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Abstract

Aim: We have recently reported that hypoxia stimulates transcription of the TrkB neurotrophin receptor in cultured cells via stabilization of hypoxia-inducible factor-1α (HIF-1α). Here we investigated whether the expression of TrkB and other neurotrophin receptors is oxygen-sensitive also *in vivo,* and explored the functional consequences of an oxygen-regulated TrkB expression.

Methods: Rats were exposed either to 21% O_2 or 8% O_2 for 6 h and TrkB was analysed by reverse transcription real-time PCR, *in situ* mRNA hybridization, and immunological techniques. The importance of the brain derived neurotrophic factor (BDNF)-TrkB pathway in the control of mechanical airway function was assessed on isolated tracheal segments from normoxic and hypoxic rats.

Results: *TrkB* transcripts were increased approx. 15-fold in the lungs of hypoxic rats, and the respiratory epithelium was identified as the site of enhanced TrkB expression in hypoxia. The TrkB ligand, BDNF, significantly increased the contractile response to acetylcholine (Ach) of isolated tracheal segments from hypoxic but not from normoxic rats. This effect of BDNF was prevented by pre-incubation of the tissue specimens with the tyrosine kinase inhibitor K252a and by mechanical removal of the TrkB containing airway epithelium. Likewise, the nitric oxide (NO) synthase inhibitor L-NAME abrogated the influence of BDNF on ACh-induced contractions of isolated tracheal segments from hypoxic rats.

Conclusion: These results demonstrate that systemic hypoxia stimulates expression of the TrkB neurotrophin receptor in the airway epithelium. Furthermore, activation of TrkB signalling by BDNF in hypoxia enhances mechanical airway contractility to ACh through a mechanism that requires NO.

Keywords: acetylcholine – airway contractility – brain-derived neurotrophic factor – hypoxia – *in situ* mRNA hybridization – neurotrophin receptors

Introduction

Neurotrophins (NTs) constitute a family of vertebrate-specific nerve growth factors (NGFs), which are best known for their role in the development and function of the nervous system [reviewed in Huang & Reichardt 2001]. NTs act via a dual receptor system comprising three high-affinity tyrosine kinase receptors, TrkA, TrkB, and TrkC, and the less selective, low-affinity p75NTR receptor. Among the different NTs, NGF preferentially binds to TrkA [Klein *et al.* 1991], brain-derived neurotrophic factor (BDNF) and NT-4/5 interact with TrkB [Klein *et al.* 1992], and NT-3 exhibits a high affinity for TrkC [Lamballe *et al.* 1991].

Recent findings indicate that NTs fulfil important functions also outside the nervous system. In this regard, it has been shown that NGFs and their receptors are expressed in airway and lung tissue [reviewed in Freund-Michel & Frossard 2008]. Trk receptors were detected in neurones, airway smooth muscle and immune cells of the respiratory system [Nassenstein *et al.* 2006]. NTs are produced by local inflammatory cells [Braun *et al.* 1998], airway epithelial cells [Fox *et al.* 2001, Pons *et al.* 2001], and airway smooth muscle cells [Freund *et al.* 2002, Kemi *et al.* 2006]. Growing evidence suggests that BDNF, acting through the TrkB receptor, can aggravate respiratory disorders associated with an inflammatory reaction, i.e. allergic asthma [Braun *et al.* 2004]. NTs are considered to modulate the airway response to allergic stimuli directly by promoting the survival and activation of eosinophils [Nassenstein *et al.* 2004] as well as indirectly by stimulating the synthesis of pro-inflammatory peptides in sensory neurones [Goedert *et al.* 1981]. Important insights into the regulation of airway reactivity can therefore be gained by a detailed analysis of the mechanisms that control the expression of NTs and their receptors in the respiratory system.

We reported recently that hypoxia-inducible factor-1 (HIF-1) stimulates TrkB expression in cultured neuroblastoma cells and non-neuronal cell lines at low oxygen tension [Martens *et al.* 2007]. Notably, HIF-1 and its molecular downstream target, vascular endothelial growth factor (VEGF), were detected in the airways of asthmatic patients [Lee *et al.* 2006] suggesting that oxygen-dependent

transcriptional regulation plays a role in the pathophysiology of this disease. In fact, enhanced airway contractility has been reported in hypoxia [Belouchi *et al.* 1999] and bronchial asthma [Fayon *et al.* 2006], but the underlying mechanisms are not thoroughly understood.

In view of the above our study served a twofold purpose. First, we aimed at investigating whether hypoxia stimulates expression of TrkB and other neurotrophin receptors also *in vivo*. And if so, we sought to explore the functional consequences of increased TrkB at low oxygen tension. We found that TrkB was indeed enhanced in the airway epithelium of hypoxic rats. Furthermore, the TrkB ligand, BDNF, increased the maximal tension of isolated tracheal segments from hypoxic rats in response to ACh. This effect of BDNF was dependent on an intact airway epithelium and the availability of nitric oxide (NO).

Materials and methods

Exposure of rats to systemic hypoxia

The studies were approved by the Local Animal Care Committee (permit no. G 0189/07) and performed in compliance with the „European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes" (Council of Europe No 123, Strasbourg 1985). Pathogen-free male Wistar rats, weighing between 250 and 350 g, were exposed in an airtight animal cage either to atmospheric oxygen (21% O_2) or normobaric hypoxia (8% O_2, 6 h). Thereafter, the animals were anaesthetized by intraperitoneal injection of urethane (1.2 g kg^{-1} body weight, Sigma, Deisenhofen, Germany), killed by exsanguination, and their tracheas quickly removed. Organs were excised and snap-frozen in nitrogen-cooled methyl butane. The lungs were instilled with 8 mL of a 1:1 mixture (in PBS) of OCT compound (Sakura Finetek, Heppenheim, Germany) and processed for cryosectioning.

Reverse transcription real-time PCR

RNA preparation and first-strand cDNA synthesis were performed as described in detail previously [Martens *et al.* 2007, Wagner *et al.* 2005]. For *BDNF* mRNA measurements, the total RNA was treated with DNaseI (Promega, Mannheim, Germany) for 30 min at 37 °C prior to reverse transcription (RT). Incubation with DNAseI was performed to avoid contamination with genomic DNA as intron-spanning primers could not be used for the amplification of *BDNF* transcripts. In addition to DNAseI treatment, PCR was performed with RNA samples that had been incubated in the absence of reverse transcriptase to test for genomic DNA contamination. Quantitative real-time PCR using SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) was carried out in a GeneAmp5700 thermocycler (Perkin Elmer, Waltham, MA, USA) with the primers listed in Table 1. Differences in transcript levels between samples from normoxic (21% O_2) and hypoxic (8% O_2) rat organs were calculated on the basis of differences in the threshold cycle (Ct) values. The Ct values for the genes of interest were subtracted by the Ct values for *β-actin* to obtain ΔCt values. Differences in mRNA between normoxic and hypoxic rats were calculated as ΔCt(normoxia) minus ΔCt(hypoxia). Assuming DNA doubling with each amplification cycle of the PCR, the x-fold difference between two samples is given by the equation $2^{ΔΔCt}$. Notably, *β-actin* mRNA levels were not significantly different between 21% O_2 and 8% O_2.

