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Neutrophil elastase-induced elastin degradation mediates macrophage influx and lung injury in 60% O2-exposed neonatal rats

Azhar Masood,1,4 Man Yi,2 Rosetta Belcastro,1 Jun Li,1 Lianet Lopez,1 Crystal Kantores,1 Robert P. Jankov,1,3,4,5 and A. Keith Tanswell1,3,4

1Lung Biology Programme, Physiology and Experimental Medicine, Hospital for Sick Children Research Institute, Toronto, Ontario, Canada; 2Department of Pediatrics and Child Health, University of Manitoba, Winnipeg, Manitoba, Canada; 3Department of Paediatrics, University of Toronto, Toronto, Ontario, Canada; 4Department of Physiology, University of Toronto, Toronto, Ontario, Canada; and 5Heart and Stroke Richard Lewar Centre of Excellence in Cardiovascular Research, University of Toronto, Toronto, Ontario, Canada

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Masood A, Yi M, Belcastro R, Li J, Lopez L, Kantores C, Jankov RP, Tanswell AK. Neutrophil elastase-induced elastin degradation mediates macrophage influx and lung injury in 60% O2-exposed neonatal rats. Am J Physiol Lung Cell Mol Physiol 309: L53–L62, 2015. First published May 1, 2015; doi:10.1152/ajplung.00298.2014.—Neutrophil (PMNL) influx precedes lung macrophage (LM) influx into the lung following exposure of newborn pups to 60% O2. We hypothesized that PMNL were responsible for the signals leading to LM influx. This was confirmed when inhibition of PMNL influx with a CXC chemokine receptor-2 antagonist, SB-265610, also prevented the 60% O2-dependent LM influx, LM-derived nitrotyrosine formation, and pruning of small arterioles. Exposure to 60% O2 was associated with increased lung contents of neutrophil elastase and α-elastic, a marker of denatured elastin, and a decrease in elastin fiber density. This led us to speculate that neutrophil elastase-induced elastin fragments were the chemokines that led to a LM influx into the 60% O2-exposed lung. Inhibition of neutrophil elastase with sivelestat or elafin attenuated the LM influx. Sivelestat also attenuated the 60% O2-induced decrease in elastin fiber density. Daily injections of pups with an antibody to α-elastic prevented the 60% O2-dependent LM influx, impaired alveologenesis, and impaired small vessel formation. This suggests that neutrophil elastase inhibitors may protect against neonatal lung injury not only by preventing structural elastin degradation, but also by blocking elastin fragment-induced LM influx, thus preventing tissue injury from LM-derived peroxynitrite formation.

neonatal lung injury; oxygen toxicity; macrophages; elastin fragments; BPD

A SERIOUS ADVERSE OUTCOME of extreme prematurity is the development of a chronic neonatal lung injury, bronchopulmonary dysplasia (BPD). The major features observed in moderate-to-severe BPD, as observed in the current era, are parenchymal thickening, arrested/impaired alveolarization (10), and pulmonary hypertension (51). The impairment of alveolarization may be sustained at least into late childhood and early adult life (11, 52). The presence of sustained pulmonary hypertension in infants with severe BPD is associated with an high mortality (1). Vascular structural remodeling is more rapid and severe in the neonate than in the adult (44). Recovery may be incomplete (31, 32, 44), have lasting effects on lung growth (10, 12, 42, 43), and lead to severe pulmonary hypertension in adult life (9, 15). There is currently no effective preventive therapy, and BPD is second only to asthma for health care costs in children (20).

