Neurotrophins induce nitric oxide generation in human pulmonary artery endothelial cells

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Aims

Members of the growth factor family of neurotrophins [NTs; e.g. brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3)] and their high-affinity receptors (tropomyosin-related kinase; Trk) and low-affinity receptors p75 neurotrophin receptor (p75NTR) have been localized to pulmonary artery (PA) in humans. However, their role is unclear. Based on previous findings of NTs and their receptors within the pulmonary endothelium, we tested the hypothesis that NTs induce nitric oxide (NO) production in pulmonary endothelial cells (ECs), thus contributing to vasodilation.

Methods and results

In human pulmonary artery ECs loaded with the NO-sensitive fluorescent dye diaminofluorescein-2, both BDNF and NT3 (100 pM, 1 nM, and 10 nM) acutely (<10 min) and substantially increased fluorescence levels in a concentration-dependent fashion (to levels comparable to that induced by 1 μM acetylcholine). NT-induced elevation of NO levels was blunted by the tyrosine kinase inhibitor K252a, the nitric oxide synthase (NOS) inhibitor NG–nitro-L-arginine methyl ester, the Ca2+ chelator 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid, and the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide. Suppression of TrkB or TrkC expression via siRNA as well as functional blockade of p75NTR prevented NT-induced NO elevation. Both BDNF and NT3 increased phosphorylation of Akt and endothelial NO synthase (eNOS). In endothelium-intact porcine PA rings, NTs increased cGMP and induced vasodilation in pre-contracted arteries.

Conclusion

These results indicate that NTs acutely modulate pulmonary endothelial NO production and contribute to relaxation of the pulmonary vasculature.

Keywords

Brain-derived neurotrophic factor • Neurotrophin-3 • Tropomyosin-related kinase • p75NTR • Lung

1. Introduction

Neurotrophins (NTs) such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT3) are growth factors conventionally associated with the nervous system, regulating growth, differentiation, and survival.¹–⁷ NTs bind two receptor types, the low-affinity pan-NT p75 neurotrophin receptor (p75NTR) receptor, and the high-affinity tropomyosin-related kinase receptors (TrkA binds NGF, TrkB preferentially binds BDNF, TrkC preferentially binds NT3).¹,²,⁵,⁸ There is now increasing evidence for Trk and p75NTR (as well as NT) expression in non-neuronal tissues,⁹–¹² including the lung¹³–²² and pulmonary vasculature.²³,²⁴ In addition to expression within tissues (e.g. neuronal tissues⁸), there is circulating BDNF as well (~15–30 ng/mL),²⁵,²⁶ raising the possibility of NT effects in different lung cell types. While there is some evidence for NT effects in the airway,¹³–¹⁵,¹⁷,²⁰,²¹ their role in the pulmonary circulation has been barely investigated.²⁷ Within human pulmonary artery (PA), immunocytochemical studies show that BDNF, NT3, and Trk receptors are particularly expressed in the intima.²⁴ In mouse pulmonary arteries, p75NTR is expressed in both endothelium and smooth muscle, with arterial rings from p75NTR knockout mice showing increased vasoconstriction.²⁷ These initial findings indirectly suggest a role for NTs in local control of vascular responses. Intimal expression of NTs and their receptors raises the exciting possibility that locally produced NTs particularly influence the endothelium; however, the underlying
mechanisms are not known. Endothelial cells (ECs) of the systemic circulation express Trk receptors, and chronic exposure of rat brain vasculature to NT3 increases endothelial nitric oxide synthase (eNOS) protein level. Based on these initial data, we hypothesized that NTs enhance nitric oxide (NO) production in the pulmonary endothelium, and contribute to vasodilation. While NO modulation could occur through increased expression of signalling proteins (e.g. eNOS or Akt), in other tissues NTs can also induce very rapid effects (seconds to minutes) via activation of Trk and other signalling mechanisms. Accordingly, we theorized that if NTs are involved in modulation of pulmonary tone, they could potentially produce NO in ECs on a rapid timescale.