SDS-PAGE and immunoblotting

SDS-PAGE was performed with membrane fractions of snap-frozen tissues as described previously [Martens *et al.* 2007, Kirschner *et al.* 2008]. The following primary antibodies were used: mouse monoclonal anti-TrkB antibody (dilution 1:250, cat.# 610101, BD Transduction Laboratories, Heidelberg, Germany) and mouse monoclonal antibody against β-actin (dilution 1:1000, cat.# MAB1501R, Chemicon, Hofheim, Germany). The anti-β-actin antibody was applied to detect differences in protein loading after membrane stripping.

In situ mRNA hybridization

Constructs for the *in vitro* transcription of full-length (*TrkB-FL*) and truncated (*TrkB-T1*) mRNA probes were generated by PCR using rat brain cDNA as a template in combination with the following primers: *rat TrkB-FL* (NCBI sequence no. NM012731.1): 5′-ATAGGATCCCACGGATGTTGCTGACCAA-3′ (forward), 5′-TGCAAGCTTGACAGGGATCTTGGTCATC-3′ (reverse); *rat TrkB-T1* (NCBI accession no. AY265419.1): 5′-ATAGGATCCTGGAGGAAGGGAAGTCTG-3′ (forward), 5′-TGCAAGCTTCAGTGGTGGTCTGAGGTTG-3′ (reverse). Amplified DNAs were cloned into the pSPT18 plasmid (Roche, Penzberg, Germany). *In vitro* transcription of digoxigenin-labelled antisense and sense RNA probes was accomplished with the digoxigenin RNA labelling kit (Roche). *In situ* mRNA hybridization was performed on 6-µm cryostat sections using a modification of our previous protocol [Kirschner *et al.* 2008]. In brief, 4% paraformaldehyde-fixed tissue sections were hybridized in 50% formamide, 5x SSC, 5x Denhardt's solution (Sigma), 100 µg ml^{-1} herring sperm DNA (Promega), 200 µg ml^{-1} yeast transfer RNA (Sigma) for 16 h at 68 °C. After several washing steps and a subsequent blocking reaction, digoxigenin was detected with alkaline phosphatase-conjugated anti-digoxigenin antibody according to the supplier's instructions (Roche). Cryosections from rat brain (*dentate gyrus*), in which we observed the characteristic expression pattern of *TrkB-FL* and *TrkB-T1,* served as positive control. Negative controls were carried out by hybridization of the tissue sections with the corresponding sense RNA probes. A total of 18 tissue sections from three normoxic and three hypoxic rats were examined.

Immunohistochemistry

Immunolabelling was performed on 4-µm paraffin sections cut from 3% paraformaldehyde-fixed lung tissue. Five-µm cryosections from rat trachea were used for haematoxylin-eosin (HE) staining. The lung sections were deparaffinized and demasked for 6 min in a pressure cooker. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol. After washing with PBS,

the sections were blocked with 5% fat-free milk (Sigma) in PBS for 30 min at room temperature. Immunohistochemistry was performed with the following antibodies (in PBS, 5% non-fat milk): rabbit polyclonal anti-TrkB antibody (dilution 1:100, cat.# ab6180, Lot# 219285, Abcam, Cambridge, UK), 1 h at room temperature and polyclonal alkaline phosphatase-conjugated goat anti-rabbit IgG (dilution 1:200, cat.# A9919, Sigma) as secondary antibody. Incubation with rabbit isotype IgGs was carried out as a negative control. Reactions were visualized with NBT/BCIP substrate (Roche). Washed and mounted slides were viewed under a light microscope (Axioplan 2, Zeiss, Jena, Germany) connected to a digital camera (Spot RT Slider, Diagnostic Instruments, Sterling Heights, MI, USA) and recorded using the Metamorph V4.1.2 software (Visitron Systems GmbH, Puchheim, Germany).

Isometric force measurements on isolated tracheal segments

Isolated rat tracheas were cut into segments including two cartilaginous rings each. The specimens were placed between two stainless steel hooks in a 5 mL water-jacketed organ chamber (Danish Myo Technology, Aarhus, Denmark). Contraction measurements were performed in PSS Mulvany's solution (pH 7.4, 37 °C) consisting of 119 mmol L^{-1} NaCl, 4.7 mmol L^{-1} KCl, 2.5 mmol L^{-1} $CaCl_2$, 1.17 mmol L^{-1} $MgSO_4$, 25 mmol L^{-1} $NaHCO_3$, 1.18 mmol L^{-1} KH_2PO_4, 0.027 mmol L^{-1} EDTA, and 5.5 mmol L^{-1} glucose, gassed with 95% O_2 / 5% CO_2. Isometrically developed contractile force was measured with a mechano-electric transducer and recorded using the Chart5 software and an ADI Powerlab amplifier (ADInstruments, Sidney, Australia). The resting force was adjusted stepwise to 0.7 g during the 20 min equilibration period. Contraction of the specimens was elicited with ACh (Sigma) which was added to the organ bath at final concentrations increasing incrementally between 10^{-9} and 10^{-3} mol L^{-1}. ACh was applied either alone or after pre-incubation with recombinant human BDNF (50 ng mL^{-1}, Alomone Labs, Jerusalem, Israel). The tyrosine kinase inhibitor K252a (100 nmol L^{-1} final concentration in 0.005% DMSO, Calbiochem/Merck, Darmstadt, Germany) was added 25 min before the administration of BDNF and ACh, respectively. Control experiments were carried out with 0.005% DMSO alone. The NO synthase inhibitor L-nitroarginine methyl ester (L-

NAME; 0.1 mmol L^{-1} final concentration in water, Sigma) was applied 15 min prior to the administration of BDNF and ACh, respectively. Tension of the preparations was calculated as the ratio between the recorded force and the narrowest width of the tracheal segments. In some experiments the airway epithelium was gently scrubbed off with a rat's whisker. Removal of the epithelial cell layer and structural intactness of the subjacent smooth muscle cells were assessed by light microscopy of HE-stained tissue sections.

Statistics

If not otherwise indicated all values are presented as means ± SD. The real-time RT-PCR data were analysed using Student's t-test. The results obtained from isometric force measurements were statistically evaluated using a two-way ANOVA with Bonferroni test as *post-hoc* test in Graph Pad Statistic 4. *P* values less than 0.05 were considered significant.

Results

Neurotrophin receptors in tissues of normoxic and hypoxic rats

To explore whether the expression of different neurotrophin receptors, particularly of the TrkB receptor, is regulated in an oxygen-dependent manner, we exposed rats either to atmospheric oxygen (21% O_2) or normobaric hypoxia (8% O_2, 6 h). This protocol was chosen, because it has previously been proved to reliably induce oxygen-regulated genes in various tissues *in vivo* [Weidemann *et al.* 2008, Willam *et al.* 2006]. Transcript levels of neurotrophin receptors were determined by a quantitative RT-PCR method based on differences in Ct values. The most striking finding of these experiments was a nearly 15-fold increase in *TrkB* mRNA in the lungs of hypoxic *vs.* normoxic rats (*Figure 1a*). TrkB transcripts were not significantly elevated by hypoxia in any other organ examined including kidney, heart, brain, liver and spleen (*Figure 1*). The TrkB ligand BDNF was also detected in lung tissue, and an approx. 2.5-fold increase (*P*<0.01) in *BDNF* mRNA was detected in the lungs

of hypoxic compared to normoxic rats (*Figure 1a*). As intron-spanning primers could not be used for the amplification of *BDNF* transcripts, the PCR was repeated with RNA samples that had been incubated in the absence of reverse transcriptase to test for contamination with genomic DNA. Notably, DNA bands reflecting BDNF were not detected with RNA samples from which reverse transcriptase had been omitted (data not shown). For comparison, we also measured the mRNA content of other neurotrophin receptors in normoxic and hypoxic tissues. The only significant effect found was a reduction in $p75^{NTR}$ transcripts in hypoxic rat lungs (*Figure 1a*).