Exposure of neonatal rats to 60% O2 for 14 days leads to a chronic lung injury, which has many of the pathological features of human BPD. These include parenchymal thickening (8, 16, 29, 39–41, 53, 54), impaired alveolarization (29, 39–41, 53), pulmonary hypertension (24–26, 28), and vascular remodeling (39–41). We recently demonstrated that the parenchymal thickening, impaired alveolarization, vascular remodeling and nitrotyrosine formation were all mediated by peroxynitrite (40). We had earlier demonstrated that nitrotyrosine formation was attenuated by macrophage depletion (25), consistent with macrophages being the primary source of nitric oxide for peroxynitrite formation. The signal(s), which lead to macrophage recruitment into the lung, in this model, have yet to be elucidated. In a previous study (29), we had identified no increase in the known macrophage chemoattractants MCP-1, MIP-1α, and MIP-1β, following exposure to 60% O2. Indirect evidence suggested that a neutrophil influx precedes the macrophage influx in this model (53). This led us to hypothesize that neutrophils initiate the signaling process by which macrophages are recruited to the lung, through generation of elastin fragments by the action of neutrophil-derived elastase. Elastin fragments are known chemokines for macrophages in an adult murine model of emphysema (18).

MATERIALS AND METHODS

In vivo procedures. All animal experiments were conducted in accordance with Canadian Council on Animal Care guidelines. Approval for animal use was obtained from the Animal Care Review Committee of the Hospital for Sick Children Research Institute. Pathogen-free timed-pregnant Sprague-Dawley rats (250–275 g) were obtained from Taconic (Germantown, NY). Rat pups (10–12/litter) were exposed to air or 60% O2 in paired chambers (BioSpheryx, Lacona, NY) for up to 14 days, as previously described (8, 16, 24–26, 28, 39–41, 53, 54). We have previously demonstrated that this
For inhibition of neutrophil influx, paired litters received either 5 μl/g of 0.1% (vol/vol) DMSO in 0.9% (vol/vol) saline (vehicle control) or 2 μl/g SB-265610 (Glaxo Smith-Kline, King of Prussia, PA), a selective CXC chemokine receptor-2 antagonist, in 5 μl/g of 0.1% (vol/vol) DMSO in 0.9% (vol/vol) saline by daily ip injection into the right iliac fossa, via a 30-gauge needle, as previously described (53). We have previously demonstrated that SB-265610 impairs the influx of neutrophils, but not macrophages, in a neonatal lung injury (37). To assess effects of neutrophil elastase inhibition, paired litters received either sivelestat (Tocris, Ellisville, MO) by daily sc injection into the nape of the neck at concentrations of 0–6 0 μg/g in 5 μl/g 0.9% (vol/vol) saline vehicle or elafin (Sigma-Aldrich, St. Louis, MO) by daily ip injection at concentrations of 0–8 μg/g in 5 μl/g 0.9% (vol/vol) saline vehicle. Because of economic considerations, the number of pups receiving elafin and the...
duration of their exposure were less than for pups receiving sivelestat. To inhibit macrophage influx, paired litters received either 0.9% (vol/vol) saline (vehicle control) or GdCl3·6H2O (Sigma-Aldrich) in 0.9% (10 μg/g and 5 μl/g ip) within 6 h of birth, as previously described (25). To determine whether circulating degraded elastin fragments were acting as macrophage chemotaxins, rat pups were injected with a goat anti-rat α-elastin polyclonal antibody (ab21608: Abcam, Cambridge, MA), with euthanasia at day 7. Based on preliminary dosimetry (data not shown) the antibody was diluted 1:5 in normal saline and injected daily ip at 5 μl/g containing 42 μg/g protein. Control animals received nonspecific goat serum at the same volume and protein content. At the termination of each exposure period, pups were killed by ether inhalation.

**Tissue preparation.** Lungs were inflated with air under a constant airway pressure of 20 cmH2O while being perfusion fixed over 12 h with 4% (wt:vol) paraformaldehyde in PBS. Lung sections were stained either with hematoxylin and eosin (Surgipath, Winnipeg, MB, Canada) or with Weigert’s resorcin-fuchsin stain (Rowley Biochemical Institute, Danvers, MA) and were then counterstained with tartrazine (Sigma-Aldrich, St. Louis, MO).

