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Reactive oxygen species are involved in BMP-induced dendritic growth in cultured rat sympathetic neurons

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ABSTRACT

Previous studies have shown that bone morphogenetic proteins (BMPs) promote dendritic growth in sympathetic neurons; however, the downstream signaling molecules that mediate the dendrite promoting activity of BMPs are not well characterized. Here we test the hypothesis that reactive oxygen species (ROS)-mediated signaling links BMP receptor activation to dendritic growth. In cultured rat sympathetic neurons, exposure to any of the three mechanistically distinct antioxidants, diphenylene iodinium (DPI), nordihydroguaiaretic acid (NGA) or desferroxamine (DFO), blocked de novo BMP-induced dendritic growth. Addition of DPI to cultures previously induced with BMP to extend dendrites caused dendritic retraction while DFO and NGA prevented further growth of dendrites. The inhibition of the dendrite promoting activity of BMPs by antioxidants was concentration-dependent and occurred without altering axonal growth or neuronal cell survival. Antioxidant treatment did not block BMP activation of SMAD 1,5 as determined by nuclear localization of these SMADs. While BMP treatment did not cause a detectable increase in intracellular ROS in cultured sympathetic neurons as assessed using fluorescent indicator dyes, BMP treatment increased the oxygen consumption rate in cultured sympathetic neurons as determined using the Seahorse XF24 Analyzer, suggesting increased mitochondrial activity. In addition, BMPs upregulated expression of NADPH oxidase 2 (NOX2) and either pharmacological inhibition or siRNA knockdown of NOX2 significantly decreased BMP-7 induced dendritic growth. Collectively, these data support the hypothesis that ROS are involved in the downstream signaling events that mediate BMP7-induced dendritic growth in sympathetic neurons, and suggest that ROS-mediated signaling positively modulates dendritic complexity in peripheral neurons.

1. Introduction

Dendrites are the primary sites of synapse formation in the nervous system (Purves and Hume, 1981; Rubin, 1985), and the length and branching pattern of dendrites are tightly correlated to neuronal function (Elston, 2000; Jan and Jan, 2001; McAllister, 2000). Therefore, understanding the signaling molecules that influence the size and complexity of the dendritic arbor is crucial for gaining insight into the function of the nervous system. Bone morphogenetic protein (BMP) family members have been shown to promote dendritic growth in hippocampal (Withers et al., 2000), cortical (Le Roux et al., 1999) and retinal ganglion neurons (Hocking et al., 2008). In sympathetic neurons, BMPs are known to not only enhance the growth of existing dendrites (Lein et al., 1996; Majdazari et al., 2013) but also to induce de novo dendritic formation (Lein et al., 1995).

The signaling pathways that mediate the dendrite promoting activity of BMPs are not well characterized. BMPR1A is required for BMP-induced dendritic growth in cultured sympathetic neurons, and genetic deletion of this receptor subunit results in significant reduction of dendritic arborization of sympathetic neurons in the adult animal (Majdazari et al., 2013). BMPRII is required for BMP-induced dendritic growth in cultured cortical neurons (Lee-Hoeflich et al., 2004). There is at least one report suggesting that the dendrite promoting activity of BMPs requires SMAD 1 activation (Guo et al., 2001). However, there are also reports that the dendrite promoting activity of BMPs may be mediated by SMAD-independent signaling pathways involving c-jun kinase or p21 kinase (Lee-Hoeflich et al., 2004; Podkowa et al., 2010, 2013). But how SMAD-dependent or independent signaling pathways ultimately enhance dendritic arborization remains unknown.

Reactive oxygen species (ROS) are byproducts of normal cellular metabolism and include superoxide ion ($O_2^{\bullet}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2). High levels of ROS have been shown

Abbreviations: BMP, bone morphogenetic protein; DPI, diphenylene iodinium; NGA, nordihydroguaiaretic acid; DFO, desferroxamine; MAP-2, microtubule-associated protein-2; NOX, NADPH oxidase; OCR, oxygen consumption rate; ROS, reactive oxygen species; SCG, superior cervical ganglia.

