Role of brain-derived neurotrophic factor in hyperoxia-induced enhancement of contractility and impairment of relaxation in lung parenchyma

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Sopi RB, Martin RJ, Haxhiu MA, Dreshaj IA, Yao Q, Jafri A, Zaidi SI. Role of brain-derived neurotrophic factor in hyperoxia-induced enhancement of contractility and impairment of relaxation in lung parenchyma. Am J Physiol Lung Cell Mol Physiol 295: L348–L355, 2008. First published May 30, 2008; doi:10.1152/ajplung.00067.2008.—Prolonged hyperoxic exposure contributes to neonatal lung injury, and airway hyperreactivity is characterized by enhanced contraction and impaired relaxation of airway smooth muscle. Our previous data demonstrate that hyperoxia in rat pups upregulates expression of brain-derived neurotrophic factor (BDNF) mRNA and protein, disrupts NO-cGMP signaling, and impairs cAMP production in airway smooth muscle. We hypothesized that BDNF-tyrosine kinase B (TrkB) signaling plays a functional role in airway hyperreactivity via upregulation of cholinergic mechanisms in hyperoxia-exposed lungs. Five-day-old rat pups were exposed to \( \geq 95\% \) oxygen or room air for 7 days and administered daily tyrosine kinase inhibitor K-252a (50 \( \mu \)g kg \(^{-1}\) day \(^{-1}\) ip) to block BDNF-TrkB signaling or vehicle. Lungs were removed for HPLC measurement of ACh or for in vitro force measurement of lung parenchymal strips. ACh content doubled in hyperoxic compared with room air-exposed lungs. K-252a treatment of hyperoxic pups restored ACh content to room air levels. Hyperoxia increased contraction and impaired relaxation of lung strips in response to incremental electrical field stimulation. K-252a administration to hyperoxic pups reversed this increase in contraction and decrease in relaxation. K-252a or TrkB-Fc was used to block the effect of exogenous BDNF in vitro. Both K-252a and TrkB-Fc blocked the effects of exogenous BDNF. Hyperoxia decreased cAMP and cGMP levels in lung strips, and blockade of BDNF-TrkB signaling restored cAMP but not cGMP to control levels. Therefore, hyperoxia-induced increase in activity of BDNF-TrkB receptor signaling appears to play a critical role in enhancing cholinergically mediated contractile responses of lung parenchyma.

cholinergically mediated contractile responses and decreased relaxant responses of airways and lung parenchymal tissues measured under in vitro and in vivo conditions (4, 16, 17, 31, 36). It remains unclear by what mechanism(s) cholinergic effects are upregulated under these conditions.

We (42) have shown the presence of brain-derived neurotrophic factor (BDNF) and tyrosine kinase B (TrkB) receptors on airway smooth muscle cells in developing rat pups and upregulation of BDNF after hyperoxic exposure. We (44) have also demonstrated expression of BDNF in airway preganglionic neurons. This raises the possibility that upregulation of neurotrophins may contribute to airway reactivity under certain pathophysiologičal states associated with neonatal lung injury, asthma, and other inflammatory airway disease. Neurotrophins and their receptors are expressed in a variety of nonneural tissue including lung (34, 42) and may play a critical role in regulation of the lower airways (44). Furthermore, BDNF acting via TrkB receptors facilitates responses of human airway smooth muscle to different contractile stimuli including ACh under in vitro conditions (32).

Recently, we (36) have demonstrated that exposure of rat pups to high oxygen concentration impairs relaxant responses of lung parenchyma through a NO-cGMP-dependent mechanism. Mhanna et al. (31) have also shown that relaxation of tracheal tissue from hyperoxia-exposed rat pups was impaired, and this was accompanied by diminished release of PGE\(_2\); and production of cAMP. Therefore, both cGMP and cAMP signaling pathways have been implicated in this process. We (43) have previously proposed that upregulation of BDNF and TrkB receptors, by hyperoxic exposure of immature rats, alters downstream regulation of intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) such that contractile responses of the airways and lung parenchyma to endogenously released ACh are increased. This is consistent with available data that the rapid excitatory activity of BDNF is via activation of TrkB receptors and that BDNF appears to stabilize excitatory postsynaptic receptors and enhance quantal excitatory neurotransmitter release probably via postsynaptic Ca\(^{2+}\) signaling (23, 32, 44).