**Immunohistochemistry.** Immunostaining was performed on lung sections of 5-μm thickness by an avidin-biotin-peroxidase complex method (19). For detection of neutrophils, a rabbit polyclonal antibody to myeloperoxidase (A0398: Dako Canada, Mississauga, ON, Canada) was applied at a dilution of 1:200 at room temperature for 1 h. For detection of μ-conprotease, a mouse anti-rat α-elastin polyclonal antibody (RA75: Elastin Products, Owensville, MO) was applied overnight at 4°C at a dilution of 1:4,000. The anti-rat α-elastin polyclonal antibody (RA75: Elastin Products, Owensville, MO) was applied overnight at 4°C at a dilution of 1:4,000. The secondary donkey polyclonal anti-goat IgG (sc-2042: Santa Cruz Biotechnology) was applied at a dilution of 1:200 at room temperature for 2 h. For detection of macrophages, a mouse anti-rat cluster of differentiation 68 (CD-68) polyclonal antibody (MCA341R: Serotec, Oxford, UK) was applied at a dilution of 1:200 at room temperature for 1 h. Lung tissue neutrophil and macrophage counts were obtained from the total fields of view of known dimensions then expressed per millimeter squared. When only intraluminal cell counts (cells adjacent to the inner walls of the distal air sacs) were assessed, values were expressed as a ratio to the tissue fraction. For detection of nitrotyrosine, a rabbit anti-nitrotyrosine polyclonal antibody (06-284: Upstate Biotechnology, Waltham, MA) was applied overnight at 4°C at a dilution of 1:100. The secondary goat polyclonal anti-rabbit IgG (sc-2040: Santa Cruz Biotechnology) was applied at a dilution of 1:200 at room temperature for 1 h. For detection of α-elastin, a goat anti-rat α-elastin polyclonal antibody (RA75: Elastin Products, Owensville, MO) was applied overnight at 4°C at a dilution of 1:4,000. The secondary donkey polyclonal anti-goat IgG (sc-2042: Santa Cruz Biotechnology) was applied at a dilution of 1:200 at room temperature for 1 h.

**Assessment of vessel pruning.** Barium-gelatin pulmonary arteriograms were performed as described by Le Cras et al. (36) and were imaged by X-ray radiography. For small vessel counts, tissue sections were stained with Hart’s elastin stain. Staining was with Weigert’s resorcin-fuchsin and counterstaining with tartrazine, as previously described (38, 39–41). Only vessels with both inner and outer elastin bands, to identify arterioles, and arbitrarily selected outside diameters of 30–80 μm, as assessed by superimposed concentric rings, were counted. In subsequent studies, related to upgrades in microscope and software, we changed our arbitrarily selected outside diameters to 20–65 μm, which we have found to be more sensitive.

**Assessment of elastin fiber density.** Threshold analysis of Hart’s-stained images was used to quantify elastin fiber density (13). The area occupied by elastin fibers was calculated by dividing the pixel fractional area for elastin by the pixel fractional area for all tissue (5, 35, 38).

**Neutrophil elastase activity.** Neutrophil elastase activity in lung homogenates was measured after incubation with fluorogenic substrate (MeOSu-AAPV-AMC; Enzo Life Sciences, Brockville, ON, Canada) as described by others (34). Briefly, 40 μl of lung homogenate was mixed with 80 μl of MeOSu-AAPV-AMC substrate to yield a final concentration 0.1 nM in 0.1 M Tris, pH 7. With use of excitation/emission wavelengths of 400/505 nm with a cutoff at 455 nm, samples were incubated in a black 96-well plate and were mixed every 5 min until being read after 30 min of incubation. Background fluorescence of the substrate alone was subtracted from individual readings, which were then corrected for protein content.