2. Methods

2.1 Human PA ECs culture

Branch pulmonary arteries were excised from lung resection samples incidental to patient surgery (typically lobectomies or pneumonectomies) normally discarded by the pathologist following diagnosis (anonymous and de-identified samples; thus not considered Human Subjects Research per Mayo Clinic IRB). Only samples from normal areas of the lung (identified by the pathologist) were used. The endothelial layer was gently scraped and cells isolated by gentle agitation. Cell culture methods are described in Supplementary material online, Methods section.

2.2 Western blot analysis

Pulmonary arterial segments and ECs from different groups (see Results) were processed for western analysis using standard techniques and quantified using gel densitometry (see Supplementary material online, Methods section).

2.3 siRNA transfection

Previously described techniques21 for siRNA transfection of cultured human cells were modified for PA ECs (see Supplementary material online, Methods for details). Briefly, ECs were transfected with non-specific or target-specific (TrkB or TrkC) siRNA using Lipofectamine. siRNA efficiency of protein suppression was confirmed by western analysis.

2.4 NO imaging

PA ECs were seeded in 8-well Labtek chambers and grown to between 50 and 75% confluence prior to imaging. Cells were loaded with either the NO-sensitive fluorescent dye 4,5 diaminofluorescein diacetate (DAF-2 DA) or the NO-insensitive DAF-4 (CalBiochem; 5 μM, 45 min at room temperature). Samples were visualized using a real-time fluorescence imaging system (MetaFluor; Universal Imaging, Downingtown, PA, USA) mounted on a Nikon Diaphot inverted microscope, with a ×40/1.3NA oil immersion lens (Fryer Instruments, Edina, MN, USA). Cells were perfused with 2.5 mM CaCl₂, HEPES-buffered Hanks Balanced Salt Solution (HBSS; Invitrogen; pH 7.40), and baseline fluorescence was established. The responses of 5–15 cells per chamber were obtained from individual, software-defined regions of interest. DAF-2 was excited at 488 nm with a Lambda 10-3 filter changer (Sutter Instruments, Novato, CA, USA) and fluorescence emissions collected at 510 nm at 0.5 Hz with a Photometric Cascade digital camera system (Roper Scientific, Tucson, AZ, USA).

2.5 Vascular contractility studies

Methodological details are provided in Supplementary material online, Methods section. Briefly, fourth generation intralobar porcine pulmonary arterial segments were isolated and, in some samples, endothelium was removed by gentle abrasion. Using an organ-bath system, isometric force generation was measured at 37°C in physiological salt solution (PSS, see Supplementary material online, Methods). Vessels were contracted with the thromboxane A₂ analogue U46619 (10 nM) and responses to NTs and ACh recorded.

2.6 Measurement of cyclic GMP

NT or ACh-induced production of cGMP was measured in both endothelium-intact and -denuded PA segments using a standard cGMP ELISA kit (see Supplementary material online, Materials).

2.7 NO calibrations

As described in Supplementary material online, Materials, NO levels as measured by DAF-2 fluorescence were empirically calibrated by the use of increasing concentrations of the short-acting NO donor MAHMA-NONOate, and then converting fluorescence to absolute NO levels.

2.8 Statistical analysis

ECS were derived from at least four patients, with at least three replications per protocol per patient. Comparisons were performed using two-way ANOVA, and Bonferroni or Dunnnett’s correction for repeated measures. At least 10 cells/group were used for imaging. Statistical significance was tested at P < 0.05. Values are reported as mean ± SE.