Therefore, in this study, we initially hypothesized that hyperoxic exposure of rat pups would increase ACh content in the lung, and the blockade of TrkB receptors would attenuate this effect. We then tested the hypothesis that blockade of BDNF-TrkB signaling would prevent the increased contractile responses and/or decreased relaxant responses that were a consequence of hyperoxic exposure. Finally, we sought to deter-

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mine a role for BDNF-TrkB signaling in modulating cGMP and cAMP pathways under these conditions. Our data strongly support the involvement of BDNF-TrkB signaling in the increased contractility of lung parenchyma observed after hyperoxic exposure in early postnatal life.

MATERIALS AND METHODS

Animals and their treatment. Sprague-Dawley rat pups were used for these experiments. The animal protocol was approved by the Institutional Animal Care and Use Committee of Case Western Reserve University, Cleveland, Ohio. On the 5th day of life, rat pups were exposed to hyperoxic (≥95% O2) or room air conditions for a period of 7 days. Rat pups from two different litters were randomly mixed and assigned to hyperoxic or room air groups (n = 8–10 per group). Pups in each group were administered once daily K-252a (a TrkB receptor blocker, 50 μg/kg “day 1” ip) or vehicle (25% DMSO in saline ranging from 16 to 22 μl volume depending on the weight of pups). We selected a relatively low dose of K-252a because we employed multiple doses to maintain a steady serum level of drug in the blood. We have not observed any visible morbidity in treated animals based on weight gain, general behavior, and gross analysis of organs at death. Hyperoxic groups were housed with their mothers in a Plexiglas chamber (38 l) and exposed to continuous flow of O2 (2 l/min) for 7 days. Mothers were rotated every 24 h between room air and hyperoxic groups to minimize the toxic effects of constant hyperoxic exposure. Oxygen concentration was monitored twice per day via oxygen analyzer (MiniOX I; MSA Medical Products, Pittsburgh, PA). The pups assigned to room air were kept in a commercial rat cage. Animals were euthanized on day 12 either by asphyxiation in CO2 or by guillotine for ACh measurement to prevent the degradation of ACh.

Estimation of ACh in lung. To study whether hyperoxic exposure increases ACh content in the lung and whether it can be prevented by TrkB receptor blockade, we measured ACh in lung using HPLC. K-252a was injected daily intraperitoneally, and on the last day of exposure it was administered 4 h before killing to provide enough time for the absorption, metabolism, and circulation of K-252a. Normal saline was administered in place of K-252a in control animals. Additionally, 10 min before death, pups were injected intraperitoneally with 0.2 ml of Ringer solution (concentration in mM: 150 NaCl, 2.4 CaCl2, 4 KCl) containing 10 μM neostigmine to block acetylcholinesterase activity and thus to avoid a differential effect of hyperoxic vs. normoxic exposure on this enzyme. The lungs were removed and frozen quickly by dipping them in an ethanol-dry ice bath (n = 13 in each group). ACh extraction was carried out using a modified method of Beley et al. (3). In brief, the lungs were weighed and homogenized using Tissue-Tearor in 50 volumes of 1 N formic acid/acetone solution. The solution was incubated in ice for 20 min, and the tissue suspension was centrifuged at 10,000 × g at 4°C for 10 min. The 0.5 ml of the supernatant was vortexed for 10 min with 2 ml of heptane/chloroform (8:1 vol/vol) to remove lipids. After 20 min of incubation in ice, the samples were centrifuged, and the organic layer was removed. Three volumes of 3-heptane containing 3 mg/ml sodium tetrathylphoron were added to the aqueous phase. After vortexing for 10 min, the samples were incubated in ice for 20 min and then centrifuged at 4°C for 10 min. Finally, 0.2 ml of the organic layer was added to 50 μl of 1 N HCl, vortexed for 10 min, and centrifuged. The organic layer was discarded, and the hydrochloric extract was dried under vacuum and stored at −80°C until analysis. The dried samples were dissolved in Ringer solution immediately before injection into the ACh/choline chromatographic system with a Bioanalytical Systems MF-9053 assay kit containing two cartridge columns that consisted of a polymeric analytical column followed by an immobilized enzyme reactor column (GBC Separations, Hubbardston, MA). The mobile phase consisted of filtered (0.2-μm Millipore cellulose filter) and helium-degassed Milli-Q water at pH 8.5 containing 50 mM Na2HPO4 and 0.2 g of EDTA per liter. Kathon (50 μl/l) was added as a bacteriostatic agent. A GBC Separations amperometric detector containing an Ag/AgCl reference electrode and platinum working electrode set at +500 mV were used. Output noise was reduced by using an active filter (Link, GBC Separations) set at 30 Hz cutoff frequency. The results were expressed as picomoles per gram lung tissue.