We then explored whether the failure of most organs to respond to low oxygen tension with changes in TrkB alluded to a tissue-specific gene regulatory mechanism, or whether this was due to confinement of tissue hypoxia to the lungs. For that purpose, mRNA levels of VEGF and glucose transporter (GLUT)-1 were measured as surrogate parameters of hypoxia because both genes are enhanced in numerous tissues upon oxygen depletion. As shown in *Figure 1g*, the mRNAs of at least one of the genes encoding *VEGF* and *GLUT-1* were significantly stimulated at 8% O_2 in any organ, indicating that hypoxia-dependent gene regulation was preserved in all tissues examined.

Several TrkB receptor isoforms with distinct physiological functions exist that are generated by alternative mRNA splicing. We used a monoclonal antibody recognizing both, the 145 kD full-length (TrkB-FL, gp145^{TrkB}) and the 95 kD truncated (TrkB-T1, gp95^{TrkB}) protein, to assess TrkB expression by immunoblotting (*Figure 2a*). The two TrkB isoforms were clearly up-regulated in hypoxic *vs.* normoxic rat lungs (*Figure 2a*). The TrkB-T1 protein (gp95^{TrkB}), which may have a passive role as BDNF scavenger [Dechant 2001] but can also act independently of TrkB-FL in inositol-1,4,5-trisphosphate-dependent calcium signalling [Rose et al. 2003], seemed more enhanced by hypoxia than the TrkB-FL (gp145^{TrkB}) isoform (*Figure 2a*). Real-time RT-PCR was performed to measure the different *TrkB* mRNA splice variants in lung tissue from normoxic and hypoxic animals. Notably, exposure of rats to 8% O_2 increased both *TrkB* transcipts approx. 14-fold, thus leaving their relative ratios (normoxia *vs.* hypoxia) unchanged (*Figure 2b*).

In situ mRNA hybridization was used to identify the cellular sites of TrkB in lung tissue at normoxia and hypoxia. Using specific RNA probes for *TrkB-FL* and *TrkB-T1,* the two transcripts were detected in basal cells of the ciliated bronchial epithelium of normoxic rats (*Figure 3a, b*). Signal intensity in the respiratory epithelium was considerably enhanced upon exposure of rats to 8% O_2 (*Figure 3a, b*) and *TrkB-FL* was observed *de novo* in alveolar epithelial cells (*Figure 3a, high-power magnifications*). Immunohistochemical staining with the use of a polyclonal antibody directed against the gp145^{TrkB} and gp95^{TrkB} proteins confirmed the airway epithelium as the site of TrkB in rat lungs (*Figure 3c*).

Contractility of isolated tracheal segments

Neurotrophins and their receptors modulate airway smooth muscle tone and may contribute to airway hyperreactivity in bronchial asthma [Nassenstein *et al.* 2006, Braun *et al.* 1998, Fox *et al.* 2001, Noga *et al.* 2002]. We therefore reasoned that activation of the BDNF-TrkB signalling pathway in response to hypoxia might impair mechanical airway function. To test this hypothesis, force measurements were performed on isometrically contracting rat tracheal segments, which permitted us to manipulate TrkB receptor activity by pharmacological means. Tissue specimens were freshly prepared from the tracheas of rats that had been kept either at 21% or 8% O_2 for 6 h to stimulate TrkB expression. Characteristic s-shaped dose-response curves of airway tension were obtained by incrementally increasing the final concentration of ACh between 10^{-9} and 10^{-3} mol L^{-1} (*Figure 4a*). Maximal tension was 8.1 ± 0.5 mN mm^{-1} and 6.6 ± 0.6 mN mm^{-1} (n=5, each) in isolated tracheal segments of normoxic and hypoxic rats, respectively (*Figure 4a*). Addition of the TrkB ligand BDNF (50 ng ml^{-1} final concentration) to the bath solution had no effect on tissue preparations from rats at 21% O_2 (*Figure 4b*), but significantly increased maximal tension in tracheal segments of hypoxic rats (*Figure 4c*). Addition of the tyrosine kinase inhibitor K252a (100 nmol L^{-1}) prevented this effect of BDNF (*Figure 4d*).

Since the TrkB neurotrophin receptor is present in airway epithelial cells (*Figure 3*), one would predict that ablation of the epithelium from hypoxic tracheal segments resulted in a failure of BDNF to increase maximal tension in the presence of ACh. We tested this by gently scrubbing off the epithelium from the tracheal segments without damaging the subjacent smooth muscle cell layer (*Figure 5c*). Importantly, the contractile response to ACh was maintained in tracheal segments without epithelium indicating functional preservation of the specimens (*Figure 5a*). However, BDNF failed to enhance ACh-induced contractions in deepithelialized preparations (*Figure 5b*).

Through the release of NO and other factors the airway epithelium has an important role in the regulation of airway contractility [reviewed in Bove & van der Vliet 2006, Jacobs & Zeldin 2001]. To assess the activity of the local NO system in normoxic and hypoxic rat lungs, we measured mRNA levels of the three NO synthase (NOS) isoforms. Transcripts of the inducible *NOS2*, which is a HIF target gene [Melillo *et al.* 1995], were increased approx. 27-fold upon exposure of rats to 8% O_2 (*Figure 6a*). Considering the strong increase of pulmonary *NOS2* mRNA in hypoxia, we explored whether NOS activity was necessary for the effect of BDNF on ACh-induced tracheal contractions. For this purpose, isolated tracheal segments of rats, that had been pre-exposed to 8% O_2 to stimulate TrkB expression, were treated with the NOS inhibitor L-NAME (0.1 mmol L^{-1}) prior to administration of BDNF and/or ACh. While L-NAME did not significantly change ACh-induced contractions in the absence of BDNF, it prevented the increase of maximal tension by BDNF (*Figure 6b*). Application of the NO donor sodium nitroprusside at gradually increasing final concentrations between 10^{-7} and 10^{-3} mol L^{-1} reduced force development in the presence of L-NAME demonstrating preservation of the NO response in our preparations (*Figure 6c*).

Discussion

Prompted by our recent finding that oxygen restriction stimulates transcription of the TrkB neurotrophin receptor in cultured neuroblastoma cells and several non-neuronal cell lines [Martens *et al.* 2007], we set out to investigate whether hypoxia regulates TrkB also *in vivo*. Further on, we aimed at exploring functional consequences that may evolve from an oxygen-stimulated expression of TrkB. Strikingly, TrkB mRNA and protein levels were increased in the lungs of hypoxic *vs*. normoxic rats, but in no other organs examined (*Figures 1, 2*). On the other hand, exposure of rats to normobaric hypoxia increased the mRNA levels of at least one of the genes for *GLUT-1* or *VEGF* indicating that gene regulation by oxygen was maintained in all tissues under investigation (*Figure 1g*). In contrast to our current results, TrkB expression in the cerebral cortex of rats was enhanced by a combination of oxygen restriction and left common carotid artery occlusion [Narumiya *et al.* 1998]. This suggests that the oxygen sensitivity of the machinery that controls TrkB expression varies among tissues and/or that other factors, in addition to oxygen depletion, contribute to the enhancement of TrkB in ischaemic rat brain. Notably, the mRNAs of the full-length TrkB receptor and a truncated splice variant, which lacks the catalytic tyrosine kinase domain, increased in parallel such that the ratio of both transcripts remained approximately the same in hypoxic *vs*. normoxic lung tissue (*Figure 2b*). This observation is conceivable if one considers that both mRNAs are transcribed from the same promoter [Barettino *et al.* 1999] and that splicing of the native *TrkB* mRNA is obviously not affected by hypoxia.