3. Results

3.1 PA expression of NTs and their receptors

Western analysis showed abundant expression of the pro-forms of BDNF and NT3, (consistent with intracellular production of NTs) in both endothelium-intact vs. -denuded arteries (Figure 1A). Complete endothelial denudation was confirmed by lack of eNOS (Figure 1A). The continued presence of NTs in such samples confirmed production by non-endothelial components of the artery. PA also expressed full-length and truncated forms of TrkB and TrkC, along with the p75NTR receptor (Figure 1B). The ratio of full-length to truncated TrkB was substantially higher than that for TrkC. Separately isolated PA ECs consistently expressed both NTs and their receptors (Figure 1C).

3.2 NT effects on Akt and eNOS phosphorylation

In PA ECs, exposure to 10 nM BDNF or NT3 (R&D Systems) for just 2 min produced significant phosphorylation (P < 0.05) of Akt at Ser473 compared with vehicle control, albeit less than that induced by 1 μM ACh (Figure 2A). As the activation of Akt leads to rapid phosphorylation of eNOS (among other targets), we determined eNOS phosphorylation at Ser1177 and found it to be significantly increased by both BDNF and NT3 (P < 0.05), to levels comparable to that by ACh (Figure 2B).

3.3 NO production in PA ECs

Details of DAF-2 validation and calibration are provided in Supplementary material online, Material.

3.3.1 Response to ACh

In DAF-2 loaded ECs, treatment with both 1 and 10 μM ACh significantly increased fluorescence after ~3 min (Figure 3A; P < 0.05 compared with vehicle control). Both the rate of fluorescence increase as well as peak fluorescence were greater with 10 μM ACh, compared with 1 μM ACh (Table 1; Figure 3A).
The sequence of events associated with NO generation in ECs is well-established (initiated by agonist-induced increase in intracellular Ca\(^{2+}\) followed by eNOS dissociation from caveolae and phosphorylation, resulting in generation of NO from L-arginine). Experiments were conducted to verify this pathway in our cell preparation. ACh-induced increase in DAF-2 fluorescence was prevented by pre-treating ECs with the eNOS inhibitor NG–nitro–L–arginine methyl ester (L-NAME) (100 \(\mu\)M, 20 min) prior to ACh stimulation (Figure 3A; \(P\), 0.05). In other cell sets, application of the Ca\(^{2+}\) chelating agent 1,2-bis(o-aminophenoxy)ethane-N,N,N\(^\prime\),N\(^\prime\)-tetraacetic acid (BAPTA) (10 \(\mu\)M, 45 min) or the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) (50 \(\mu\)M) blunted ACh-induced changes in DAF-2 fluorescence (Figure 3A; \(P\) < 0.05 for L-NAME, BAPTA, and PTIO).

### 3.3.2 Response to BDNF and NT3

Exposure to 100 pM, 1 nM, or 10 nM BDNF or NT3 significantly increased NO levels (compared with vehicle control) in a concentration-dependent fashion (Figure 3B and C; \(P\) < 0.05 for vehicle and concentration). Compared with either MAHMA-NONOate or 1 or 10 \(\mu\)M ACh, the kinetics of fluorescence change were slower (8–10 min for NTs; Table 1). At the highest NT concentration, fluorescence levels at 15 min were comparable to that induced by 1 \(\mu\)M ACh (Figures 3A vs. B and C). There were no significant differences in the extent of NO production between BDNF and NT3 at any concentration (Figure 3B and C).