Lung parenchymal strip preparation. Lungs were removed from rat pups, and lung parenchymal strips were prepared as described earlier (36). Strips were placed in oxygenated Krebs-Henseleit (KH) physiological solution (concentration in mM: 118.2 NaCl, 25 NaHCO3, 4.6 KCl, 1.2 KH2PO4, 1.2 MgSO4, 2.5 CaCl2 and 10 dextrose, pH 7.4). Lung parenchymal strips of 1-mm thickness were obtained by sectioning the fresh left lung of each animal in a parasagittal plane using a vibratome (Vibratome 1000 Plus Sectioning System). The lung parenchymal strips were transferred to tissue baths (Radnoti Glass Technology, Monrovia, CA) containing 15 ml KH solution and continuously oxygenated with a gas mixture (95% O2-5% CO2) at 37°C. It was not possible to process all lung parenchymal strips on the same day; therefore, some animals were killed after being returned to room air for 24 h, such that exposure to hyperoxia was always of 7 days duration. No differences were observed in the contraction or relaxation of lung parenchymal strips of animals that were killed either immediately or 1 day after cessation of high O2 exposure.

Physiological studies. One end of each lung parenchymal strip was anchored at the bottom of the tissue bath, and the other end was connected to a force displacement transducer (FT-03C; Grass Instruments, Quincy, MA) as we have described previously (36). An initial load of 0.2 g was used. The suspended lung parenchymal strips were rinsed every 20 min with KH solution and allowed to equilibrate for 60 min to establish a steady baseline. Lung parenchymal strip tension was measured by PowerLab/4SP (ADInstruments) and recorded using Chart 5.0 software. Electrical field stimulation (EFS) was applied through platinum electrodes to these lung parenchymal strips at various voltages [2–20 V alternating current (AC) at 60 Hz] for 10 s at 2-min intervals to induce contraction. The contraction of lung parenchymal strips was determined by measuring of the amplitude from baseline to the peak, and this force was expressed in milligrams. To demonstrate that the increase in contraction was due to BDNF, in a separate set of experiments, lung strips from animals breathing room air (n = 6) were incubated with exogenous BDNF (25 ng/ml; Sigma, St. Louis, MO) for 60 min, and contraction in response to EFS was measured. In another series of experiments, Trk receptors were blocked by incubating the strips from room air breathing animals (n = 6) with K-252a (1 μM) for 30 min before incubation with BDNF (25 ng/ml for 60 min). As K-252a has some cross-reactivity with other Trk receptors, and no specific TrkB receptor blocker is available, we also used a TrkB-specific molecule (TrkB-Fc chimeric protein, 1.5 μg/ml for 30 min; Sigma) in place of K-252a (n = 4) to sequester BDNF, and contraction was recorded in response to EFS.

