to be unusually leaky in several model systems (16, 24, 52). Therefore, the vasculature of the chronically inflamed airway mucosa of rats infected with *M. pulmonis*, in which angiogenesis and microvascular remodeling are features, would be expected to be leaky under baseline conditions. When we examined this issue in infected Fischer 344 (F-344) rats, no significant increase in baseline leakage was detected (41). However, the method used in that study was calibrated for the extensive leakage evoked by substance P rather than for the modest increase in baseline leakage.

Address for reprint requests and other correspondence: D. M. McDonald, Cardiovascular Research Institute, Box 0130, Rm. S-1363, Univ. of California, San Francisco, CA 94143-0130 (E-mail: dmcd@itsa.ucsf.edu).

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Several factors are likely to participate in the leakage from newly formed and remodeled vessels. An increase in the luminal surface area due to microvascular proliferation or remodeling would itself favor more extravasation. Increased vessel caliber would also favor leakage to the extent that it decreased resistance and increased the transmural driving force. In addition, factors that impair the removal of extravasated plasma via lymphatics and other routes would tend to increase the accumulation of plasma constituents in the tissue.

Alterations in endothelial barrier function would amplify the effects of the other changes in the microvasculature. Endothelial gaps, fenestrae, transcytotic vesicles, vesiculo-vascular organelles, and defects in the endothelial monolayer all may contribute to plasma leakage in pathological conditions, accompanied by angiogenesis and microvascular remodeling (15, 23, 49). However, the relative contributions of these factors have been difficult to sort out in complex conditions such as chronic inflammation and tumors. In normal vessels, plasma leakage caused by histamine, bradykinin, substance P, or 5-hydroxytryptamine (5-HT) results in large part from the formation of focal gaps between endothelial cells (6, 36, 39, 40). Therefore, in the present study, we began to determine the mechanism of leakage in chronic inflammation by examining the contribution of endothelial gaps.

Our approach was to reexamine the issue of whether baseline plasma leakage increases in the airways after *M. pulmonis* infection. We reasoned that in airway disease of long duration, even small amounts of sustained baseline plasma leakage could have functionally important consequences (28). Therefore, we designed a study in which smaller amounts of leakage could be detected than in an earlier study by McDonald et al. (41), and we compared the amount of baseline leakage in the infected airways of two strains of rat, F-344 and Wistar. Next, we examined the mechanism of leakage in infected rats through a mechanism involving endothelial gaps.

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We compared the amount of baseline plasma leakage in the airways of F-344 and Wistar rats infected with *M. pulmonis* for 4 wk. The accumulation of Evans blue in the trachea over 5, 30, or 120 min was measured as in a study of leakage during the late-phase response to antigen (8). Leaky vessels were identified in three ways: vessel labeling with the particulate (5- to 200-nm) tracer Monastral blue (39), vessel labeling with biotinylated *Ricinus communis* agglutinin I (RCA I) lectin (57), and staining of endothelial cell borders with silver nitrate (39). The question of whether endothelial gaps could explain the baseline leakage in infected rats was also addressed by examining the luminal surface of the microvasculature with scanning electron microscopy (6). Reversibility of the baseline leakage by β₂-agonists was tested in infected rats treated with aerosolized salmeterol (8, 61). Specificity of the heightened response of the remodeled microvasculature to substance P was examined by comparing the leakage evoked by platelet-activating factor (PAF) and 5-HT, which cause leakage without activating appreciable neurogenic inflammation (30, 55). We found that *M. pulmonis* infection increased baseline leakage in the airways of Wistar rats through a mechanism involving endothelial gaps. This finding was novel and differed from earlier studies in which our laboratory could not detect an increase in baseline plasma leakage after *M. pulmonis* when measured over 5 min in F-344 rats (41) and in mice (58).

**MATERIALS AND METHODS**

**Animals.** Male pathogen-free Wistar rats and F-344 rats (Charles River Laboratories, Hollister, CA) were housed in microisolator cages under barrier conditions. Wistar rats were 36–40 days old and 126–150 g body weight on arrival, and F-344 rats were 51–57 days old and 151–175 g body weight on arrival. All experimental procedures were approved by the Committee on Animal Research of the University of California, San Francisco.