The precise signalling pathways that account for the exquisite oxygen sensitivity of TrkB in the respiratory system remain elusive at present. In this regard, we reported recently that HIF-1 activates transcription of the TrkB gene through interacting with three of several binding motifs in the *TrkB* promoter [Martens *et al.* 2007]. As the HIF-1 protein accumulates in numerous tissues under hypoxia, stimulation of the *TrkB* promoter by HIF-1 alone cannot explain the unique oxygen susceptibility of TrkB expressing cells in the lungs. Instead, one would rather postulate the existence of additional, more tissue-specific co-factors, which act synergistically with HIF-1 controlling TrkB expression.

One candidate could be the Wilms' tumour transcription factor, Wt1, which stimulates the *TrkB* promoter *in vitro* and *in vivo* [Wagner *et al.* 2005] and which is distributed in a tissue-restricted manner including broncho-epithelial cells [Marcet-Palacios *et al.* 2007].

Evidence has been provided that NTs and their receptors are present in different lung compartments and play a role in certain pathophysiological states such as asthma, allergy and lung cancer [Hoyle 2003, Lommatzsch *et al.* 2003, Nockher & Renz 2003, Ricci *et al.* 2004]. Consistent with a previous study [Yao *et al.* 2006], we detected alternatively spliced TrkB transcripts and proteins in the bronchial epithelium of normal adult rats. TrkB in these cells was markedly enhanced upon acute hypoxic exposure (*Figure 3*). Likewise, mRNA content of the TrkB ligand BDNF was significantly increased in rat lungs at 8% O_2 (*Figure 1a*). Earlier investigations showed that contractile airway function is sensitive to changes in tissue oxygenation, but yielded controversial results regarding the effects of cholinergic agonists [Belouchi *et al.* 1999, Clayton *et al.* 1999, Roux *et al.* 2002]. The reasons for these discrepancies are unclear at present but may be related to the complex effects of chronic hypoxia on calcium-contraction coupling in airway smooth muscle cells [Roux *et al.* 2002].

HIF-1α was recently discovered in the airway system of asthmatic patients [Lee *et al.* 2006] where it may accumulate due to the release of cytokines [Hellwig-Burgel *et al.* 1999] and/or impaired oxygenation of the thickened airway mucosa [Li & Wilson 1997]. Interestingly, stimulation of HIF-1α increased the contractile response of cultured trachea strips to ACh suggesting that HIF-1 downstream pathways are associated with airway hyperreactivity [Chachami *et al.* 2007]. Our novel data demonstrate that activation of the BDNF-TrkB signalling pathway upon acute hypoxic treatment of rats sensitized isolated tracheal segments to the action of ACh. This conclusion is based on the following observations: First, the TrkB ligand BDNF significantly increased maximal force development in response to cholinergic stimulation of tracheal segments from hypoxic rats (*Figure 4c*). Second, this effect of BDNF, which did not occur in preparations from normoxic rats (*Figure 4a*), could be prevented with the tyrosine kinase inhibitor K252a (*Figure 4d*). Third, consistent with

the stimulation of TrkB expression in broncho-epithelial cells by hypoxia (*Figure 3*), removal of the airway epithelium from the *in vitro* preparations abrogated the enhancement of ACh-induced tracheal contractions by BDNF (*Figure 5*).

The results of several studies suggest that NTs influence mechanical airway reactivity directly by increasing the intracellular calcium concentration in smooth muscle cells [Prakash *et al.* 2006] and through indirect mechanisms, i.e. by changes in sensory nerve activity [Nassenstein *et al.* 2006, de Vries *et al.* 2006]. Our data identify the airway epithelium as another target of BDNF in the control of airway contractility. Local mediators that are released from bronchial epithelial cells and act on airway smooth muscle tone include NO [reviewed in Bove & van der Vliet 2006], leukotrienes and prostaglandins [reviewed in Jacobs & Zeldin 2001]. Exposure of rats to 8% O_2 significantly increased pulmonary *NOS2* mRNA indicating activation of the NO system at low oxygen tension (*Figure 6a*). Furthermore, the NOS inhibitor L-NAME abolished the enforcement of ACh-induced tracheal contractions by the TrkB ligand, BDNF (*Figure 6b*). Thus, the capacity of BDNF to modulate mechanical airway contractility required NOS activity. It is noteworthy, that BDNF augmented histamine-evoked airway contractions and diminished the level of NO in exhaled air of guinea pigs *in vivo* [Bennedich Kahn *et al.* 2008]. Moreover, BDNF facilitated the contractility of lung parenchymal strips through a NO-cGMP-dependent mechanism [Sopi *et al.* 2007, Sopi *et al.* 2008]. However, in the two latter studies BDNF enhanced lung parenchyma contractility as a consequence of hyperoxia rather than oxygen restriction as seen in our investigation [Sopi *et al.* 2007, Sopi *et al.* 2008]. It is currently unclear, why the BDNF-TrkB pathway can modulate airway contractility in a similar manner under two seemingly opposing conditions (hyperoxia *vs.* hypoxia). In contrast to Sopi *et al.* who used tissue specimens from newborn animals [Sopi *et al.* 2008], we performed our experiments with adult rats. Therefore, it can be speculated whether the susceptibility of the BDNF-TrkB receptor system to oxygen varies with age. In any case, the combined results of our present work and earlier studies suggest that a reduced ability to release NO is one mechanism that accounts for increased airway contractility during elevated BDNF levels. However, such possibility is

seemingly in contrast with the failure of L-NAME to enhance ACh-induced hypoxic airway contractions in the absence of BDNF (*Figure 6b*). In fact, this could point to the possibility that BDNF, rather than lowering NO release from the airway epithelium directly, enhances ACh-dependent tracheal smooth muscle tone in hypoxia through a second, yet unknown mechanism that requires NO.

In conclusion, our findings suggest that enhanced expression of the TrkB receptor in hypoxia contributes to increased airway contractility. While our data support a close relationship between BDNF and NO in the regulation of airway contractility, additional pathways for the action of BDNF likely exist that warrant further investigations. These results qualify the BDNF-TrkB signalling system as a potential drug target in conditions of airway hyperreactivity.

Acknowledgements

Competing interests

The authors declare that they have no competing interests.