Relaxation responses of lung parenchymal strips were performed on tissues from additional animals. A cumulative concentration-response curve was made to find a concentration of bethanechol (Sigma) that elicited 50–75% of maximal response in lung parenchymal strips. A concentration of 10–4 M bethanechol was found to be the optimal to elicit 50–75% of a maximal response. EFS were applied to these preconstricted lung parenchymal strips at various voltages (5–50 V AC at 60 Hz) for 10 s at 2-min intervals to induce relaxation. The relaxation of strips after EFS was expressed as a percentage of preconstricted state for each strip as previously described by us (36), and compared between animals treated with vehicle or K-252a.

cAMP and cGMP assay in lung parenchymal strips. cAMP and cGMP were measured in acetylated samples through a competitive enzyme immunoassay (EIA) method using a cAMP or cGMP EIA kit as per the manufacturer’s instructions (Cayman Chemicals, Ann Arbor, MI). The detection limit of the assay was 0.1 pmol/ml. Lung parenchymal strips incubated in KH buffer but not EFS-stimulated,
from room air or hyperoxic rat pups either injected intraperitoneally with K-252a daily or vehicle, were obtained, stabilized, and snap-frozen (n = 10 per group). Frozen lung parenchymal strips were thawed in chilled 5% TCA, homogenized (10% wt/vol), and centrifuged, and supernatant was collected. TCA was extracted using water-saturated ether, and residual ether was removed by heating samples at 70°C for 10 min. Samples and standards were acetylated by adding 4 M KOH (1:5) and acetic anhydride (0.25:5) followed by vortexing. Samples were then diluted to 1:25 and 1:50 with EIA buffer. Fifty microliters from samples or standards per well were added to ELISA plates ( precoated with mouse monoclonal anti-rabbit antibodies and blocked with blocking agent) along with cAMP- or cGMP-acetylcholinesterase conjugate and cAMP- or cGMP-specific rabbit antibodies for 18-h incubation at 4°C. After incubation, the ELISA plate was washed with washing buffer and then developed with Ellman’s reagent for 90 min. The product of this enzymatic reaction was read at 405-nm wavelength. The concentrations of cAMP or cGMP were expressed in picomoles per gram tissue (wet weight).

Statistical analysis. The results of our experiments are expressed as means ± SE. Statistical significance was determined by two-way ANOVA with repeated measurements to determine the effect of EFS on contraction and/or relaxation responses between groups. To analyze differences between individual voltages, post hoc comparison via Tukey-Kramer multiple comparisons test was used. ACh, cAMP, and cGMP contents were compared between groups using Student’s t-test. In all cases, P < 0.05 was considered statistically significant.

RESULTS

Effect of K-252a administration on ACh production in lung. ACh levels measured by HPLC were significantly greater in lungs from rat pups exposed to hyperoxia than from animals breathing room air (41 ± 5 vs. 23 ± 2 pmol/g tissue, n = 13 per group; P < 0.01). TrkB receptor blockade by K-252a (50 μg/kg ip daily for 7 days) reduced the ACh levels in hyperoxia-exposed lungs (hyperoxia vs. hyperoxia + K-252a: 41 ± 5 vs. 20 ± 2 pmol/g tissue; P < 0.01) but had no effect on ACh levels in room air pups (room air vs. room air + K-252a: 23 ± 2 vs. 22 ± 2 pmol/g tissue, Fig. 1).

Effect of TrkB receptor blockade on hyperoxia-induced contractile responses of lung parenchymal strips. Hyperoxia (n = 10) overall increased the contractile responses significantly compared with room air (n = 10; P < 0.001). As shown in Fig. 2A, the responses in hyperoxia-exposed pups were significantly greater than from room air pups at 6–20 V.

To determine whether BDNF-TrkB signaling is involved in hyperoxia-induced hyperreactivity, when K-252a was administered daily, the hyperoxia-induced increase in contractile responses of lung parenchymal strips in response to EFS remained at room air levels. In hyperoxic animals treated with K-252a (n = 10), the contractile responses were significantly diminished at ≥8 V compared with hyperoxic control animals (n = 10; P < 0.001; Fig. 2B). There was no effect of K-252a on contractile responses of lung parenchymal strips obtained from animals exposed to room air (n = 10) compared with room air controls (n = 10, Fig. 2C).

