Biochimica et Biophysica Acta 1833 (2013) 2953–2960

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

TRPC3 regulates release of brain-derived neurotrophic factor from human airway smooth muscle

Pawan K. Vohra [a,1], Michael A. Thompson [a,1], Venkatachalem Sathish [a,b], Alexander Kiel [a], Calvin Jerde [a], Christina M. Pabelick [a,b], Brij B. Singh [c], Y.S. Prakash [a,b,*,1]

[a] Department of Anesthesiology, Mayo Clinic, 200 First St SW, Rochester, MN 55905, USA
[b] Department of Physiology & Biomedical Engineering, Mayo Clinic, 200 First St SW, Rochester, MN 55905, USA
[c] Department of Biochemistry and Molecular Biology, University of North Dakota, 264 Centennial Dr, Grand Forks, ND 58202, USA

ARTICLE INFO

Article history:
Received 9 January 2013
Received in revised form 21 July 2013
Accepted 23 July 2013
Available online 27 July 2013

Keywords:
Neurotrophin
Lung
Inflammation
Asthma
Tropomyosin-related kinase
Signaling

ABSTRACT

Exogenous brain-derived neurotrophic factor (BDNF) enhances Ca^{2+} signaling and cell proliferation in human airway smooth muscle (ASM), especially with inflammation. Human ASM also expresses BDNF, raising the potential for autocrine/paracrine effects. The mechanisms by which ASM BDNF secretion occurs are not known. Transient receptor potential channels (TRPCs) regulate a variety of intracellular processes including store-operated Ca^{2+} entry (SOCE; including in ASM) and secretion of factors such as cytokines. In human ASM, we tested the hypothesis that TRPC3 regulates BDNF secretion. At baseline, intracellular BDNF was present, and BDNF secretion was detectable by enzyme linked immunosorbent assay (ELISA) of cell supernatants or by real-time fluorescence imaging of cells transfected with GFP–BDNF vector. Exposure to the pro-inflammatory cytokine tumor necrosis factor-alpha (TNFα) (20 ng/ml, 48 h) or a mixture of allergens (ovalbumin, house dust mite, *Alternaria*, and *Aspergillus* extracts) significantly enhanced BDNF secretion and increased TRPC3 expression. TRPC3 knockdown (siRNA or inhibitor Pyr3; 10 μM) blunted BDNF secretion, and prevented inflammation effects. Chelation of extracellular Ca^{2+} (EGTA; 1 mM) or intracellular Ca^{2+} (BAPTA; 5 μM) significantly reduced secreted BDNF, as did the knockdown of SOCE proteins STIM1 and Orai1 or plasma membrane caveolin-1. Functionally, secreted BDNF had autocrine effects suggested by phosphorylation of high-affinity tropomyosin-related kinase TrkB receptor, prevented by chelating extracellular BDNF with chimeric TrkB-Fc. These data emphasize the role of TRPC3 and Ca^{2+} influx in the regulation of BDNF secretion by human ASM and the enhancing effects of inflammation. Given the BDNF effects on Ca^{2+} and cell proliferation, BDNF secretion may contribute to altered airway structure and function in diseases such as asthma.

1. Introduction

Primarily recognized and described in the nervous system, neurotrophins (NTs) are growth factors important for neuronal development and function [1–3]. There is now increasing evidence that NTs and their receptors are expressed in non-neuronal tissues including the airway [4–9], but their function is under investigation. We previously reported that brain-derived neurotrophic factor (BDNF), and its high-affinity tropomyosin-related kinase (TrkB) as well as low-affinity pan-NT p75NTR receptors are expressed by human airway smooth muscle (ASM) [6,7], and that exogenous BDNF enhances $[Ca^{2+}]_i$ responses to agonist [7] and cell proliferation [10]. Furthermore BDNF potentiates the effects of pro-inflammatory cytokines such as tumor necrosis factor (TNFα) [6,10,11]. While these effects of exogenous BDNF are clearly relevant to inflammatory airway diseases such as asthma, characterized by enhanced airway contractility, ASM cell proliferation and remodeling, the sources and mechanisms of regulation of BDNF in the airway are not clear.