**Drugs.** Evans blue (30 mg/ml; J. T. Baker, Phillipsburg, NJ) dissolved in sterile 0.9% NaCl (saline) was injected intravenously in a dose of 1 ml/kg. Monoalba blue pigment (3% suspension of copper phthalocyanine blue pigment BW-431-P in saline; Chemicals and Pigments Department, E. I. duPont deNemours, Wilmington, DE) was injected intravenously in a dose of 1 ml/kg. Salmeterol (GlaxoWellcome Research and Development, Medicines Research Centre, Stevenage, UK) was dissolved in glacial acetic acid and sterile phosphate-buffered saline (PBS; Sigma, St. Louis, MO) to a concentration of 5 mg/ml, and the solution was adjusted to pH 6.0 with 4 M NaOH. Dilutions of this stock with sterile PBS (0.5, 1.0, and 5.0 mg salmeterol/ml) were used to generate the aerosols. Substance P (50 μg/ml; Peninsula Laboratories, Belmont, CA) and 5-HT (220 μg/ml; Sigma) were dissolved in saline containing 0.005 M acetic acid and then diluted with the acidified saline to give the desired dose (0.1, 0.3, 0.5, and 1 μg/kg of substance P; 50 μg/kg of 5-HT) in 1 ml/kg administered intravenously. PAF (PAF<sub>C53</sub>; 1 mg/ml; Sigma) was dissolved in saline containing 0.005 M acetic acid and 1% bovine serum albumin (BSA; Sigma), diluted with saline to 1 μg/ml, and injected intravenously in a dose of 1 ml/kg. Anesthetics included ketamine (50 mg/kg; Sanofi Winthrop Pharmaceuticals, New York, NY) and xylazine (2 mg/kg; Phoenix Pharmaceuticals, St. Joseph, MI) injected intramuscularly together or pentobarbital sodium (50 mg/kg; Nembutal, Abbott Laboratories, North Chicago, IL) injected intraperitoneally, with supplements as needed.

**Inoculation with *M. pulmonis* organisms.** Rats were lightly anesthetized with ketamine and xylazine and then inoculated with 100 μl of *M. pulmonis* culture medium into each nostril on each of 3 consecutive days. The culture medium, provided by Julie Gibbs-Erwin (University of Alabama at...
Birmingham, Birmingham, AL), contained 5.3 × 10^5 colony-forming units/ml of strain 5782C. After inoculation, the rats were housed three per microisolator cage for 4 wk.

Measurement of plasma leakage. Evans blue (30 mg/kg) was injected over 10 s into a femoral vein of pentobarbital sodium-anesthetized rats 5, 30, or 120 min before the vascular perfusion. The vasculature of rats was perfused with acidified fixative (1% paraformaldehyde in 0.05 M citrate buffer, pH 3.5) for 2 min at a pressure of 120–140 mmHg via a blunt 13-gauge needle inserted through the left ventricle into the ascending aorta for 5 min at a pressure of 120–140 mmHg. Alternatively, immediately after fixation, the vasculature of rats was perfused with a biotinylated lectin (see Staining of microvasculature and localization of leaky vessels with lectins), which confirmed that few vessels contained trapped blood after the perfusion. The tracheae were removed, blocked, and weighed. Evans blue was extracted in formamide (Sigma, over night at 60°C) and measured by spectrophotometry at 610 nm (41). The Evans blue content of the solution was calculated from a standard curve and is expressed in nanograms of Evans blue per trachea or per milligram of tracheal wet weight.

For the substance P, PAF, and 5-HT experiments, the injection of Evans blue preceded the injection of the mediator by 30 s. The mediator was injected into a femoral vein of pentobarbital sodium-anesthetized rats over 20 s, and the rats were perfused 5 or 30 min later. Substance P-treated animals that died <30 min after the injection were perfused immediately.

Exposure to salmeterol aerosol. Unanesthetized rats in a wire net restrainer were exposed nose only for 10 min to an aerosol of salmeterol (0.5, 1.5, or 5.0 mg/ml) or vehicle (PBS, pH 6.0) generated by an ultrasonic nebulizer (particle size 1–3 μm; model 5000D, DeVilbiss, Somerset, PA) (8). The nebulizer basin was filled with the salmeterol solution in sterile PBS, and the air intake of the nebulizer was attached to a tank delivering type 1 grade breathing air flowing at 250 ml/min. The rats were removed from the restrainer immediately after the exposure. Four and one-half hours after the treatment, the rats were anesthetized with pentobarbital sodium, and Evans blue (30 mg/kg) was injected intravenously; 30 min later, the rats were perfused with fixative as in the other studies in which plasma leakage was measured (see Measurement of plasma leakage).