Effect of TrkB receptor blockade on EFS-induced relaxation of preconstricted lung parenchymal strips. Consistent with our previous observation, hyperoxia significantly reduced the EFS-induced relaxation responses of preconstricted lung parenchymal strips (n = 10) compared with lung parenchymal strips obtained from animals exposed to room air (P < 0.001; n = 10). The differences in relaxation responses were significantly greater at ≥30 V compared with animals exposed to room air (Fig. 3A). K-252a treatment to hyperoxic animals restored the relaxation responses to the room air level. In hyperoxic animals treated with K-252a (n = 10), the relaxation was overall significantly greater compared with hyperoxic controls (P < 0.001), and these differences were significant at ≥30 V (Fig. 3B). Administration of K-252a (n = 10) did not produce any changes in relaxation responses of lung strips of room air-exposed animals (n = 10, Fig. 3C).

Effect of exogenous BDNF and blockade of BDNF-TrkB signaling in vitro on contraction of lung parenchymal strips. To demonstrate that BDNF played a key role in the increased contraction, the lung strips from room air-exposed animals were incubated with exogenous BDNF. As expected, the overall contractile responses of lung strips incubated with 25 ng/ml BDNF were significantly higher (n = 6; P < 0.01) compared with the controls. As shown in Fig. 4, the contractile responses of lung strip treated with exogenous BDNF were significantly greater than controls at 8–20 V. When the strips were first incubated with K-252a to block BDNF-TrkB signaling, the addition of BDNF did not increase the contraction (n = 6; P < 0.001), rather the values dropped below the control values. These differences were significant at ≥10 V compared with BDNF alone vs. K-252a + BDNF (Fig. 4). Similarly, when BDNF-TrkB signaling was blocked by sequestering BDNF by a prior incubation of strips with TrkB-Fc chimeric protein, the addition of BDNF did not increase the contraction, rather the values again dropped below the control values (n = 4; P < 0.001). These differences were significant at ≥10 V compared with BDNF alone vs. TrkB-Fc + BDNF.

Effect of TrkB receptor blockade on cAMP and cGMP production in lung parenchymal strips. We sought to determine whether upregulation of BDNF-TrkB signaling during hyperoxia might decrease cAMP or cGMP content. In lung strips from room air pups, the concentration of cGMP was significantly higher (29.3 ± 6.7 pmol/g, n = 10) compared with the hyperoxic pups (8.3 ± 0.9 pmol/g; P < 0.01; n = 10, Fig. 5A). When K-252a was administered daily along with the hyperoxic exposure, the cGMP level was not changed (9.2 ± 1.26 vs. 8.3 ± 0.9 pmol/g; hyperoxic + K-252a vs. ACh production in lungs. ACh production was higher (P < 0.01) in hyperoxia (H, n = 13)-exposed lungs compared with room air (RA; n = 13) controls. This effect was reversed by the administration of tyrosine kinase inhibitor K-252a (K; 50 μg·kg⁻¹·day⁻¹ ip for 7 days) in hyperoxic animals (H + K; n = 13). K did not have any effect on RA animals (RA + K; n = 13).

Fig. 1. Effect of hyperoxia and tyrosine kinase B (TrkB) receptor blockade on ACh production in lungs. ACh production was higher (P < 0.01) in hyperoxia (H; n = 13)-exposed lungs compared with room air (RA; n = 13) controls. This effect was reversed by the administration of tyrosine kinase inhibitor K-252a (K; 50 μg·kg⁻¹·day⁻¹ ip for 7 days) in hyperoxic animals (H + K; n = 13). K did not have any effect on RA animals (RA + K; n = 13).
hyperoxic, \( n = 10 \) each group). No change was also observed when K-252a was administered in room air-exposed pups \((31.8 \pm 3.6 \, \text{pmol/g}, \, n = 9 \) vs. \(29.3 \pm 6.7 \, \text{pmol/g}; \) room air + K-252a vs. room air, \( n = 10 \), Fig. 5A).