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**Fig. 3.** Lung tissue of d-7 rat pups exposed to air (A and C) or 60% O2 (B and D) and treated with daily vehicle (A and B) or SB-265610 (C and D). Exposure to 60% O2 was associated with an apparent increase in immunoreactive nitrotyrosine (brown stain), which was reversed by treatment with SB-265610. Bar = 100 μm.
As previously described (19), exposure to 60% O₂ for 7 days resulted in an apparent macrophage influx into lung tissue (Fig. 2B), as assessed by CD-68 staining, compared with lung tissue from air-exposed pups (Fig. 2A). Inhibition of a neutrophil influx, by daily injections of SB-265610 (53), also resulted in a significant attenuation of the 60% O₂-induced macrophage influx (Fig. 2D) but had no apparent effect on the macrophage content of lung tissue from air-exposed pups (Fig. 2C). These qualitative assessments were confirmed on quantitative assessments of macrophage density per unit area (Fig. 2E). Based on our previous observation that vessel loss in 60% O₂ was mediated by macrophage-derived peroxynitrite (40), we anticipated that the SB-265610-mediated inhibition of macrophage influx would also both limit macrophage-derived protein nitration and prevent vessel loss. As assessed by immunohistochemistry, there was an increase in nitrotyrosine immunoreactivity following exposure to 60% O₂ (Fig. 3) compared with air-exposed lung tissue (Fig. 3A). Treatment with SB-265610 had no apparent effect on air-exposed lung tissue (Fig. 3C), but it reduced nitrotyrosine formation in lung tissue exposed to 60% O₂ (Fig. 3D). Preliminary observations of pulmonary

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Fig. 6. Rat pups were exposed from birth to air (white bar) or 60% O2 (black bars) or 60% O2 after receiving a GdCl₃·6H₂O injection on day 1 of life (gray bar) and were euthanized on day 7 after receiving increasing doses of daily sc sivelestat or saline vehicle. Lung tissue macrophage content was assessed by cell CD-68 immunoreactivity. Exposure to 60% O₂ increased lung macrophage content relative to air-exposed pups. This 60% O₂-induced macrophage influx was reduced by treatment with sivelestat in a dose-dependent fashion with values not significantly different from control values in air at doses ≥10 μg/g. *P < 0.05 by 1-way ANOVA compared with all other groups. Values are means ± SD for lung sections from 4 average-sized pups, each from different litters.

artery angiograms suggested that the apparent vessel pruning observed with 60% O₂ (Fig. 4B), compared with air-exposed pups (Fig. 4A), was attenuated by treatment with SB-265610 (Fig. 4C). This qualitative assessment was confirmed on quantitative assessments of small vessel density per unit area (Fig. 4D). We have previously reported that treatment with SB-265610 protects against 60% O₂-induced impairment of alveologenesis (53).

We speculated that the chemoattractant responsible for the neutrophil-dependent influx of macrophages would be neutrophil elastase-generated elastin fragments (18) and, as a first step, tested lung homogenates for their content of α- elastin, a marker for denatured elastin. As assessed by immunohistochemistry, there was an apparent increase in α-elastin in the lungs of rat pups exposed to 60% O₂ for 7 days (Fig. 5B) relative to those exposed to air (Fig. 5A). This impression was confirmed by slot blot analysis (Fig. 5C), which, when quantified, demonstrated a marked increase in lung content of α-elastin, normalized to β-actin, following exposure of rat pups to 60% O₂ (Fig. 5D). To determine whether neutrophil elastase was responsible for initiating the 60% O₂-induced macrophage influx, we examined the effect of a neutrophil elastase inhibitor, sivelestat (21, 30), on macrophage influx. As shown in Fig. 6, we observed a dose-dependent decrease in 60% O₂-induced macrophage influx with sivelestat treatment. At a sivelestat dose of 10 μg·g⁻¹·day⁻¹ the lung tissue macrophage density with 60% O₂ was not significantly different from that observed with air, or from pups exposed to 60% O₂ and treated with gadolinium chloride to deplete lung macrophage content (25).

To confirm that this observed effect of sivelestat could be attributed to an effect on neutrophil elastase, rather than to some other unanticipated action, we used a second neutrophil elastase inhibitor, elafin (48, 55), to confirm that inhibition of neutrophil elastase attenuated macrophage influx. As shown in Fig. 7, treatment with elafin also inhibited 60% O₂-mediated macrophage influx in a dose-dependent fashion. Based on economic considerations, subsequent studies were performed using sivelestat, at a dose of 10 μg·g⁻¹·day⁻¹, rather than elafin as the neutrophil elastase inhibitor.