Staining of microvasculature and localization of leaky vessels with lectins. Monastral blue pigment (30 mg/kg) was injected into a femoral vein; 28 min later, biotinylated Lycopersicon esculentum lectin (250 μg in 500 μl of PBS-BSA; Vector Laboratories, Burlingame, CA) was injected intravenously. Two minutes later, fixative (1% paraformaldehyde and 0.5% glutaraldehyde in PBS, pH 7.4) was perfused via the ascending aorta for 5 min at a pressure of 120–140 mmHg. Alternately, immediately after fixation, the vasculature was stained with lectin by perfusion of 1) 50 ml of PBS over 1 min, 2) 50 ml of PBS plus 1% BSA over 1 min, 3) 50 ml of biotinylated RCA I lectin (10 μg/ml in PBS-BSA; Vector Laboratories) over 1 min, and 4) 50 ml of PBS-BSA over 1 min. All tracheae were removed, opened lengthwise, pinned flat in plastic dishes coated with Sylgard (Dow Corning, Midland, MI), and permeabilized by overnight incubation in PBS containing 0.3% Triton X-100 (Sigma) at room temperature to facilitate penetration of subsequent reagents. The pinned tracheae were incubated for 22–24 h with avidin-peroxidase complex (Vector Laboratories) diluted 1:200, washed for 2 h with 50 mM Tris buffer (pH 7.4) containing 1% Triton X-100, exposed for 5 min to 0.05% 3,3’-diaminobenzidine (Sigma) in Tris buffer, and reacted for 10 min with 0.05% 3,3’-diaminobenzidine and 0.01% H2O2 in Tris buffer (all steps done at room temperature). Tracheae were rinsed thoroughly with water, flattened during dehydration with increasing concentrations of ethanol, cleared in toluene, and mounted whole in Permount (57).

Silver nitrate staining of endothelial cell borders. Rats anesthetized with pentobarbital sodium were perfused with fixative (1% paraformaldehyde and 0.5% glutaraldehyde in 0.075 M cacodylate buffer, pH 7.4) as in the lectin experiments and were then perfused with five solutions in rapid succession: 0.9% NaCl for 2 min, 5% glucose for 10 s, 0.2% AgNO3 for 7 s, 5% glucose for 10 s, and fixative for 1 min (39). The tracheae were removed, and the silver was developed by exposure to light. The tracheae were flattened during dehydration with ethanol and mounted in Permount.

Scanning electron microscopy. The tracheae were fixed by perfusion as for silver nitrate staining, removed, and immersed in 2.5% glutaraldehyde in cacodylate buffer for at least 2 h. Next, the tracheae were embedded in 10% agarose, and 100-μm-thick vibratome cross sections were cut to expose the luminal surface of the mucosal blood vessels (6). In brief, the sections were rinsed with buffer, immersed in cacodylate-buffered 2% tannic acid for 24 h at room temperature, rinsed with buffer, and then immersed in 2% OsO4 in 0.1 M cacodylate buffer for 2 h at 4°C. After dehydration with ethanol, the sections were infiltrated with 100% t-butanol and freeze-dried under vacuum. Sections were sputter-coated with 4–16 nm of gold-palladium alloy. The luminal surface of the vessels was photographed with a JEOL JSM-840A scanning electron microscope.

Immunohistochemistry. The expression of 5-HT2A receptors on endothelial cells in the tracheae was examined with immunohistochemical methods similar to those used for localizing substance P receptors (10). The primary antibody was a rabbit polyclonal antibody raised to a synthetic peptide sequence corresponding to the NH2-terminal amino acids 22–41 of the rat 5-HT2A receptor (21). Tracheae from three infected and three pathogen-free Wistar rats were fixed by vascular perfusion of 4% paraformaldehyde, immersed in the same fixative for 2 h, infiltrated with 30% sucrose overnight, and then frozen in optimum cutting temperature compound. The brain was used as a positive control. Cryostat sections 80 μm in thickness were incubated in 5% normal goat serum in PBS-0.3% Triton for 1 h and then in the 5-HT2A receptor antibody (1:200 and 1:500; DiaSorin, Stillwater, MN) in PBS-0.3% Triton for 16 h at room temperature. After a wash in PBS, the sections were incubated for 2 h with indocyanine (Cy3)-conjugated goat anti-rabbit IgG secondary antibody (1:200; Jackson ImmunoResearch, West Grove, PA), washed in PBS, mounted in Vectashield (Vector Laboratories), and examined by fluorescence microscopy. The primary antibody was omitted from the incubation mixture as a negative control.