Similarly, the concentration of cAMP was significantly higher in the lungs of room air-exposed pups \((484.8 \pm 26.5 \, \text{pmol/g}, \, n = 9)\) compared with the hyperoxic pups \((330.0 \pm 52.3 \, \text{pmol/g}; \, P < 0.05; \, n = 9)\). When K-252a was administered daily along with the hyperoxic exposure, the cAMP level recovered to room air levels \((460.6 \pm 19.1 \, \text{vs.} \, 484.8 \pm 26.5 \, \text{pmol/g}; \) hyperoxic + K-252a, \( n = 8 \) vs. room air, \( n = 9 \). There was no significant change in cAMP level in room air animals treated with K-252a; \( n = 8 \) (Fig. 5B).

**DISCUSSION**

Neurotrophins comprise a family of polypeptide growth factors including NGF, BDNF, NT-3, and NT-4/5. They function through their corresponding high-affinity Trk receptor subtypes as well as a common low-affinity receptor, p75NTR (13, 33). We (44) have demonstrated that airway preganglionic neurons in the brainstem express BDNF mRNA and protein, suggesting a role for BDNF in airway neural control. Neurotrophins and their Trk receptors are also expressed in nonneuronal tissues such as pulmonary structures (28, 34). Previously, we (42) have shown that hyperoxic exposure to neonatal rats increased the mRNA and protein of BDNF as well as TrkB receptors in lungs. We have now demonstrated a role for BDNF-TrkB signaling in modulation of contractile function of peripheral airways exposed to hyperoxia in early life.

Neurotrophins, including BDNF, are known to have profound effects on development, differentiation, synapse formation and stabilization, structural and functional neural plasticity, as well as maintenance and repair of the nervous system in vertebrates by regulating signaling pathways (14, 19, 26, 29, 33, 38, 46). Additionally, findings obtained through an array of techniques in normal and transgenic animals provide insight into the modulatory mechanisms of BDNF at central synapses (5, 7, 12). BDNF signaling evokes both short- and long-term periods of enhanced synaptic transmission, acting pre- and postsynaptically (38). BDNF-producing neurons respond to stimulation with increased synthesis and elevated release of BDNF (1, 2, 38, 40), which, in turn, enhances quantal release of neurotransmitters (38) such as glutamate. The elevated levels of BDNF expression and activity of glutamatergic receptors (21, 27) facilitate glutamatergic synaptic transmission (12, 25) and unmask silent synapses (20). With this background, we sought to explore whether BDNF-TrkB signaling may contribute to enhanced cholinergic responsiveness of intrapulmonary airway smooth muscle after the hyperoxic exposure that we (42) have shown to increase BDNF content at that site. This line of investigation is consistent with recent data that incubation of human airway smooth muscle cells with BDNF increased responses of \([\text{Ca}^{2+}]_i \) and incubation of human bronchial strips increased force responses to ACh, histamine, caffeine, and bradykinin. Blockade of TrkB receptors by administering K-252a decreased \([\text{Ca}^{2+}]_i \) responses to ACh and caffeine (32).

We observed that ACh content in the lungs of intact animals was increased in response to hyperoxic exposure. This increase in ACh content was accompanied by an increase in contractile...
responses of in vitro preparation of lung parenchymal strips. In intact animals, BDNF-TrkB signaling may participate in a centrally induced increase in cholinergic outflow to the airways by enhancing ACh production under conditions of hyperoxic exposure. BDNF release may cause rapid excitation of neurons via activation of high-affinity TrkB receptors, producing postsynaptic long-term potentiation. In addition, BDNF co-released with ACh at the postganglionic site may cause a long-lasting enhancement of excitatory synaptic transmission and increased cholinergic outflow to airway bronchomotor units (44). The influence of BDNF from central sources can be excluded from contributing to the physiological responses observed in our in vitro preparation of lung strips as they do not have central connections. However, in intact animals, these central influences cannot be ignored, as BDNF can be anterogradely transported via axonal terminals of airway-related preganglionic neurons and thus provide an additional influence on airway hyperreactivity. Enhanced contractility of lung parenchymal strips in vitro in response to hyperoxia may be due to increased BDNF that increases 