Hart’s staining of lung tissue suggested that vehicle-treated pups exposed to 60% O₂ had a reduction in long elastin fibers in the parenchyma (Fig. 8B) relative to vehicle-treated and air-exposed pups (Fig. 8A). This appeared to be reversed by treatment with sivelestat (Fig. 8D). Sivelestat had no apparent effect on air-exposed pups (Fig. 8C). At this dose (Fig. 8E), sivelestat attenuated the reduction in lung fiber density induced by exposure to 60% O₂, as assessed by threshold analysis, consistent with a 60% O₂-induced macrophage influx being downstream of elastin degradation by neutrophil elastase. This was further examined by directly assessing lung neutrophil elastase activity (Fig. 8F). Exposure to 60% O₂ increased lung neutrophil elastase activity, relative to values for lung homogenates from air-exposed control pups, which was attenuated by concurrent treatment with sivelestat. Treatment of 60% O₂-exposed rat pups with sivelestat for 14 days attenuated the 60% O₂-mediated inhibition of alveologenesis, as assessed by secondary crest-to-tissue ratio (Fig. 9A), but did not attenuate the 60% O₂-mediated inhibition of small vessel formation, as assessed by small vessel density-to-tissue ratio (Fig. 9B).

Lastly, to confirm that products of elastin degradation were the chemokine(s) responsible for the macrophage influx following exposure to 60% O₂, we treated pups with an antibody to α-elastin for 7 days. Relative to air-exposed and vehicle-injected pups (Fig. 10A), exposure of vehicle-injected pups to 60% O₂ resulted in an apparent increase in lung macrophage content (Fig. 10B). Treatment with the antibody to α-elastin had no apparent effect on macrophage content in air-exposed pups (Fig. 10C) but did appear to reduce the macrophage content in pups exposed to 60% O₂ (Fig. 10D). These qualitative assessments were confirmed by direct cell counts (Fig. 10E). As previously described, exposure to 60% O₂ for 14 days impaired alveologenesis, as reflected in a reduced secondary
Fig. 8. Rat pups were exposed to air or 60% O₂ from birth and were euthanized on day 14 after receiving daily sivelestat at 10 µg·g⁻¹·day⁻¹ sc or saline vehicle. Exemplars of Heart’s-stained tissue to show elastin in lung tissue from an air-exposed and vehicle-treated pup (A), a 60% O₂-exposed and vehicle-treated pup (B), an air-exposed and sivelestat-treated pup (C), and a 60% O₂-exposed and sivelestat-treated pup (D). Exposure to 60% O₂ caused an apparent reduction in parenchymal long elastin fibers, which appeared to be restored by treatment with sivelestat. E: lung tissue elastin fiber density was assessed by densitometric analyses of Hart’s-stained tissue. Exposure to 60% O₂ decreased lung tissue elastin fiber density in vehicle-treated pups (o:v), which was attenuated by concurrent treatment with sivelestat (o:s). Relative to vehicle-treated pups in air (a:v), sivelestat had no effect on elastin fiber density in air-exposed pups (a:s). F: exposure to 60% O₂ increased lung homogenate neutrophil elastase activity in vehicle-treated pups (o:v), which was attenuated by concurrent treatment with sivelestat (o:s). Relative to vehicle-treated pups in air (a:v), sivelestat had no effect on lung neutrophil elastase activity in air-exposed pups (a:s). AU, arbitrary units. *P < 0.05 by 1-way ANOVA compared with all other groups. Values are means ± SD for lung sections from 4 average-sized pups, each from different litters, or homogenates from 4 different litters.