Statistical analysis. Values are expressed as means ± SE. Experimental groups consisted of 4–14 rats. The significance of differences between groups was assessed by analysis of variance (ANOVA) and Bonferroni-Dunn or Fisher’s protected least significant difference test for multiple comparisons. Differences between groups were considered significant when P was <0.05.
RESULTS

Increased baseline leakage after *M. pulmonis* infection. In pathogen-free rats, the amount of Evans blue leakage in the trachea under baseline conditions (no stimulus) was about the same regardless of whether the dye accumulation time (dye injection to perfusion) was 5, 30, or 120 min (Fig. 1A). As found previously (41), there was about twofold the corresponding pathogen-free value for 30 min (11 ± 1 ng/mg).

*Effect of increased tracheal mass on leakage measurement.* *M. pulmonis* infection was accompanied by significant increases in tracheal weight: 26% increase in F-344 rats (58 ± 2 mg in infected rats vs. 46 ± 1 mg in pathogen-free rats) and 40% increase in Wistar rats (79 ± 4 mg in infected rats vs. 56 ± 1 mg in pathogen-free rats). The contribution of tissue growth to the elevated leakage values, assuming that increased tissue mass was accompanied by a corresponding increase in vascularity, was examined by comparing the amount of leakage scaled for tissue weight (in ng/mg trachea; Fig. 1A) with the unscaled values expressed per organ (in ng/trachea; Fig. 1B).

This comparison showed that the increased baseline leakage after infection resulted both from the heightened leakiness of the remodeled microvasculature and from the expansion of the microvasculature accompanying the increased tissue mass. In infected F-344 rats, the amount of leakage measured over 30 min was significantly larger than in pathogen-free rats regardless of how the data were expressed, and the scaled values correlated closely with the unscaled values ($R^2 = 0.94$ by simple regression analysis; Fig. 1). The scaled values at 30 min were 138% larger than corresponding pathogen-free values, whereas the unscaled values were 227% larger (Fig. 1). Similar results were obtained in infected Wistar rats despite the greater increase in tracheal weight and the significantly larger amount of leakage ($R^2 = 0.89$ by regression of scaled and unscaled values; Fig. 1B). The scaled values at 30 min were 355% larger than the pathogen-free values, and the unscaled values were 554% larger (Fig. 1). The increased tissue mass after infection was responsible for the difference between the scaled values and the unscaled values. Based on a comparison of the scaled and unscaled values in both strains, increased vessel leakiness would account for $\sim 60\%$ of the augmented leakage, and increased vascularity accompanying the enlarged tissue mass would account for the remaining 40%.

Wistar rats were used for the remainder of the experiments because of their unusually large amount of baseline leakage.

*Mechanism of baseline leakage after *M. pulmonis* infection.* The tracheal microvasculature of Wistar rats undergoes extensive remodeling after *M. pulmonis* infection (13). Consistent with such previous observations, we found that the simple, repetitive pattern of

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**Fig. 1.** Baseline plasma leakage in tracheas of Fischer 344 (F-344) and Wistar rats at 5, 30, and 120 min after intravenous injection of Evans blue. Intravascular dye was washed out by vascular perfusion of fixative. Evans blue leakage was scaled for tissue weight (A) and unscaled (B). Values are means ± SE; n = 5–8 rats/group. Baseline leakage was increased after infection in both F-344 rats and Wistar rats, but the increase was significantly larger in Wistar rats. Significantly different ($P < 0.05$ by analysis of variance with Bonferroni-Dunn multiple comparison test) from corresponding value for: *pathogen-free rats; †F-344 rats. ‡Significantly different from corresponding pathogen-free value, $P < 0.05$ by analysis of variance with Fisher’s protected least significant difference test but not by Bonferroni-Dunn test.
mucosal vessels in pathogen-free Wistar rats was replaced by tortuous new and remodeled vessels (Fig. 2, A and B). Abundant adherent leukocytes were located within the larger remodeled vessels. The severity of these changes was greatest in the rostral trachea and tended to decrease caudally.

The walls of some newly formed or remodeled vessels had scattered foci of Monastral blue extravasation (Fig. 2B). Patches of extravasated blue tracer were found both in tortuous new vessels the size of capillaries and in enlarged remodeled venules (Fig. 2B). Linear accumulations of extravasated tracer were present in some vessels, as would be expected for leaky sites along endothelial cell borders. Heavily labeled vessels were found in parts of the rostral trachea where the microvascular remodeling was most severe, but most vessels in the caudal trachea, where the remodeling was more mild, had little or no leakage of Monastral blue.