### 3.3.3 Effect of NO scavenging and eNOS inhibition

To demonstrate that BDNF and NT3 exposure results in NO generation via Ca\(^{2+}\)-mediated eNOS activation, ECs were treated with the Ca\(^{2+}\) chelator BAPTA (10 \(\mu\)M) or the NOS inhibitor L-NNAME (100 \(\mu\)M) prior to addition of either BDNF or NT3 (10 nM). Incubation of ECs with either agent (BAPTA or L-NNAME) significantly reduced the subsequent increase in NT-induced NO (Figure 4A; \(P\) < 0.05). To further verify production of NO by 10 nM BDNF or NT3,
Figure 3 Effect of NTs on NO production in PA ECs. (A) In ECs loaded with the fluorescent NO indicator diaminofluorescein (DAF-2), quality control studies were performed using 1 or 10 \( \mu \text{M} \) ACh. Inhibition of intracellular \( \text{Ca}^{2+} \) by the chelator BAPTA, eNOS using L-NAME, or NO itself using PTIO, prevented elevation of DAF-2 fluorescence by ACh, confirming the \( \text{Ca}^{2+} \)-eNOS pathways in production of NO, as well as the specificity of DAF-2 for NO. Separately, lack of ACh effects on NT receptors relevant to NO production was verified (tyrosine kinase inhibitor K252A and p75NTR neutralizing antibody). (B) BDNF or (C) NT3 (100 pM, 1 nM, or 10 nM) produced significant increases in NO levels in a concentration-dependent fashion. Compared with either MAHMA-NONOate (not shown) or ACh, the time of onset and rate of increased fluorescence was significantly slower (Table 1); however, the total DAF-2 fluorescence at 15 min induced by 10 nM BDNF or NT3 was comparable to 1 \( \mu \text{M} \) ACh. There were no significant differences in the extent of NO production between BDNF and NT3 at any concentration; vehicle treatment indicates exposure to HBSS only. Left panels are representative tracings, right panels are bar graph summaries. Values are means ± SE. Asterisk indicates significant NT (or ACh) effect compared with vehicle, dollar denotes significant concentration effect, and hash denotes significant inhibitor effect (\( P < 0.05 \)).
ECs were pre-incubated and continually perfused with the NO scavenger PTIO (50 μM) during addition of NTs. As with ACh, PTIO significantly diminished DAF-2 fluorescence (Figure 4A; P < 0.05).

### 3.3.4 Effect of NT receptor blockade

While there are currently no specific blockers of Trk receptors, previous studies have used the tyrosine kinase inhibitor K252a to block NT action.20,29–31 In pulmonary ECs, pre-exposure to 100 nM K252a for 30 min significantly reduced DAF-2 fluorescence increases with subsequent 1 or 10 nM BDNF or NT3 exposure (Figure 4B; P < 0.05). Blocking p75NTR was accomplished by incubating ECs for 30 min with a neutralizing antibody (1 μg/mL, Upstate/Millipore) prior to NT exposure. The antibody partially (but not significantly) blocked NT-induced increase in DAF-2 fluorescence (Figure 4B). Pretreatment with K252a or p75NTR antibody did not significantly affect DAF-2 fluorescence alone or the response to MAHMA-NONOate or 10 μM ACh (Figure 3A).

### 3.3.5 Effect of NT receptor siRNA

In TrkB or TrkC siRNA-transfected cells loaded with DAF2, 10 nM BDNF or NT3 produced significantly lower fluorescence rate increases (P < 0.05), compared with Lipofectamine alone as well as compared with non-specific siRNA (Figure 4C). There was no substantial difference in NT-induced NO levels in cells treated with Lipofectamine vs. medium only (i.e. no Lipofectamine). In TrkB siRNA-transfected cells, NO production by 10 nM NT3 was somewhat lower than that in Lipofectamine exposed cells; however, NT3 continued to produce an increase in NO levels (Figure 4C; P < 0.05). Conversely, in TrkC siRNA-transfected cells, NO production by 10 nM BDNF was not affected (Figure 4C; P < 0.05).

### 3.4 Functional effect of acute NT exposure

In endothelium-intact porcine PA rings at optimal length and constricted with U46619, addition of 10 nM BDNF or NT3 resulted in significant relaxation (P < 0.05; Figure 5A) that initiated within 2 min and stabilized by 8 min. There was no significant difference between BDNF vs. NT3 effects, although responses to NT3 tended to be lower. The extent of NT-induced relaxation was ~30–40% of that induced by ACh in endothelium-intact rings (Figure 5). Removal of endothelium completely abolished both NT- and ACh-induced relaxation (Figure 5A inset). However, direct, endothelium-independent vasodilatation by 10 mM papaverine (PPV) was retained.