Fig. 9. Rat pups were exposed to air or 60% O₂ from birth and were euthanized on day 14 after receiving daily sivelestat at 10 µg·g⁻¹·day⁻¹ sc or saline vehicle. A: lung tissue alveolar development was assessed by quantifying the secondary crest-to-tissue ratio. Exposure to 60% O₂ decreased the secondary crest-to-tissue ratio in vehicle-treated pups (o:v), which was attenuated by concurrent treatment with sivelestat (o:s). Relative to vehicle-treated pups in air (a:v), sivelestat had no effect on secondary crest-to-tissue ratio in air-exposed pups (a:s). B: vascular development was assessed by quantifying the small vessel-to-tissue ratio. Exposure to 60% O₂ decreased small vessel density in vehicle-treated pups (o:v), which was not attenuated by concurrent treatment with sivelestat (o:s). Relative to vehicle-treated pups in air (a:v), sivelestat had no effect on small vessel density in air-exposed pups (a:s). *P < 0.05 by 1-way ANOVA compared with all other groups; $P < 0.05 by 1-way ANOVA compared with vehicle-treated control group. Values are means ± SD for lung sections from 4 average-sized pups, each from different litters, or homogenates from 4 different litters.
crest density, and small vessel formation as assessed by small vessel density, compared with air-exposed pups. Treatment with the antibody to α-elastin protected against both the 60% O₂-mediated impairments of alveologenesis (Fig. 11A) and small vessel formation (Fig. 11B).

Because an increase in macrophage content of the lung parenchyma could theoretically be due either to an increased recruitment of macrophages to the lung or to an arrest of migration into the distal air sacs, we assessed intraluminal macrophage and neutrophil counts in isolation. The patterns of changes in cell number with the SB-265610 and anti-α-elastin antibody interventions (Table 1) were similar to those observed with total lung cell counts, consistent with 60% O₂-induced recruitment, rather than arrested migration. In contrast, the pattern with the sivelestat intervention differed from that observed with total cell counts, in that an effect of sivelestat on intraluminal AM content following exposure to 60% O₂ was observed.

**DISCUSSION**

Exposing neonatal rats to 60% O₂ from birth for 14 days leads to parenchymal thickening (8, 16, 29, 38–41, 53, 54), impaired alveolarization (29, 38–41, 53), pulmonary hypertension (24–26), and vascular remodeling (39–41). Influxes of both neutrophils (53) and macrophages (25) are required for these pathological processes to occur. The final mediator of these 60% O₂-induced processes is peroxynitrite (40). Peroxynitrite is formed from the reaction of superoxide with nitric oxide. This reaction is diffusion limited in a gaseous phase and is extremely rapid in an aqueous phase (6). In the 60% O₂-induced neonatal rat lung injury, the primary source of nitric oxide for peroxynitrite formation is the macrophage (25), thus explaining the critical role that macrophages play in the development of the injury. The role played by the neutrophil has been less clear. One possibility has been that neutrophils supply superoxide for peroxynitrite formation, although macrophages alone generate significant amounts of superoxide in 60% O₂-induced neonatal rat lung injury (53), and a contribution from neutrophils may not be required. Alternatively, it may be neutrophil-derived elastase that mediates the critical role played by neutrophils. A protective effect on lung growth of an elastase inhibitor, instilled into the tracheas of oxygen-exposed and ventilated mouse pups, has been described by others (17). This protective effect was attributed
vascular development was assessed by quantifying the small vessel-to-tissue ratio. Exposure to 60% O2 decreased small vessel density in control serum-treated pups. Relative to control serum-treated pups in air (a:c), the anti-α-elastin antibody had no effect on secondary crest-to-tissue ratio in control serum-treated pups (o:c), which was attenuated by concurrent treatment with the anti-α-elastin antibody (o:ab). Relative to control serum-treated pups in air (a:c), the anti-α-elastin antibody had no effect on small vessel density in air-exposed pups (a:ab). *P < 0.05 by 1-way ANOVA compared with all other groups. Values are means ± SD for lung sections from 4 average-sized pups, each from different litters.

to a direct effect of neutrophil elastase on the elastin network of the lung.