Regions of extravasated RCA I lectin demarcated many leaky vessels in the tracheas of infected Wistar rats (Fig. 2, C and D). Patches of RCA I lectin bound to the extracellular matrix were scattered throughout the infected tracheas and were much more abundant than sites of Monastral blue labeling. No leakage of Monastral blue or RCA I lectin was found in tracheas of pathogen-free rats (Figs. 2, A and C).

Additional abnormalities in remodeled vessels were visible after endothelial cell borders were stained with silver nitrate (Fig. 2, E and F). Dotlike silver deposits, corresponding to endothelial gaps seen by other methods (42), were found at endothelial cell borders in some remodeled vessels in infected rats (Fig. 2F). These dots were not uniformly distributed in the trachea, and many vessels had few or none. No silver dots were found in the vessels of pathogen-free rats (Fig. 2E). Moreover, examination of the luminal surface of remodeled vessels by scanning electron microscopy revealed that the borders of some endothelial cells were loosely overlapped or were interrupted by gaps <1 μm in diameter (Fig. 2H). Monastral blue particles were trapped in some of the gaps (Fig. 2H). Tracheal vessels in pathogen-free rats did not have these features (Fig. 2G). The gaps had the same appearance as observed in earlier studies of leakage evoked by substance P (6, 39) but were less numerous and not as uniformly distributed.

Reduction in baseline leakage by salmeterol. Exposure to an aerosol of salmeterol for 10 min had no consistent effect on baseline leakage in the tracheas of pathogen-free Wistar rats but caused a dose-related decrease in baseline leakage in infected rats (Fig. 3). Reductions of 45, 54, and 60% in Evans blue accumulation over 30 min were found in infected tracheas after the three concentrations of salmeterol (0.5, 1.5, and 5 mg/ml, respectively). The reductions produced by all three concentrations were significant (Fig. 3). Despite these reductions, the amount of leakage in the infected rats was still significantly greater than the corresponding leakage in pathogen-free rats except with the highest concentration of salmeterol (5 mg/ml; Fig. 3). Exposure to an aerosol of the PBS vehicle caused a small (~30%), insignificant reduction in baseline plasma leakage in infected rats but not in pathogen-free rats (Fig. 3).

Increased sensitivity to substance P-induced leakage in infected rats. As previously reported (39), an intravenous injection of substance P produced an abnormally large amount of plasma leakage in the tracheae of infected rats (Fig. 4). Dose-response experiments in Wistar rats showed that leakage produced by doses of 0.5 μg/kg (60% increase) and 1.0 μg/kg (94% increase) was significantly greater than baseline leakage (Fig. 4). These doses produced significantly less leakage in pathogen-free rats (Fig. 4).

Unexpectedly, infected Wistar rats were so sensitive to substance P that intravenous doses of 0.5 μg/kg or greater caused apnea and death of more than half of the animals tested (Fig. 5). Breathing stopped ~10 min (range 5–17 min) after the injection. An abnormally large amount of leakage occurred in the tracheae even when the animals died prematurely, thereby shortening the period for extravasation to <30 min. An Evans blue concentration of 130 ng/mg was found in one rat that died 13 min after a substance P dose of 1 μg/kg (Fig. 5). In pathogen-free rats, the mean value 30 min after this dose was 64 ± 7 ng/mg. At autopsy, the Airways of the affected rats were filled with fluid and mucus. Because all rats survived for at least 5 min, problems were not evident in the animals studied 5 min after substance P. None of the pathogen-free rats had breathing difficulties during the 30-min period after substance P.

The amount of leakage produced by substance P (1 μg/kg) was increased in infected Wistar rats at both 5 min (32% more leakage than with same dose in pathogen-free rats) and 30 min (55% more leakage than in...
pathogen-free rats; Fig. 6). Similar amounts of leakage were found 5 min after 5 μg/kg doses of substance P (data not shown).

Lack of hypersensitivity to PAF and 5-HT in infected rats. The specificity of the exaggerated effect of substance P on M. pulmonis-infected rats was examined by measuring the leakage produced by PAF and 5-HT. The doses tested (PAF, 1 μg/kg iv; 5-HT, 50 μg/kg iv) caused about as much leakage as the 1 μg/kg dose of substance P. However, PAF and 5-HT were strikingly unlike substance P in two ways. First, neither PAF nor 5-HT caused more leakage in the infected rats than in the pathogen-free rats (Fig. 6). The amount of leakage produced by PAF in the infected rats was the same or slightly less than that in the pathogen-free rats at both 5 and 30 min. Similarly, the amount of leakage produced by 5-HT at 5 min was the same in the pathogen-free and infected rats (Fig. 6). Second, neither PAF nor 5-HT caused breathing problems or death in any of the infected rats. The effect of PAF was notable in this regard because the amount of leakage at 30 min (range 56–104 ng/mg) overlapped the values for substance P (range 72–131 ng/mg), suggesting that the amount of leakage alone was not responsible for the death of animals after substance P.