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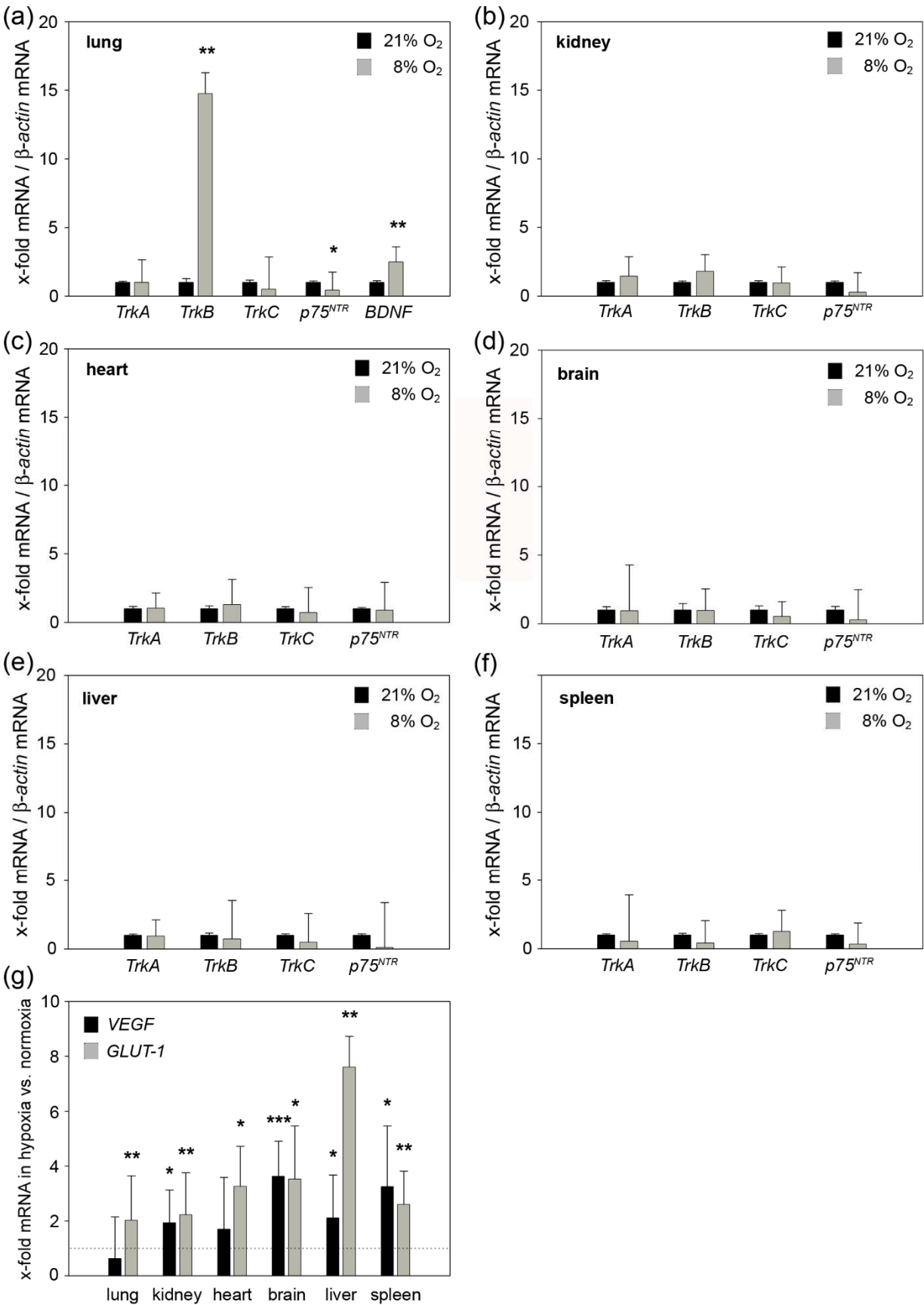
Figures and tables