The classic understanding is that NTs such as nerve growth factor (NGF) and BDNF are secreted from nerve endings and activate both Trk and p75NTR receptors, leading to altered cell growth and other functions [12–16]. BDNF has now been shown to be expressed by several airway cell types including epithelium, immune cells, nerves and even ASM [5,17–20]. Furthermore, sputum and/or bronchoalveolar lavages (BAL) from patients with asthma, allergic rhinitis or chronic cough show elevated levels of BDNF [17,21–24] suggesting that the different airway cell types may actually secrete BDNF. Here, Kemi et al. [20]

Abbreviations: ASM, airway smooth muscle; BAL, bronchial alveolar lavage; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; BDNF, brain-derived neurotrophic factor; NT, neurotrophins; ELISA, enzyme linked immunosorbent assay; GFP, green fluorescent protein; NGF, nerve growth factor; ROI, region of interest; siRNA, small interference ribose nucleic acid; SOCE, store-operated calcium entry; STIM1, stromal interactive molecule one; TLR, toll-like receptor; TNFα, tumor necrosis factor-alpha; TrkB, tropomyosin-related kinase; TRPC, transient receptor potential channel

* Corresponding author at: 4-184 W Jos SMH, Mayo Clinic, Rochester, MN 55905, USA. Tel.: +1 507 255 7481; fax: +1 507 255 7300.
E-mail address: Prakash.ys@mayo.edu (Y.S. Prakash).

[1] Equal contributions.

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http://dx.doi.org/10.1016/j.bbamcr.2013.07.019

previously found BDNF in the extracellular medium of human ASM cells. While secretory mechanisms for BDNF have been examined in neurons, and involve vesicular release [25], the mechanisms underlying BDNF secretion by non-neuronal cells, particularly in the lung, are not clear. The relevance of understanding BDNF secretion in the airway lies in the increasing recognition that local growth factor production can have pleiotropic effects in the context of inflammation, especially given the evidence that a range of airway cells express NT receptors and can thus respond to local factor production [5].

In neuronal tissues, a major stimulus for NT secretion is elevation in $[Ca^{2+}]_i$ typically following electrical stimulation leading to vesicular release [26]. While it is likely unnecessary for ASM cells to rapidly secrete NTs, vesicular secretion pathways do exist in airway cells [27], and are regulated by a number of complex mechanisms. The mechanisms by which BDNF release occur in non-neuronal tissues are unknown. The highly regulated, vesicular release of neurons [25,28] may not be relevant in ASM, and it is beyond the scope of any single study to examine the many complex secretory pathways that may exist in ASM. However, $[Ca^{2+}]_i$ is known to modulate secretion in general, and of BDNF in neurons and vasculature [29,30]. Thus mechanisms that regulate $[Ca^{2+}]_i$ in ASM may play a role in modulating BDNF secretion. In this regard, in non-ASM cells, recent studies suggest a role for canonical transient receptor potential (TRPC) channels in fluid secretion and in exocytosis of other intracellular proteins [31]. We have previously demonstrated that human ASM expresses a variety of TRPC channels, but TRPC3 is particularly important for modulating store-operated Ca^{2+} entry (SOCE) [32]. Relevant to airway disease, inflammatory cytokines such as TNFα increase TRPC3 expression [32,33] while BDNF modulates TRPC3 function in human ASM [6]. In non-neuronal cells, [34,35] TRPC3 has been shown to regulate secretion, but its non-SOCE role in ASM is not known. Accordingly, in the present study, we hypothesized that TRPC3 is important in BDNF secretion by ASM, and tested this hypothesis in human ASM in the context of enhanced BDNF secretion in the presence of inflammation.

2. Material and methods

2.1. Human ASM cells

Previously described techniques for isolating human ASM cells from lung samples incidental to patient thoracic surgery were used [6,7]. Briefly, under a protocol approved by the Mayo Clinic Institutional Review Board, surgical lung specimens of patients undergoing pneumenectomies or lobectomies for focal, non-infectious disease were obtained from surgical pathology at St. Marys Hospital of the Mayo Clinic, immediately following diagnosis by the pathologist. To avoid confounding issues relating to airway pathology, samples were limited to those from patients without clinical diagnoses of asthma, COPD or fibrosis. With the pathologist's aid, normal areas of 3rd to 6th generation bronchi were identified, dissected free of adventitia, and placed in cold Hanks' balanced salt solution (HBSS, Invitrogen, Carlsbad, CA) supplemented with 10 mM HEPES and 2 mM Ca^{2+}. Under microscopy, the epithelium was denuded in these bronchial rings, the ASM layer removed by blunt dissection, minced and ASM cells enzymatically dissociated. Cells were seeded into culture flasks or other chambers for experimentation, and maintained under usual culture conditions in phenol red-free DMEM/F-12 (Invitrogen) supplemented with 10% FBS. Prior to experimentation, cells were serum starved for 48 h and only cells of passages 1–3 were used. Maintenance of ASM phenotype with subculture was verified as previously described [36] and involved verification of smooth muscle actin and myosin but absence of fibroblast markers (fibroblast surface protein) or epithelial markers (E-cadherin).