Because of these findings, we sought to determine whether the immunoreactivity for 5-HT receptors that mediate leakage differed in pathogen-free and infected Wistar rats. However, no specific immunoreactivity for 5-HT2A receptors was detected in sections of tracheas from either group of rats. Yet, as a positive control, subsets of cortical neurons were lightly stained in sections of brain from both pathogen-free and infected rats.

DISCUSSION

This study found that the microvasculature of the tracheal mucosa of Wistar and F-344 rats infected with M. pulmonis was abnormally leaky in the absence of other stimuli. Compared with the airways of pathogen-free rats, where the small amount of baseline leakage and the baseline clearance were in equilibrium, the airways of infected rats accumulated two to five times as much Evans blue tracer over a period of 30 min. The
increased baseline leakage appeared to come from newly formed and remodeled blood vessels as shown by vascular labeling with Monastral blue, binding of extravasated RCA I lectin, staining of endothelial cell borders with silver nitrate, and direct observation by scanning electron microscopy. Gaps between endothelial cells and increased vascular surface area were likely to be involved. The experiments showed that the baseline leakage was reduced by the inhalation of salmeterol. They also confirmed that substance P produces significantly more leakage in infected rats than in pathogen-free rats. However, neither PAF nor 5-HT produced more leakage in infected rats. These results suggest that the remodeled microvasculature is abnormally leaky under baseline conditions and overresponds to substance P but may not have a generalized hypersensitivity to inflammatory mediators.

Chronic leakage from vessels in M. pulmonis-infected rats. These experiments sought to determine whether plasma leakage in the airways was increased after M. pulmonis infection by using the accumulation of Evans blue dye as an index. Previously, it seemed that little leakage occurred after infection in the absence of stimuli that trigger neurogenic inflammation (38, 41). The leakage was quantified by allowing Evans blue to circulate in the blood for as long as 120 min to prolong the period for extravasation and elucidate the balance between leakage and clearance of the extravasated dye from the trachea.

Measurements of extravasated Evans blue are considered a fair representation of extravasated albumin and hence plasma (51). The dye binds spontaneously and avidly to albumin (32, 48). When injected into the blood, most of the free dye is bound to albumin within 2 min, provided the amount of dye is below saturation as in the present experiments (53, 62).

M. pulmonis infection initiates an airway disease that proceeds in a rostral to caudal direction. The amount of angiogenesis and vascular remodeling shows a similar gradient in severity, and the vessels in the rostral part of the trachea leak more than those in the caudal part (5, 13).
Factors contributing to increased baseline plasma leakage. Several factors are likely to contribute to the sustained plasma leakage in the airways of rats with *M. pulmonis* infection. First, angiogenesis, which is a prominent feature of the airway remodeling produced by the infection, increases the vascular surface area and probably creates leaky vessels because altered permeability is a common feature of newly formed blood vessels. This feature has been observed in healing wounds (52), regions of implanted or transgenically overexpressed angiogenic factors (49, 59), and tumors (16, 23, 24). Second, the remodeled vessels are bathed in a sea of inflammatory mediators, cytokines, and growth factors that are likely to promote vessel leakiness and may increase the sensitivity of the microvasculature to other leak-producing substances (5, 13). For example, vascular endothelial growth factor, also known as vascular permeability factor, causes vessel growth and increases permeability (16). Third, the abundant adhering and migrating leukocytes in the microvasculature of the infected tracheas are likely to contribute to the leakage (60). Last, a reduction in the activity of enzymes that degrade inflammatory mediators would promote leak-producing actions of inflammatory peptides (9).