Figure 1: *Panels (a)-(f):* Messenger RNA levels of neurotrophin receptors in different organs of rats. Animals were exposed for 6 h either to 21% or 8% O_2, and mRNAs measured by real-time RT-PCR. Relative transcript content in normoxic and hypoxic organs were determined on the basis of differences in threshold cycle (Ct) values. Ct values for the genes of interest were subtracted by the Ct values for *β-actin* to obtain ΔCt values. Differences in mRNA levels between normoxic and hypoxic rats were calculated as ΔCt(normoxia) minus ΔCt(hypoxia). The primers used for PCR amplification were not selective for alternatively spliced *TrkB* transcripts. Fold increases in mRNA were obtained using the equation $2^{\Delta\Delta Ct}$. *Panel (g)*: Relative messenger RNA content of vascular endothelial growth factor (*VEGF*) and glucose transporter-1 (*GLUT-1*) in different organs of hypoxic (8% O_2, 6 h) *vs.* normoxic (21% O_2) rats. The dotted line indicates the mRNA level at 21% O_2 (=1). Note, that the transcripts of at least one of the genes encoding VEGF and GLUT-1 were significantly increased by hypoxia in all organs examined. Values are means ± SD of n=5 normoxic and hypoxic animals, each (*$P<0.05$, **$P<0.01$, ***$P<0.001$, Student's t-test).
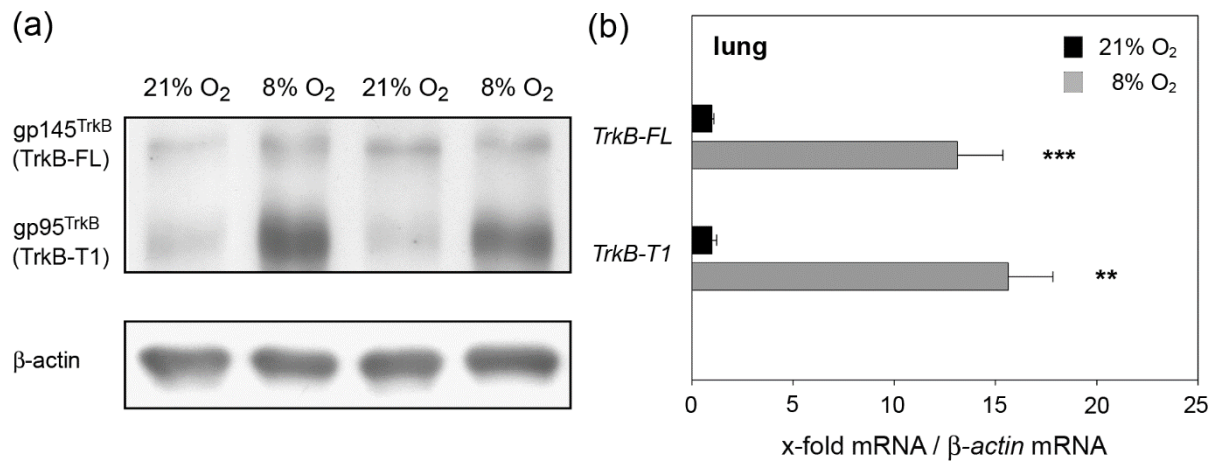
Figure 2: Expression of TrkB in lung tissue of rats exposed either to 21% or 8% O_2. *Panel (a)*: Representative immunoblot carried out on different samples obtained from two normoxic and hypoxic rats, each. The monoclonal antibody recognised both, the 145 kD full-length (gp145[TrkB]) and the 95 kD truncated (gp95[TrkB]) TrkB protein. Equal protein loading is revealed by the similar intensities of the β-actin bands. *Panel (b)*: Messenger RNA of the full-length *TrkB* neurotrophin receptor (*TrkB-FL*) and a truncated splice variant (*TrkB-T1*) which lacks the intracellular tyrosine kinase domain, in lung tissue of normoxic and hypoxic rats. Values are means ± SD of n=5 normoxic and hypoxic animals, each (**$P<0.01$, ***$P<0.001$, Student's t-test).
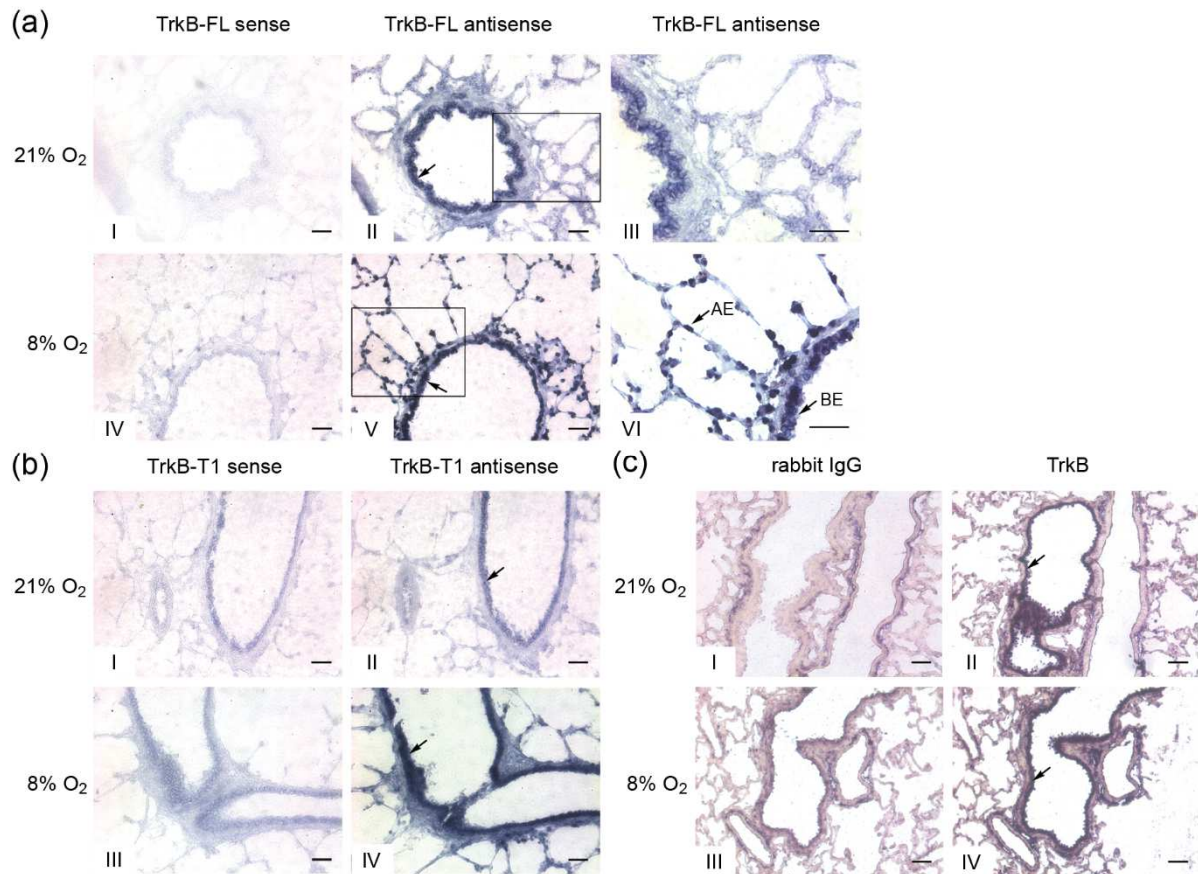
Figure 3: *In situ* mRNA hybridisation of the full-length TrkB neurotrophin receptor (TrkB-FL, *Panel (a)*) and a splice variant, which lacks the intracellular tyrosine kinase domain (TrkB-T1, *Panel (b)*) on lung tissue sections of normoxic (21% O_2) and hypoxic (8% O_2) rats. Detection of the RNA probes was accomplished with an alkaline phosphatase-conjugated anti-digoxigenin antibody. Hybridisation with corresponding sense RNA probes served as negative control. Arrows mark specific hybridisation signals in the bronchial epithelium. Higher magnifications of the boxed areas (II, V) are shown in micrographs III and IV. AE, alveolar epithelial cell. The micrographs are representative for the 18 tissue sections that were analysed from 3 normoxic and hypoxic animals, each. Scale bars indicate 100 µm. Immunostaining of TrkB on lung cryosections of normoxic and hypoxic rats (*Panel (c)*). Experiments were performed with a polyclonal antibody reacting with both, the gp145^{TrkB} and the gp95^{TrkB} proteins. Incubation of the tissue sections with normal rabbit IgG served as negative control. The primary antibody was detected with horseradish peroxidase-conjugated anti-rabbit IgG. Arrows indicate TrkB immunoreactivity of the bronchial epithelium. The micrographs shown are representative for the 18 lung sections that were examined from 3 normoxic and hypoxic animals, each. Scale bars indicate 100 µm.
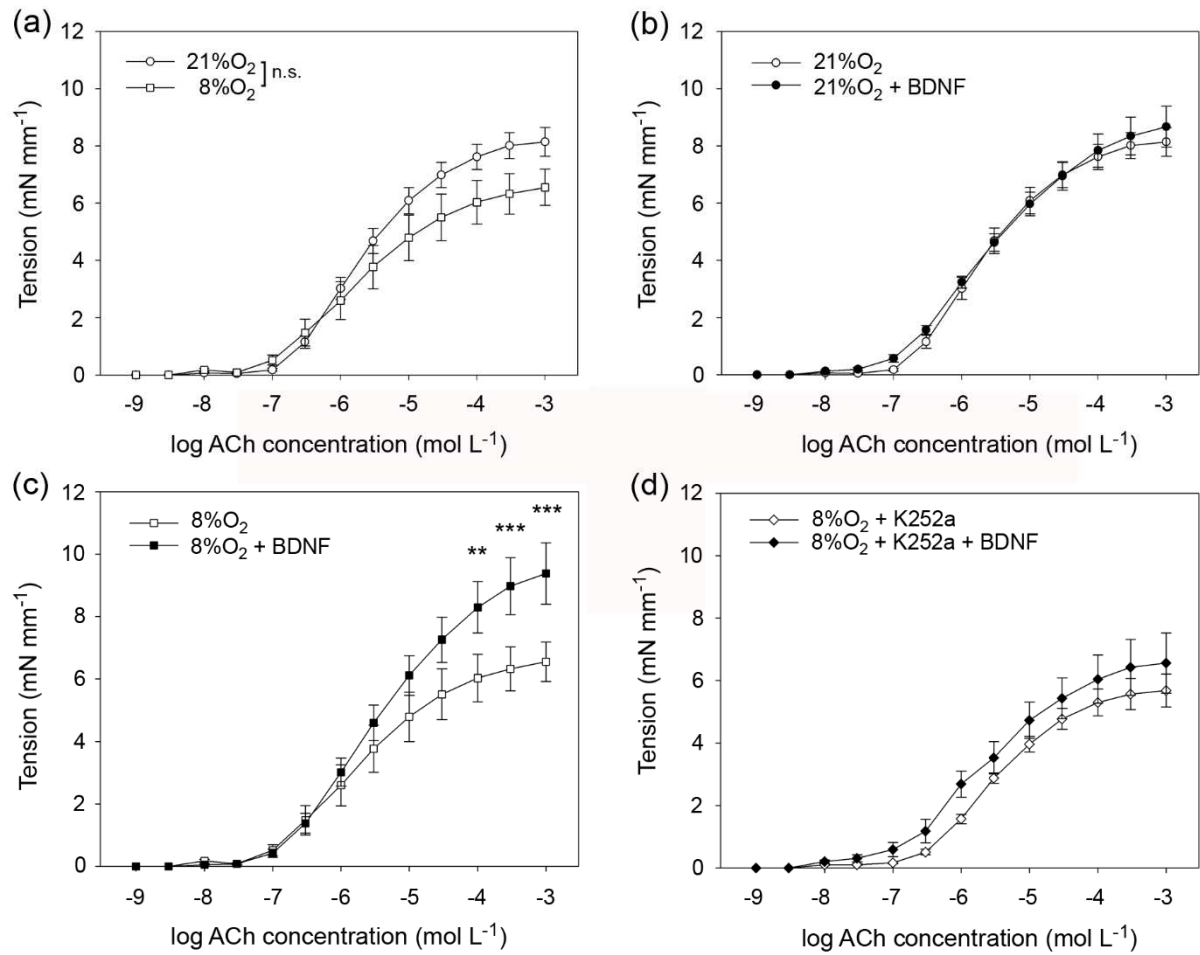