2.2. Western analysis

Standard SDS-PAGE (Criterion Gel System; Bio-Rad, Hercules, CA; 4–15% gradient gels) and PVDF membrane (Bio-Rad) transfer techniques were used. Membranes were blocked with milk in TBS with 0.1% Tween-20 and blotted. GAPDH was used as a loading control. For secondary antibody detection, either horseradish peroxidase-conjugated antibodies with chemiluminescence substrate or far-red fluorescent dye conjugated antibodies were used. Blots were imaged on a Kodak Image Station 4000MM (Carestream Health, New Haven, CT) or a LiCor OdysseyXL system, and quantified using densitometry.

For immunoprecipitation studies of phosphorylated proteins, ASM cell lysates in RIPA buffer supplemented with protease inhibitor cocktail were immunoprecipitated using phosphotyrosine antibody 4G10 (Millipore, 06-427) and immunocomplexes captured using catch release kit (Millipore, 17–500) per manufacturer instructions. Thereafter, samples were subjected to SDS-PAGE, PVDF membrane transfer and immunoblotted against TrkB antibody (Neuromics, GT15080) with antibody-reactive bands detected as above.

2.3. ELISA

Sandwich ELISA for BDNF in cell supernatants was performed (R&D Systems, Minneapolis, MN) using standard, manufacturer-provided protocols. Initial ELISA of undiluted supernatants suggested BDNF levels at the lower limits of detection of the kit (<20 pg/ml). Therefore, the supernatants were concentrated 12-fold prior to ELISA, and the measurements were corrected post hoc. Colorimetric quantification of BDNF levels was performed on a FlexStation3 microplate reader at 450 nm (wavelength correction set to 540 nm) and compared to a standard curve based on manufacturer-supplied recombinant BDNF. Confirmation of BDNF in the supernatant was performed using Western analysis of the same concentrated samples (Millipore Centriprep).

2.4. Pro-inflammatory stimulation

Cells made serum-free for at least 24 h were exposed to medium only, 20 ng/ml TNFα or a mixture of allergens (10 μg each of ovalbumin, and of extracts from *Alternaria alternata*, *Aspergillus fumigatus* and *Dermatophagoides farinae* (house dust mite)) for 24 h. For inhibition or siRNA studies, pro-inflammatory stimuli followed such interventions.

2.5. siRNA transfection

ASM cells at ~50% confluence were made serum- and antibiotic-free for 24 h before transfection with 300 pmol of siRNA against TRPC3, Orai1, STIM1 or caveolin-1 (Ambion-Applied Biosystems, Austin, TX) or with a HA-tagged TRPC3 overexpression plasmid using Lipofectamine reagent (Invitrogen, Carlsbad, CA). Scrambled siRNA was used to verify the lack of off-target effects. Six hours after transfection, fresh growth medium was added and cells were grown overnight. Serum deprivation was then maintained for 48 h followed by experimentation.

2.6. GFP–BDNF transfection, imaging, and analysis

Serum-free ASM cells at ~50% confluence were transfected with 300 pM of a GFP–BDNF vector [30,37,38] using Lipofectamine reagent. An empty vector as well as GFP alone was used for transfection controls. Samples were visualized using a Nikon real-time fluorescence imaging system, with a 100×/1.3 NA oil immersion lens, standard GFP filters and a high-sensitivity 14-bit digital camera. GFP–BDNF transfected cells were exposed to vehicle, TNFα (as above) or Pyr3 and then acutely exposed to 1 μM of the bronchoconstrictor agent acetylcholine (ACh) and images were acquired at 1 Hz.

Image analysis was performed using Nikon Elements software. In image sets of GFP–BDNF transfected ASM cells, peri-membranous regions showing punctate fluorescence were identified, and small regions of interest (ROIs) were drawn. At least 10 ROIs that included the plasma membrane (i.e. covered extracellular and intracellular regions) were drawn allowing measurements of fluorescence changes representing