responses of in vitro preparation of lung parenchymal strips. In intact animals, BDNF-TrkB signaling may participate in a centrally induced increase in cholinergic outflow to the airways by enhancing ACh production under conditions of hyperoxic exposure. BDNF release may cause rapid excitation of neurons via activation of high-affinity TrkB receptors, producing postsynaptic long-term potentiation. In addition, BDNF co-released with ACh at the postganglionic site may cause a long-lasting enhancement of excitatory synaptic transmission and increased cholinergic outflow to airway bronchomotor units (44). The influence of BDNF from central sources can be excluded from contributing to the physiological responses observed in our in vitro preparation of lung strips as they do not have central connections. However, in intact animals, these central influences cannot be ignored, as BDNF can be anterogradely transported via axonal terminals of airway-related preganglionic neurons and thus provide an additional influence on airway hyperreactivity. Enhanced contractility of lung parenchymal strips in vitro in response to hyperoxia may be due to increased BDNF that increases [Ca\(^{2+}\)]. (32).

Increased contractile responses of lung strips occurred in room air-exposed animals with the addition of exogenous BDNF under in vitro conditions. When BDNF-TrkB signaling was blocked by K-252a, addition of exogenous BDNF did not increase the contraction in response to EFS. Similarly, when BDNF was sequestered using TrkB-Fc chimeric protein, addition of exogenous BDNF also did not increase the contraction of strips. Use of TrkB-Fc chimeric protein to sequester BDNF gave comparable findings to TrkB receptor blockade with K-252a supporting our use of this blocker to inhibit BDNF effects on lung contractility. Interestingly, by the addition of K-252a or
TrkB-Fc chimeric protein, the contractile responses to EFS dropped below the control values. The most probable explanation of this finding is that these agents also blocked the responses of endogenous BDNF. We did not observe such an effect of K-252a administration under in vivo conditions (Fig. 2C). This may reflect differences in delivery of the blocker to the lung tissue or biological compensatory mechanisms operating in vivo vs. in vitro. These experiments provide strong evidence that BDNF contributes to the increase in contractility of lung strips from hyperoxic rat pups.

We acknowledge that K-252a is not a specific blocker for only TrkB receptors and can also inhibit other Trk receptors. Furthermore, in glia cells, BDNF-mediated activation of truncated T1 isoform of TrkB that is not inhibited by K-252a has been reported to control the release of Ca^{2+} from intracellular stores through G protein and inositol trisphosphate-dependent pathway (35). Thus other Trk receptors, truncated TrkB receptors, and pathways downstream to BDNF may also contribute to the contraction of strips under in vivo condition. Our previous data indicate that airway smooth muscle cells are the major source for the increased production of BDNF in response to hyperoxia in our model (42). Furthermore, we observed that exposure to hyperoxia not only induced increased contractile responses, but also impaired relaxant responses in rat pup lungs. Both of these physiological functions are important for homeostatic regulation of airway patency during postnatal maturation. Increased airway contractility and impaired relaxation may possibly limit the exposure of distal alveolar units to connective tissue and interstitial cells with varying contractile properties (15). We speculate that changes we have observed in strips are therefore reflective of changes in airway smooth muscle structures.