Figure 4: Tension of isometrically contracting tracheal segments in the presence of ACh (ACh). Tissue specimens were prepared from rats immediately after exposure to 21% O_2 or 8% O_2 to stimulate TrkB expression. Tension (y-axis) was calculated as the ratio of the developed force to the narrowest width of the tissue rings. Increasing concentrations of ACh (10^{-9} to 10^{-3} mol L^{-1}) elicited s-shaped dose-response curves in preparations from normoxic and hypoxic rats (*Panel (a)*). Pre-incubation with the TrkB ligand, BDNF (50 ng ml^{-1}), significantly enhanced the contractile response to higher concentrations of ACh in hypoxic (*Panel (c)*) but not in normoxic (*Panel (b)*) preparations. This effect of BDNF was prevented by pretreatment of the tracheal segments with the tyrosine kinase inhibitor K252a (100 nmol L^{-1}, *Panel (d)*). Each experimental protocol was performed with 5 tracheal segments from 5 different animals. Values are presented as means ±SEM of n=5 rats. Statistical significances are indicated (**$P<0.01$, ***$P<0.001$, two-way ANOVA). n.s., not significant.
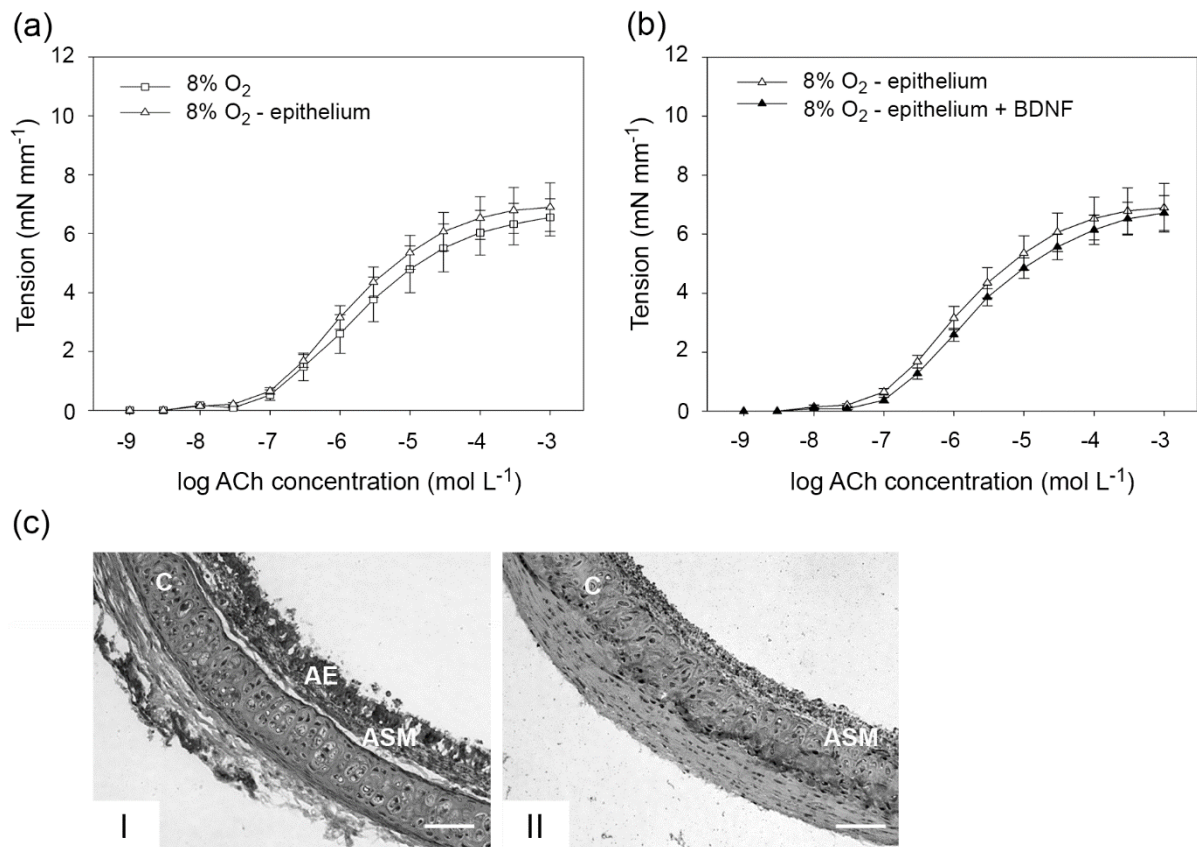
Figure 5: *Panel (a)*: Contractile response to ACh of isolated hypoxic tracheal segments with intact epithelium and after deepithelialization. *Panel (b)*: Failure of the TrkB ligand, BDNF (50 ng/ml), to augment ACh-induced isometric contractions of hypoxic tracheal segments after mechanical removal of the epithelium. Five tracheal segments from 5 different animals were analysed under each experimental condition. Values are means ± SEM of n=5 rats ($P>0.05$, two-way ANOVA). *Panel (c)*: Representative histomorphology (HE-staining) of isolated rat tracheal segments with intact epithelium (I) and after mechanical deepithelialisation (II). Scale bars indicate 200 µm. AE, airway epithelium; ASM, airway smooth muscle; C, cartilage. Each experimental protocol was performed with 5 tracheal segments from 5 different animals. Values are presented as means ±SEM of n=5 rats. Statistical significances are indicated (**$P<0.01$, ***$P<0.001$, two-way ANOVA). n.s., not significant.
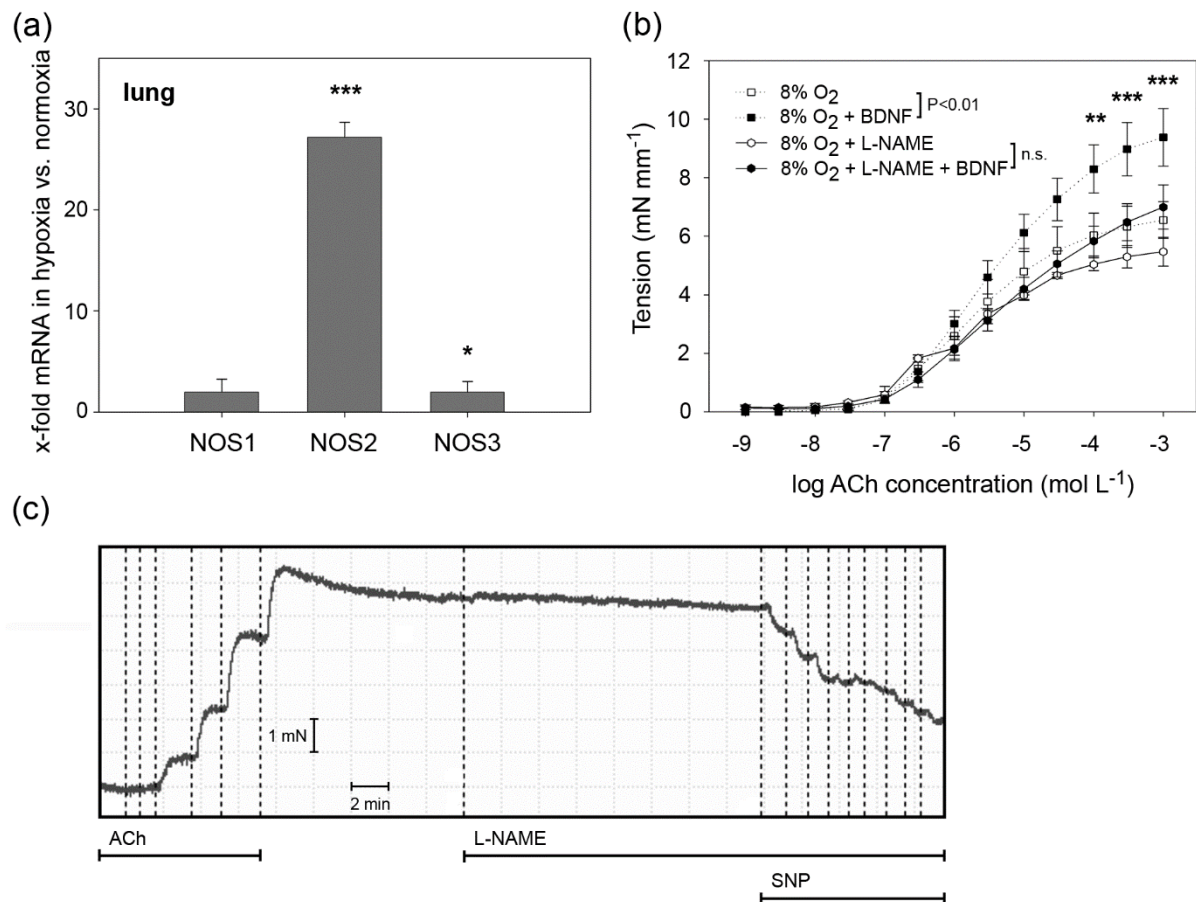
Figure 6: *Panel (a)*: Messenger RNA levels of the three NO synthase (NOS) isoforms in rat lungs. Animals were exposed either to 21% O_2 or 8% O_2 for 6 h, and tanscripts measured by real-time RT-PCR. Relative mRNA content in normoxic and hypoxic organs were determined on the basis of differences in threshold cycle (Ct) values as described above. Values are means ± SD of n=5 normoxic and hypoxic animals, each (*$P<0.05$, ***$P<0.001$, Student's t-test). *Panel (b)*: Contractile response to ACh of isolated tracheal segments pretreated with the NOS inhibitor L-NAME (0.1 mmol L^{-1}, 15 min). Contraction curves obtained in the absence of L-NAME are shown for comparison. Note, that L-NAME prevented the effect of BDNF of ACh-induced tracheal contractions ($P>0.05$, two-way ANOVA). Five tracheal segments from 5 different hypoxic animals were analysed under each experimental condition. Values are means ± SEM. **$P<0.01$ and ***$P<0.001$ between BDNF-treated and untreated preparation in the absence of L-NAME. *Panel (c)*: Original recording of the mechanical force of an isometrically contracting isolated tracheal segment from hypoxic rat. Shown are the effects of stepwise increasing concentrations of ACh (between 10^{-8} and 3×10^{-6} mol L^{-1}), L-NAME (0.1 mmol L^{-1}) and the NO donor sodium nitroprusside (SNP, between 10^{-7} and 10^{-3} mol L^{-1}). Vertical dotted lines in bold mark the application of L-NAME and the gradual increases of ACh and SNP, respectively.