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**Table 1 Dynamic parameters of NO production**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Onset (seconds after addition)</th>
<th>Rate of rise (GL/s)</th>
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<tr>
<td>Vehicle</td>
<td>n/a</td>
<td>0.77 ± 0.51&lt;sup&gt;abc&lt;/sup&gt;</td>
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<tr>
<td>1 μM ACh</td>
<td>153.45 ± 18.56&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.13 ± 0.85&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>10 μM ACh</td>
<td>139.70 ± 14.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.79 ± 0.57&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>100 nM MAHMA-NONOate</td>
<td>60.20 ± 7.83&lt;sup&gt;b&lt;/sup&gt;</td>
<td>34.60 ± 9.78&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>10 μM Sulpho NONOate</td>
<td>n/a</td>
<td>0.65 ± 0.46&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>100 μM BDNF</td>
<td>555.67 ± 54.73&lt;sup&gt;b,c,d&lt;/sup&gt;</td>
<td>0.86 ± 0.17&lt;sup&gt;c,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>1 nM BDNF</td>
<td>161.86 ± 22.56&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.60 ± 0.62&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10 nM BDNF</td>
<td>196.58 ± 62.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.50 ± 0.53&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>100 μM NT3</td>
<td>355.40 ± 41.25&lt;sup&gt;b,c,d&lt;/sup&gt;</td>
<td>0.58 ± 0.35&lt;sup&gt;b,c,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>1 nM NT3</td>
<td>120.42 ± 26.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.64 ± 1.90&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10 nM NT3</td>
<td>191.92 ± 33.70&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.29 ± 0.97&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

Values are means ± SE; n > 3 for all experiments.
<sup>a</sup>Indicates significant difference from MAHMA-NONOate.
<sup>b</sup>Indicates significant difference from 10 μM ACh.
<sup>c</sup>Indicates significant difference from 1 μM ACh.
<sup>d</sup>Indicates significant concentration effect (vs. 10 μM ACh; or vs. 10 nM BDNF or NT3) (P < 0.05).

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**Figure 4** Mechanisms of NT-induced NO production in ECs. (A) Pre-exposure to BAPTA, L-NAME, or PTIO significantly blunted the effect of BDNF or NT3 on NO production. (B) Inhibition of tyrosine kinases by 100 nM K252a (blocking Trk receptor effects) nearly abolished NT effects, while a p75NTR functional antibody (1 μg/mL) diminished the effect of 10 nM BDNF or NT3, albeit to a smaller extent. The inhibitors by themselves had negligible effects on DAF-2 fluorescence. (C) Suppression of TrkB receptor expression (verified by western analysis, not shown) blunted the effects of BDNF and NT3 (10 nM) on NO production compared with non-specific siRNA, albeit with a much greater effect on BDNF. Conversely, TrkC receptor suppression substantially blunted NT3 effects; however, this suppression had little or no effect on BDNF signalling. Vehicle treatment indicates exposure to HBSS only. Values are means ± SE. Asterisk indicates significant NT effect. Hash indicates significant effect of inhibitors or siRNA (P < 0.05).
3.4.1 Effect on tissue cGMP
In segments of human PA pre-incubated with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 10 μM for 20 min), exposure to BDNF or NT3 (100 pM and 1 nM, either NT) resulted in increased cGMP compared with vehicle control (Figure 5C).

4. Discussion
In this study, we present novel data on the acute (likely non-genomic) effects of the NTs BDNF and NT3 on human PA ECs, demonstrating that both NTs rapidly induce NO generation in as little as 6–8 min. This effect is mediated by phosphorylation of Akt and eNOS, and functionally results in vasodilation. NT effects appear to be mediated predominantly via high-affinity TrkB and TrkC receptors (for BDNF and NT3, respectively), with partial involvement of the low-affinity pan-NT p75NTR receptor. An impressive finding is that both NTs can induce NO levels comparable to that by well-recognized agonists such as ACh. These novel data suggest that NTs, potentially derived from multiple sources such as the PA smooth muscle as well as the endothelium itself (which expresses BDNF and NT3) can potentially modulate vasodilator function Figure 6.