The amount of Evans blue that accumulates in the trachea is determined by the permeability of the endothelium in concert with the luminal surface area of the microvasculature, transmural driving force, and rate of dye removal via the lymphatics. Angiogenesis and remodeling of the microvasculature that occurs after *M. pulmonis* infection would tend to increase the amount of leakage by expanding the vascular surface area and, in doing so, the number of endothelial gaps. The contribution of increased vascular surface area to the unscaled values, expressed as the amount of leakage per organ (e.g., ng dye/trachea), would be larger than to the scaled values for tissue mass (e.g., ng dye/mg trachea), which would tend to compensate for the angiogenesis in the thickened airway mucosa. Although the amount of leakage was larger in infected rats regardless of how the data were expressed, the scaled values in both strains were ~40% smaller than the unscaled values. Therefore, increases in vascular leakiness and surface area are both likely to contribute to the augmented leakage. A comparison of the relative sizes of the scaled and unscaled values suggests that the former contributes ~60% and the latter 40%.

Similarly, the driving force generated by the hydrostatic and osmotic pressure gradients could change in chronic inflammation. Indeed, one aspect of the vascular remodeling is an enlargement of the vessels upstream of venules, but the key variables are difficult to measure in the airways of living animals (56). Hydrostatic pressures have been measured in tracheal vessels of normal rats (4) and are changed by vasoactive drugs such as papaverine and norepinephrine (3), but to our knowledge, there are no data on how the vascular pressure profiles change in chronic inflammation. Vasodilatation could increase the driving force for leakage. For example, vasodilating prostaglandins and calcitonin gene-related peptide increase plasma leakage produced in the airways and skin by substance P and leukotrienes (11, 63). In contrast, vasodilatation leading to systemic hypotension as produced by sodium nitroprusside may decrease driving force and have no effect on leakage (20).

If the clearance of extravasated proteins through the lymphatics was impaired in chronic inflammation, the equilibrium between leakage and clearance could be altered to favor the accumulation of tracer in the tissue. In pathogen-free rats, a consistently small amount of extravasated dye was present at all time points, indicating a low rate of leakage and an ongoing balance between leakage and clearance via entry into the airway lumen and lymphatics (17). By comparison, much more dye accumulated in the airways of infected rats, and the amount of tracer continued to increase for at least 30 min after the injection. This finding indicates that leakage exceeded clearance during this period. The accumulation over time made it possible to detect at 30 min a rate of leakage that was not evident previously at 5 min (41). In chronically inflamed mouse ears, the lymphatics seem to proliferate (47), but to our knowledge, there are no detailed studies of changes in airway lymphatics in the presence of chronic inflammation.

Cellular mechanism of change in endothelial barrier function. Regardless of the contributions of increased vascular surface area and hemodynamic factors, some of the leakage observed in the present study must be due to changes in endothelial barrier function. Increases in endothelial permeability were indicated by the extravasation of the particulate tracer Monastral blue and leakage of RCA I lectin from vessels fixed by vascular perfusion.

Multiple pathways across the endothelium could contribute to the extravasation of Evans blue. Intercellular gaps, transendothelial channels, fenestrae, transcytotic vesicles, and vesiculovascular organelles are among the potential routes (15, 43, 49, 54). Of these, the current study focused on intercellular gaps because evidence from previous studies (36, 42) showing their contribution to plasma leakage caused by a variety of stimuli. The presence of endothelial gaps was documented in the present study by direct visualization of the luminal surface of the endothelium by scanning electron microscopy, which revealed focal openings of <1 μm in the endothelium of newly formed and remodeled blood vessels in infected rats but not in pathogen-free rats. Supportive evidence was also obtained by silver nitrate staining of the endothelium.

Although endothelial gaps are a reasonable candidate for leakage after *M. pulmonis* infection, they were not nearly as numerous or as uniformly distributed in the remodeled vessels of infected tracheas as they are in normal vessels made leaky by substance P, where >16 focal gaps can form around each endothelial cell (6, 39). This is not surprising considering that the rate of leakage was much less in chronically inflamed airways than after substance P.
Additional experiments will be needed to determine the relative contributions of endothelial gaps and other pathways in the augmented plasma leakage after *M. pulmonis* infection. The present study did, however, show that protein-size tracers (Evans blue bound to albumin, $\sim 60$ kDa; RCA I lectin, $\sim 120$ kDa), which have a comparatively long circulation time, leaked more than the particulate tracer Monastral blue (5–200 nm). This observation suggests that most of the routes for plasma leakage are smaller than 5 nm or are open intermittently.