Table 1: Primer sequences used for real-time RT-PCR of the respective genes.

mRNA	NCBI accession no.	Single strand sequence
rat TrkB-F	M55293.1	5'-GTGGAGGAAGGGAAGTCTGTG-3'
rat TrkB-R		5'-CAGTGGTGGTCTGAGGTTGGA-3'
rat TrkB T1-F	AY265419.1	5'-CTCCGTAAAGCAGGTTCATC-3'
rat TrkB T1-R		5'-TTCTGCTATTAGCTGGCCTC-3'
rat TrkB FL-F	NM012731.1	5'-ATCACCAACAGCCAGCTCAAGC-3'
rat TrkB FL-R		5'-CCTCCACACAGACACCGTAGAACTTGAC-3'
rat TrkA-F	NM021589.1	5'-CGGTCTGGTGACTTCGTTGA-3'
rat TrkA-R		5'-GGTCCTCAAACTCCAGGCGT-3'
rat TrkC-F	NM182809.2	5'-ACTGAGATCAATTGCCGGCG-3'
rat TrkC-R		5'-GATGGTCAGCTTCTGGAGTC-3'
rat p75^{NTR}-F	NM033217.3	5'-GCAGCACCATCGGTCTGCGGA-3'
rat p75^{NTR}-R		5'-CGGAGCCAACCAGACCGTGTGTG-3'
rat BDNF-F	EF125690.1	5'-GAGCGTGTGTGACAGTATTAG-3'
rat BDNF-R		5'-GTAGTTCGGCATTGCGAGTTC-3'
rat β-actin-F	NM031144.2	5'-ATGGTGGGTATGGGTCAGAA-3'
rat β-actin-R		5'-GGGGTGTTGAAGGTCTCAAA-3'
rat GLUT-1-F	BC061873	5'-GTCCTATCTGAGTATCGTGGCCATC-3'
rat GLUT-1-R		5'-AAGGTCCGGCCTTTGGTCTCAGGAA-3'
rat VEGF-F	NM001110334.1	5'-GTACCTCCACCATGCCAAGT-3'
rat VEGF-R		5'-CTGCATGGTGATGTTGCTT-3'
rat NOS1-F	NM_052799.1	5'-CAAGGCCCACATGGACAGGC-3'
rat NOS1-R		5-GGTGGCATACTTGACATGGTTACA-3'
rat NOS2-F	AY211532.1	5'-ATGGCTTGCCCCTGGAAGT-3'
rat NOS2-R		5'-TATCCGAGGTGGCCTTGTGGTG-3'
rat NOS3-F	AJ011115.1	5'-CAGTGAAGATCTCTGCCT-3'
rat NOS3-R		5'-GCCCGACATTTCCAT-3'