4.1 NT signalling
NTs, a family of small growth factors most commonly associated with nerve cells and tissues, signal via high-affinity, NT-specific Trk receptors (TrkA, -B,-C) with picomolar binding coefficients, and low-affinity p75NTR which binds all NTs. Preferential binding of BDNF to TrkB (with partial affinity for NT3), and NT3 to TrkC, has previously been established. In addition to tissue NT levels, there is ample evidence for circulating NTs, suggesting that both local and remote sources of NTs can mediate their effects within a tissue, depending on receptor expression.

Much of the work on NT signalling has focused on the ‘growth factor’ aspect, which involves long-term consequences mediated via altered gene and protein expression. Nonetheless, in neuronal tissues, there is now evidence for relatively rapid effects of NTs that occur in the seconds-minutes timeframe, and involve Trk receptors. These rapid effects involve modulation of plasma membrane receptors, cationic channels (e.g. Ca$^{2+}$ influx and voltage-gated Na$^{+}$ channels), and other mechanisms. NT binding results in Trk receptor autophosphorylation and triggering of downstream enzymes and intracellular signalling cascades, such as MAP kinases and PI3/Akt. The consequences of p75NTR binding are less well understood, especially in non-neuronal tissues, but these receptors may work in concert with Trk receptors in mediating NT effects.

4.2 NTs in the lung
There is increasing evidence that NTs as well as Trk and p75NTR receptors are widely distributed in non-neuronal tissues, including skeletal and smooth muscle and peripheral tissues. Immunocytochemical and other evidence suggests that both NTs and their receptors are expressed by a wide variety of component lung cells including nerves, immune cells, epithelium, smooth muscle, fibroblasts and, relevant to this study, the pulmonary vasculature. However, functional information is limited, with most work in the lung focusing on immune cell function, and exacerbation of sinusitis and asthma. The focus has again been on genomic effects of NTs: however, we and others have previously shown that some NTs (such as BDNF) can rapidly increase [Ca$^{2+}$], in human airway smooth muscle, while others (NT3) can decrease [Ca$^{2+}$], following exposure over several minutes. Accordingly, there is precedent for rapid non-genomic effects of NTs in the lung, relevant to this study.

There has been only limited examination of NT signalling in the vasculature, especially the pulmonary circuit. Both NGF and BDNF are involved in cardiovascular development, in pathological conditions such as ischaemia, and in systemic angiogenesis. Acting via Trk receptors, NGF, and BDNF increase eNOS expression and affect...
cellular survival via MAPK and PI3/Akt cascades as well as increased EC proliferation and migration.\textsuperscript{12,54–56} While these data indicate an effect of NTs on ECs, such effects again reflect the ‘growth factor’ aspect of NT function, i.e. slower, likely genomic actions.

In the pulmonary circuit, immunocytochemical studies of extrapulmonary branches of human PA show NGF, BDNF, and NT3 expression especially in the intima and adventitia (with lesser reactivity in the vascular smooth muscle media).\textsuperscript{24} The results of the present study demonstrating BDNF and NT3 expression in human pulmonary ECs are consistent with this previous work. An important aspect from both studies is the expression of both ligand and receptor within the same cell type (i.e. ECs) strongly suggesting of a functional role for NTs in local control of vascular responses via autocrine or paracrine effects (hence our focus on the endothelium). Pro-BDNF and -NT3 were readily detectable in PA EC lysates, indicating that in addition to other sources of NTs,\textsuperscript{22,24} these NTs are synthesized and then most likely secreted by ECs. Here, it is important to emphasize the expression of the functional full-length Trk receptors in human pulmonary ECs (Figure 1), as opposed to truncated Trk forms that are likely intracellular and do not mediate these NT effects.\textsuperscript{57}