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to have deleterious effects on cells including lipid peroxidation, DNA damage and cell death (Valko et al., 2007), and have been implicated in neurodegenerative diseases and cellular senescence (Furukawa et al., 2007; Jenner, 2003; Jomova et al., 2010). However, there is growing evidence that ROS can also act as signaling molecules under normal physiologic conditions. ROS have been shown to be involved in Ca^{2+}-dependent signaling downstream of many growth factors, and are known to activate transcription factors such as NF-κB (Rhee, 2006; Valko et al., 2007). ROS are required for neurogenesis in the central nervous system and have been shown to modulate synaptic plasticity in the hippocampus (Hongpaisan et al., 2004; Kennedy et al., 2012).

In this study, we test the hypothesis that ROS are involved in BMP-induced dendritic growth in sympathetic neurons. This hypothesis derives from the following observations: (1) ROS are important for neurite outgrowth in PC12 cells downstream of NGF stimulation or under hyperoxic conditions (Katoh et al., 1997; Suzukawa, 2000); (2) c-jun kinase and p21 kinase, which have been implicated in SMAD-independent mechanisms of BMP-induced dendritic growth (Podkowa et al., 2010, 2013), are also known to function upstream of ROS signaling in various cell types (Valko et al., 2007); (3) in non-neuronal cells, BMP-2 has been shown to activate NADPH oxidase, one of the enzymes that is important for production of ROS (Liberman et al., 2011; Simone et al., 2012); and (4) various isoforms of NADPH oxidase, the enzyme responsible for ROS production, are present in neonatal sympathetic neurons, in sympathetic ganglia and in sensory ganglia (Cao et al., 2009; Hilburger et al., 2005). Collectively, these data suggest a potential role for ROS signaling during BMP-induced dendritic growth in sympathetic neurons, and the data from this study support this hypothesis.

1.1. Experimental methods

1.1.1. Materials

Recombinant human bone morphogenetic proteins (BMPs) were generously provided by Curis (Cambridge, MA, USA). Nordihydroguaiaretic acid (NGA), desferroxamine (DFO), diphenyleneiodonium (DPI), cytosine-β-D arabinoside (Ara-C), 2,4-dinitrophenol (DNP) xanthine, xanthine oxidase, buthionine sulfoximine (BSO) and tertiary butyl H_2O_2 were obtained from Sigma Aldrich Corporation (St. Louis, MO). β-nerve growth factor was obtained from Harlan Laboratories (Indianapolis, IN). Thr101 and Ebselen, which are specific NOX2 inhibitors, were obtained from Millipore (Billerica, MA). Other tissue culture media components, gel electrophoresis supplies, DCF-DA and Mitosox™ were purchased from Life Technologies (Grand Island, NY).

1.1.2. Animals

All procedures involving animals were performed according to protocols approved by the Institutional Animal Care and Use Committees at the University of Buffalo (Buffalo, NY) or the University of California, Davis (Davis, CA). Timed-pregnant Holtzman rats were purchased from Harlan Laboratories (Indianapolis, IN) and timed-pregnant Sprague Dawley rats were purchased from Charles River Laboratories (Hollister, CA). All rats were housed individually in standard plastic cages in a temperature (22 ± 2 °C) controlled room on a 12 h reverse light–dark cycle. Food and water were provided ad libitum. Dams and pups were humanely euthanized prior to harvesting of superior cervical ganglia (SCG) from the pups for culture; no experimental manipulations were performed prior to euthanasia.

1.1.3. Tissue culture and transfection

Sympathetic neurons were dissociated from the SCG of perinatal (embryonic day 20 — postnatal day 1) Holtzman or Sprague Dawley rats according to previously described methods (Ghogha et al., 2012). Comparable results were obtained using cultures derived from the two different rat strains (data not shown). Cells were plated onto glass coverslips (Bellco Glass, Vineland, NJ) pretreated with poly-D-lysine (100 μg/ml, BD Biosciences, San Jose, CA) and maintained in serum-free medium containing NGF (100 ng/ml), bovine serum albumin (BSA, 500 μg/ml), bovine insulin (10 μg/ml) and human transferrin (10 μg/ml). To eliminate non-neuronal cells, the cultures were treated with the antimitotic Ara-C (1–2 μM) for 48 h beginning 24 h after plating. Experimental treatments were begun after the non-neuronal cells were eliminated.