In the experiments reported above, we describe an alternative or complementary explanation for the effect of neutrophil elastase on neonatal lung growth and development. In the 60% O2-induced neonatal rat, a significant neutrophil influx into the lung preceded a significant macrophage influx. The macrophage influx and macrophage-dependent peroxynitrite formation were attenuated by blocking the neutrophil influx, indicating that the macrophage influx was neutrophil dependent. The neutrophil influx was accompanied by an increase of neutrophil elastase activity in lung tissue. This increased elastase activity initiates the macrophage influx, as confirmed by interventions with two different elastase inhibitors. Both elastase inhibitors are likely to have effects other than inhibition of elastase activity; hence our use of two different inhibitors to make the observed effects less likely to be due to alternative side effects. The increase of tissue elastase activity was associated with an increase in elastin degradation, as assessed by the measurement of lung tissue α-elastin content and a reduction of elastin fiber density, the latter being reversible with an elastase inhibitor intervention. We cannot exclude some contribution to the elastase pool from macrophages (47) or vascular smooth muscle cells (22), both of which can liberate elastase, which could be inhibited by sivelestat and elafin. These beneficial effects of elastase inhibitors on 60% O2-mediated lung injury are consistent with our results in an earlier study in which treatment with another elastase inhibitor, α1-antitrypsin, was protective in this model (33). Although a subsequent study, in which preterm human infants were treated with α1-antitrypsin, failed to show a significant reduction in the incidence of BPD (49), this failure of effect may have been due to the unanticipated very rapid rate at which α1-antitrypsin is cleared in the newborn (50). In an earlier study, macrophage depletion protected against the 60% O2-mediated decrease in vessel density (25). That treatment with sivelestat protected against the 60% O2-mediated increase in lung macrophage content and the concurrent impairment of alveologenesis, but not the impairment of small vessel formation, was an unexpected finding for which we currently have no adequate explanation.

A number of chemotactic elastin-derived peptides have been described (VGAMPG, VGMAPG, VGSLPG, VGLSPG) that bind to antibodies against α-elastin (14), and such an antibody has been used by others to prevent macrophage recruitment and lung injury in an animal model of emphysema (18). We took the same approach in the 60% O2-mediated neonatal lung injury, injecting animals with the same antibody to α-elastin that had been used for the slot blot analysis, and observed an attenuation of the 60% O2-induced macrophage influx, consistent with elastin-derived fragments being the responsible macrophage chemokine(s). In contrast to our findings with sivelestat, treatment with the antibody to α-elastin protected against

![Graph A](image1.png)

**Fig. 11.** Rat pups were exposed to air or 60% O2 from birth and were euthanized on day 14 after receiving daily injections of 1:5 dilutions of a control serum or an antibody to α-elastin. A: lung tissue alveolar development was assessed by quantifying the secondary crest-to-tissue ratio. Exposure to 60% O2 decreased the secondary crest-to-tissue ratio in control serum-treated pups (o:c), which was attenuated by concurrent treatment with the anti-α-elastin antibody (o:ab). Relative to control serum-treated pups in air (a:c), the anti-α-elastin antibody had no effect on secondary crest-to-tissue ratio in air-exposed pups (a:ab). B: vascular development was assessed by quantifying the small vessel-to-tissue ratio. Exposure to 60% O2 decreased small vessel density in control serum-treated pups (o:c), which was attenuated by concurrent treatment with the anti-α-elastin antibody (o:ab). Relative to control serum-treated pups in air (a:c), the anti-α-elastin antibody had no effect on small vessel density in air-exposed pups (a:ab). *P < 0.05 by 1-way ANOVA compared with all other groups. Values are means ± SD for lung sections from 4 average-sized pups, each from different litters.

Table 1. Intraluminal counts for PMNL and AM after a 14-day exposure of neonatal rats to air or 60% O2