**Mechanism of reduction in baseline plasma leakage by salmeterol.** We sought to learn whether salmeterol could reduce the abnormal baseline leakage associated with chronic inflammation in the airways. This seemed to be a reasonable question because $\beta_2$-agonists are commonly used in the treatment of chronic airway disease and are known to inhibit plasma leakage evoked by a variety of stimuli including antigen, substance P, bradykinin, and PAF (7, 8, 55, 61). Dose-response studies showed that salmeterol treatment significantly reduced baseline leakage in infected Wistar rats. This reduction was obtained in the same dosage range used to abolish allergen-induced leakage in pathogen-free rats (8). The 5-h delay from treatment to leakage measurement accommodated the relatively slow onset of action of salmeterol (8). However, the highest dose (5 mg/ml), which completely eliminated the late-phase leakage, produced a 60% reduction in baseline leakage in the infected airways. Because the present study examined the effect of only one treatment with salmeterol, a logical next step would be to assess the efficacy of repeated treatments in this system.

Salmeterol is a long-acting $\beta_2$-agonist with effects on airway smooth muscle cells, mast cells, epithelial cells, nerves, and endothelial cells (29). This class of drug can decrease plasma leakage by inhibiting the formation of endothelial gaps (7); blocking the release of inflammatory mediators from sensory nerves, thereby reducing neurogenic inflammation (1); or reducing the release of leak-producing inflammatory mediators from mast cells (27). All of these mechanisms could contribute to the reduction in baseline leakage in chronically inflamed airways.

**Selective sensitivity of vessels to NK$_1$ receptor agonists.** Previous studies (5, 38, 41) have documented the abnormally large effect of substance P and capsaicin on plasma leakage from the airway microvasculature of *M. pulmonis*-infected rats. The sensitivity of the vessels to substance P appears to result from an increased expression of NK$_1$ receptors on endothelial cells of remodeled vessels (5). The present studies extended this work by determining whether this heightened sensitivity was specific to substance P. We addressed this issue by comparing the effect of substance P with that of PAF and 5-HT. PAF, through its action on PAF receptors, causes leakage through a purely nonneurogenic mechanism (30, 55). 5-HT-induced leakage, mediated by 5-HT$_2$ receptors (22), has a small neurogenic component (2). In the present study, doses of PAF and 5-HT were chosen to give an amount of leakage in pathogen-free rats comparable to substance P. We did not use bradykinin or histamine because they can potently activate both neurogenic and nonneurogenic pathways (55).

Our results showed that substance P had an augmented response in infected rats, but PAF and 5-HT did not. 5-HT and PAF receptors may have been down-regulated or inactivated after *M. pulmonis* infection, similar to changes found in 5-HT receptor activity in the brain and PAF receptor activity in mesangial cells (18, 44). We were unable to test this possibility by immunohistochemistry because 5-HT$_{2A}$ receptor immunoreactivity was not detected in the trachea of either pathogen-free or infected rats. The level of endothelial cell expression of 5-HT$_{2A}$ receptors, which mediate 5-HT-induced plasma leakage in the rat trachea (19, 45, 46), was evidently below the threshold for immunohistochemical detection by the method we used, whereas subsets of cortical neurons were stained as previously described (21).

The selective potentiation of the action of substance P came as a surprise to us because we had suspected that the chronic inflammatory changes would be accompanied by a generalized sensitization to inflammatory mediators. Indeed, this may still be the case, but PAF and 5-HT apparently are not among the mediators that evoke an augmented response.

The substance P doses of 0.5 $\mu$g/kg and 1.0 $\mu$g/kg iv used in the present study were also used in previous studies in which no deaths occurred (e.g., Refs. 11, 37). What differed in the present study was the prolongation of the circulation time from 5 to 30 min to match the timing of measurements of baseline leakage. Because all of the deaths occurred more than 5 min after the injection of substance P, we could not have observed this outcome in previous studies that ended at 5 min (11, 37). We suspect that the deaths were due to airway obstruction caused by substance P-induced edema and mucus secretion in combination with mucosal thickening and narrowing of the airway lumen due to the infection (25, 50).

In conclusion, chronic inflammation produced by *M. pulmonis* infection is accompanied by sustained plasma leakage into the airway mucosa. The amount of leakage is much greater in Wistar rats than in F-344 rats, probably because of more extensive angiogenesis and remodeling of the airway microvasculature after infection. The leakage results from gaps between endothelial cells, increased vascular surface area, and probably other changes in the newly formed and remodeled blood vessels. Treatment with the $\beta_2$-agonist salmeterol can reduce but not eliminate the leakage. The remodeled vessels are abnormally sensitive to substance P but not to PAF or 5-HT, suggesting that the infection leads to a selective sensitivity of airway vessels to NK$_1$ receptor agonists.

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