In our earlier study, we (36) observed that hyperoxia down-regulates NO-cGMP signaling. Therefore, we expected that BDNF might inhibit the NO-cGMP pathway in response to hyperoxic exposure. However, we observed that blockade of BDNF using TrkB receptor inhibitors restored the contractile and relaxant responses to room air levels without affecting cGMP. This indicates that BDNF acts to impair relaxation through a NO-cGMP-independent pathway or on molecules downstream to cGMP. Consistent with our findings, Bonthius et al. (8) have also demonstrated that BDNF does not use a nitric oxide pathway to signal neurotrophic and neuroprotective effects against alcohol toxicity in cerebellar granule cell cultures. Canossa et al. (11) have shown that, instead of downregulation of the NO pathway by BDNF, NO itself downregulates BDNF secretion in cultured hippocampal neurons (11). Therefore, the observed hyperoxia-induced downregulation of NO signaling may serve to increase BDNF levels in lungs.

Mhanna et al. (31) have shown that hyperoxia decreased relaxation of tracheal smooth muscle of rat pups via impairment of the cAMP-prostaglandin pathway. This is consistent with our current observation that hyperoxic exposure reduced cAMP levels in lung parenchymal strips. In the current study, blockade of TrkB receptors restored cAMP levels in hyperoxic animals to room air levels, suggesting that BDNF may inhibit the cAMP pathway so contributing to decreased relaxation and increased contraction of lung strips. BDNF may inhibit the cAMP/PKA pathway, leading to decreased relaxation and increased contraction of lung strips, as cAMP is known to activate PKA, which phosphorylates several membrane and/or intracellular proteins promoting airway smooth muscle relaxation and bronchodilation of contracted airways (24, 37).

We (42) have used the lung parenchymal strip model to characterize the role of BDNF in increased contraction and impaired relaxation of peripheral airway smooth muscle tissue, in which we observed BDNF overexpression after hyperoxic exposure. In vitro study of lung parenchymal strips has several advantages over in vivo physiological study. These include the ability to characterize the behavior of distal peripheral airways under carefully controlled conditions in the absence of circulating elements. The proportion of rat parenchymal strip occupied by alveolar wall, blood vessel, and airway has been proposed to be qualitatively similar to corresponding areas in human lung strips, although there will clearly be differences between human and rat tissue (6). Nevertheless, using lung parenchymal strips has some potential limitations. In particular, parenchymal strips contain various anatomic constituents including alveolar, bronchial, and blood vessel walls as well as connective tissue and interstitial cells with varying contractile properties. However, rodent parenchymal strips have been shown to provide a useful measure of actual tissue mechanical properties (15). We speculate that changes we have observed in strips are therefore reflective of changes in airway smooth muscle structures.
Peripheral sources of BDNF such as from immune cells may also contribute to hyperreactivity of lungs due to hyperoxic exposure and thus link airway inflammation and airway hyperreactivity. High levels of BDNF have been detected in the cells from bronchoalveolar lavage fluid of ovalbumin-sensitized and aerosol-challenged mice (9). However, Braun et al. (9) have shown that BDNF has a direct effect on airway smooth muscle cells rather than an indirect effect via inflammatory cells. In addition to inflammatory cells, high levels of BDNF in hyperoxic lungs could also be linked to oxygen free radicals that activate redox-sensitive transcriptional factors (i.e., c-Fos, c-Jun, Egr-1, and NF-κB; Ref. 30). It has been shown that immediate-early genes such as c-Fos regulate expression of BDNF, both in vivo and in vitro (45). Therefore, overexpression of BDNF and contractility as a consequence may be a protective mechanism against hyperoxia-induced cytotoxicity (41).

In summary, we have implicated BDNF-TrkB signaling in increased airway hyperreactivity as a consequence of neonatal hyperoxic exposure. The BDNF pathway contributed to increased ACh production in the hyperoxia-exposed lung, enhanced contractility of lung parenchymal strips, and impaired relaxation via cAMP signaling. Future studies are needed to characterize the basic cellular and molecular mechanisms whereby BDNF modulates these responses. Pharmacological modulation of BDNF-TrkB receptor signaling may serve as a potentially useful line of investigation in treatment of airway hyperreactivity elicited by pathophysiological states such as neonatal hyperoxic exposure.

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