NOX2, NOX4 and control siRNAs (Santa Cruz Biotechnology, Dallas, TX) were labeled with Cy5 using the Label IT siRNA labeling kit (Mirus Bio, Madison, WI). The labeled siRNAs (40 μM) were frozen at −20 °C prior to transfection. Sympathetic neurons were transfected using the GenMute™ siRNA transfection kit for primary neurons (SignaGen Laboratories, Rockville, MD) as described below. Following elimination of non-neuronal cells, sympathetic neurons derived from E21 rat pups were incubated with the complex containing GenMute™ transfection reagent and either control (90 pmol), NOX2 (90 pmol) or NOX4 (90 pmol) siRNA in growth medium at 37 °C in 5% CO2 incubator. After 4–5 h, the cultures were rinsed and maintained in normal growth medium. At 24 h after transfection, BMP-7 was added to cultures as described below and dendritic morphology was assessed in 50 to 70 Cy5-labeled cells per condition and compared to cells that were exposed to the transfection reagent alone (no siRNA).

1.1.4. Quantification of dendritic morphology

Following 3 days of treatment as described in the figure legend, cultures were fixed with 4% paraformaldehyde, immunostained and visualized by indirect fluorescence using previously described methods (Ghogha et al., 2012). Antibody against MAP-2 (1:5000, Millipore, Billerica, MA) or non-phosphorylated forms of M and H neurofilament subunits (SMI-32 at 1:5000, Millipore, Billerica, MA) were used to visualize dendrites. The fluorescent images were acquired using the Nikon Eclipse E400 fluorescent microscope and SPOT camera. Dendritic morphology was quantified in digitized images of neurons immunopositive for nonphosphorylated neurofilaments or MAP-2 using Metamorph software (Universal Imaging, West Chester, PA) and Image J freeware (NIH). Approximately 50 neurons per coverslip were analyzed from 2 to 3 coverslips per treatment. Experiments were replicated at least twice using cultures derived from independent dissections. Data are expressed as the mean ± SEM and statistically significant treatment-related differences were identified by one way ANOVA using a p value of 0.05 with differences between treatment groups identified using *post hoc* Tukey's test.

1.1.5. Western blotting

The effects of the antioxidants on axonal growth were assessed using a monoclonal antibody against the phosphorylated forms of M and H neurofilaments (1: 1000, SMI-31, Millipore, Billerica, MA). NADPH oxidase proteins levels were assessed using polyclonal antibodies against Nox2 or Nox4 (1: 200, Abcam, Cambridge, MA). GAPDH was used as a loading control and protein levels of GAPDH were assessed using a mouse or rabbit monoclonal antibody against GAPDH (Life Technologies, Grand Island, NY, Cell Signaling, Danvers, MA). After 3 days of experimental treatment, SCG cultures were lysed in a buffer containing 20 mM HEPES — pH 7.4, 150 mM sodium chloride, 1% Triton X-100, 10% glycerol, 2 mM EGTA and 1× Protease Inhibitor cocktail (Millipore, Billerica, MA). Lysates were heated to 70 °C in 4× LDS sample buffer (Life Technologies, Grand Island, NY), centrifuged and the supernatant from each sample separated by electrophoresis on a 4–12% Bis–Tris gel (Life Technologies, Grand Island, NY) and then immunoblotted onto a nitrocellulose membrane (Life Technologies, Grand Island, NY). The blots were incubated in Tris buffered saline (TBS) at pH 7.4 containing 5% dried nonfat powdered milk (Safeway, Phoenix, AZ) for 1 h, followed by overnight incubation at 4 °C with primary antibody. The blots were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000 in 5% dried milk in TBS) and visualized by chemiluminescence using the ECL Plus Western Blotting substrate (Thermo

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