<table>
<thead>
<tr>
<th>Intervention</th>
<th>a:c</th>
<th>a:i</th>
<th>o:c</th>
<th>o:i</th>
<th>a:c</th>
<th>a:i</th>
<th>o:c</th>
<th>o:i</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sivelestat</td>
<td>1.34*</td>
<td>0.88*</td>
<td>2.51*</td>
<td>1.46*</td>
<td>2.34</td>
<td>0.70*</td>
<td>3.18</td>
<td>2.56</td>
</tr>
<tr>
<td>Anti-α-elastin</td>
<td>1.54</td>
<td>1.34</td>
<td>2.90</td>
<td>3.78*</td>
<td>3.88*</td>
<td>1.02</td>
<td>4.88</td>
<td>3.88</td>
</tr>
<tr>
<td>SB-265610</td>
<td>0.31</td>
<td>0.25</td>
<td>0.22</td>
<td>0.31</td>
<td>0.25</td>
<td>0.22</td>
<td>0.25</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Air exposed pups were treated with a specific intervention (a:i) or a control intervention (a:c). Oxygen-exposed pups were also treated with a specific intervention (o:i) or a control intervention (o:c). Exposure to 60% O2 increased intraluminal for neutrophil (PMNL) and alveolar macrophage (AM) counts, which effect was attenuated by the specific interventions. The observed patterns of intraluminal counts for PMNL and AM did not differ from those observed for total lung neutrophil or macrophage counts described elsewhere, except that sivelestat had an effect on intraluminal AM counts following exposure to 60% O2, which had not been observed for total counts. Counts are expressed as a ratio to tissue fraction. All data are presented as means ± SD. *P < 0.05 vs. all other groups by 1-way ANOVA; n = 4 average-sized pups per group, each from different litters.
the impairment of both alveologenesis and small vessel formation induced by exposure to 60% O$_2$.

The studies described above provide the missing link in the putative chain of events that leads to impaired alveologenesis and pulmonary hypertension following exposure of the neonatal rat to 60% O$_2$. The initiating event is an increase in the cytokine IL-1$\beta$ (29), which is an upstream regulator of the neutrophil chemokine CINC-1, which mediates neutrophil recruitment (53). As described above, release of neutrophil elastase results in the production of elastin fragments, which are chemotactic for macrophages. Macrophages are the source of nitric oxide for peroxynitrite formation (25). Peroxynitrite acts on arachidonic acid to form F$_2$-isoprostanes, which are contributors to impaired alveologenesis (40), both through an upregulation of the growth inhibitor TGF$\beta$1 and through nitration and inactivation of growth factor receptors (3). A schematic summarizing these steps has recently been published elsewhere (3).

How do the mechanisms described above for the lung injury observed in the 60% O$_2$-exposed neonatal rat apply to the human infant developing BPD? Both manifest impaired alveologenesis, parenchymal thickening, pulmonary hypertension, and evidence of pulmonary inflammation. There is a widely held view that BPD is a disease caused by pulmonary inflammatory cell influx and inflammatory cell-mediated tissue injury (4). Pulmonary inflammation is associated with evidence of oxidative and nitrative stress in both neonatal rats exposed to 60% O$_2$ (3, 27, 40, 53) and human infants developing lung injury (2, 45). A cause-and-effect relationship between nitrination and lung injury has been established in the 60% O$_2$-exposed neonatal rat, but such a relationship has yet to be tested in human infants. This may not happen in the near future because of the potential toxicity of the current generation of heavy metal-containing peroxynitrite decomposition catalysts.

Of the other potential targets for intervention identified in the 60% O$_2$-exposed neonatal rat that could be applicable interventions for human infants, two appear to have potential. Transgenic mice overexpressing IL-1$\beta$ develop a BPD-like lung injury and pulmonary inflammation mediated by the increased expression of neutrophil and macrophage chemokines (7). Antagonism of IL-1$\beta$ binding to its receptor protects neonatal rats against the lung injury induced by 60% O$_2$ (29). These findings must be tempered by the recent appreciation that the signaling pathways induced by inflammation in mice and humans are very different (46) and that, despite the increase in IL-1$\beta$ observed in serum and tracheal aspirates of human infants developing BPD (23), there is as yet no evidence to show that IL-1$\beta$ is a critical mediator of BPD. Lastly, we believe that antiprotease therapy has been inadequately studied in human infants developing BPD. The clinical trial of $\alpha_1$-antitrypsin did not show a significant benefit, but there was a trend toward an effect (49), despite inadequate dosing (50).

**REFERENCES**

19. Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and

**AUTHOR CONTRIBUTIONS**


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**DISCLOSURES**

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


45. Shapiro SD, Griffin DL, Gilbert DJ, Jenkins NA, Copeland NG, Zani ML, Baranger K, Guyot N, Dallet-Choisy S, Moreau T.