4.3 NT-induced NO generation

The present study is the first to demonstrate rapid NO generation following NT exposure in ECs with the functional consequence of inducing PA vasodilation. NT-induced NO generation appears to involve previously established cascades of elevated $[\text{Ca}^{2+}]_i$, eNOS phosphorylation (via Akt), and activation. A significant finding was that NT-induced NO release (even at pM NT concentrations) was comparable to that by well-recognized vasodilators such as ACh and ATP (not shown) known to work via the endothelium. Here, it is important to emphasize that NT concentrations used in our study are in the physiological range.\textsuperscript{25,35}

NT-induced NO production appears to largely involve Trk receptors, as evidenced by the pM concentrations required, as well as the effects of K252a and siRNAs targeting TrkB and TrkC. Suppression of TrkB expression partially blunted NT3 effects, while TrkC suppression had no effect on BDNF-induced NO generation: this may reflect the partial affinity of TrkB and NT3, especially at the 10 nM NT concentration used in siRNA protocols. The role of p75NTR in NT-induced NO production is less clear, as the inhibitory effect of

Figure 6 Schematic representation of NT effects on EC-derived NO. As with known inducers of endothelial NO (such as ACh or ATP), NTs (such as BDNF and NT3) increase Ca$^{2+}$ in PA ECs and Akt phosphorylation, which then induces dissociation (and activation) of eNOS from caveolae, with subsequent production of NO. Such NT-induced NO production involves activation of Trk receptors (TrkB for BDNF and to a lesser extent NT3; TrkC for NT3), but may also involve the low-affinity p75NTR.
the functional p75NTR antibody on BDNF or NT3-induced NO production suggests NT activation of the low-affinity receptor. p75NTR is typically considered to play a role in cell proliferation and apoptosis, and has not been shown to participate in non-genomic effects; however, p75NTR interactions with Trk receptors in facilitating rapid NT effects cannot be ruled out.

4.4 Methodological issues
We used the fluorescent indicator DAF-2 DA\(^59\) to measure NO, which is sensitive to low concentrations of NO (lower detection limit 5 \(\text{mM}\)), specific in reactivity (donors of NO\(_2\) or NO\(_3\) had virtually no effect on DAF-2 fluorescence),\(^{59,61}\) and non-saturating in the physiological range of NO (17 \(\text{nM}\); upper detection limit of 200 \(\text{nM}\)).\(^{61,62}\) In our opinion, this technique is comparable in sensitivity to a Griess-reagent based evaluation of nitrate/nitrite production, but provides real-time information on dynamics of NO production.

4.5 Physiological relevance
A significant finding in our study was that both NTs induced PA relaxation, albeit not to the same extent as ACh. Consistent with the cellular work on NO production, NT-induced vasodilation was abolished by removal of endothelium, highlighting the importance of endothelium in mediating rapid NT effects. Of further evidence that NTs induce physiologically relevant levels of NO in ECs is the accumulation of cGMP within segments of endothelium-intact human PA. Interestingly, cGMP levels were comparable between NT- vs. ACh-exposed samples, but NT-induced vasodilation was smaller compared with ACh. We interpret this result in the context of ongoing studies where we have found that NTs also directly enhance intracellular Ca\(^{2+}\) in PA smooth muscle (Meuchel, Thompson, Prakash, unpublished observations). Accordingly, the net effect of NTs on vascular tone is likely determined by relative contribution of NT effects on endothelium (NO generation, vasodilation) vs. smooth muscular tone is likely determined by relative contribution of NT effects on endothelium (NO generation, vasodilation) vs. smooth muscle tone.

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Conflict of interest: none declared.

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Supplementary material
Supplementary material is available at Cardiovascular Research online.

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References


