Neuroscience Letters 595 (2015) 54–59

Contents lists available at ScienceDirect

Neuroscience Letters

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

Research article

Map kinase and PKC signaling pathways modulate NGF-mediated apoE transcription

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HIGHLIGHTS

- NGF increases apoE protein levels and transcription in PC12 cells.
- NGF-mediated apoE transcription requires the activation of MAP kinase and PKC.
- Inhibition of NOS attenuates but does not prevent NGF-induced apoE transcription.

ARTICLE INFO

Article history:
Received 2 October 2014
Received in revised form 16 March 2015
Accepted 18 March 2015
Available online 19 March 2015

Keywords:
Nerve growth factor (NGF)
Apolipoprotein E (apoE)
Signal transduction
Nitric oxide (NO)
PC12 cells

ABSTRACT

The present study assessed the mechanisms by which nerve growth factor (NGF) increased the level of apolipoprotein E (apoE) in PC12 cells. NGF (50 ng/mL) significantly increased apoE protein levels following 72 h of treatment. Similarly NGF increased luciferase activity in cells transfected with a luciferase reporter construct containing a 500 bp fragment of the apoE promoter, indicating NGF-induced apoE expression is regulated, at least in part, at the level of transcription. The non-selective nitric oxide synthase (NOS) inhibitor N^{ω}-nitro-L-arginine methylester (L-NAME; 20 mM) did not attenuate the NGF-mediated increase in luciferase activity, while the inducible NOS inhibitor s-methylisothiourea (S-MIU; 2 mM) partially attenuated this action of NGF. Inhibition of MAP kinase activation with 50 μM U0126 or pre-treatment with the PKC inhibitor bisindolylmaleimide 1 (BIS-1; 10 μM) prevented the NGF-mediated activation of the apoE promoter. Pre-treatment with the phospholipase C (PLC) inhibitor U73122 (5 μM) partially inhibited the NGF-induced increase in luciferase activity while the Akt inhibitor LY294002 (10 μM) had no effect. These data suggest NGF-induced apoE transcription requires MAP kinase and PKC activation and that these TrkA signaling pathways may be modulated by NO.

1. Introduction

The *apolipoprotein E* (*apoE*) gene encodes an essential protein associated with lipid transport and metabolism (reviewed in [1]) that has been implicated in Alzheimer's and other diseases [2]. In the brain apoE modulates neuron health and function by contributing to processes such as neurite growth, maintenance and repair [3–6] and the aggregation, clearance and metabolism of beta-amyloid (Aβ) [7–11], however, its effectiveness in these roles is often dependent on apoE genotype.

In humans, the major apoE isoforms are derived from three alleles, ϵ2, ϵ3 and ϵ4 (reviewed in [1,12]). Compared to individuals that are not ϵ4 carriers, the presence of the ϵ4 allele is associated with increased risk of coronary artery disease (reviewed in [13]) and the development of late-onset Alzheimer's disease (AD; reviewed in [14]). Although apoE genotype contributes to AD risk and ϵ4 carriers have increased Aβ levels compared to non-carriers [15] the role of apoE in Aβ accumulation is complex.

Studies have demonstrated that Aβ and apoE interact [16–18] however conflicting results have been reported regarding the effect of apoE on Aβ aggregation, clearance and degradation. ApoE was found to decrease Aβ fibril formation [19] suggesting elevations in apoE could be neuroprotective. However, the level and stability of Aβ oligomers was shown to be increased by apoE4 [20,21] suggesting elevations in apoE, particularly the E4 isoform, could have detrimental effects. With respect to clearance, apoE was reported

Abbreviations: ANOVA, analysis of variance; AD, Alzheimer's disease; AP-1, activator protein 1; APP, amyloid precursor protein; apoE, apolipoprotein E; BIS-1, bisindolylmaleimide 1; ChaT, choline acetyltransferase; L-NAME, N^{ω}-nitro-L-arginine methylester; LRP1, low density lipoprotein receptor-related protein 1; NGF, nerve growth factor; NOS, nitric oxide synthase; PLC, phospholipase C; SEM, standard error of the mean; S-MIU, s-methylisothiourea; TBS, tris-buffered saline.

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http://dx.doi.org/10.1016/j.neulet.2015.03.032
0304-3940/

to enhance neuronal uptake of soluble Aβ [22] but interfere with its uptake into astrocytes [23] and transport across the blood-brain barrier [24]. In multiple studies apoE was shown to enhance Aβ degradation [10,25,26], which was also influenced by apoE isoform [10]. Although both the isoform and level of apoE are important in modulating Aβ levels, little is known about the factors that regulate apoE expression. Interestingly, nerve growth factor (NGF), which enhances cognitive function in AD patients [27], was reported to modulate apoE expression [28]. Cloning and characterization of human and rodent apoE promoters has revealed that transcription is a key component in the regulation of apoE expression [29–31], suggesting NGF could alter apoE transcription.

NGF binds to TrkA and alters the expression of several genes (reviewed in [32]), including several that are associated with apoE. We determined previously that NGF enhances choline acetyltransferase (ChAT) expression in PC12 cells [33,34]. ChAT deficits, consistently found in AD, are greater in $\epsilon 4$ carriers compared with non $\epsilon 4$ carriers [35]. Similarly, apoE interacts with and may modulate the processing of amyloid precursor protein (APP) [36], regulates tau phosphorylation [37] and is a ligand for the low density lipoprotein receptor-related protein 1 (LRP1) receptor (reviewed in [38]). NGF increases the levels of all of these genes and these increases are attenuated by NGF-TrkA signaling pathway and/or nitric oxide synthase (NOS) inhibitors [39–42]. Similar mechanisms could also regulate the NGF-mediated changes in apoE levels. Therefore the present study first determined the effect of NGF on apoE protein levels and promoter activation and then assessed the effects of NOS and selective NGF-TrkA signaling pathway inhibitors on NGF-induced apoE promoter activation.

2. Material and methods

2.1. Cell culture and treatments

PC12 cells were maintained, plated and treated with 50 ng/mL NGF (Harlan Bioproducts for Science, Indianapolis, IN, USA) as described previously [39–41]. Cells were harvested at 24 h intervals for a maximum of 72 h for protein analysis or after 24 h for promoter analysis. Vehicle-treated (1 μL 0.02% acetic acid/mL media) cells collected at each time point were used as controls. The effects of NOS (Sigma–Aldrich, St. Louis, MO, USA) or TrkA signaling pathway (Calbiochem, San Diego, CA, USA) inhibitors on the NGF-mediated increase in luciferase activity was evaluated by pre-treating PC12 cells with one the following inhibitors 1 h prior to the addition of NGF: 20 mM N^{ω}-nitro-L-arginine (L-NAME), 2 mM s-methylisothiourea (S-MIU), 50 μM U0126, 10 μM LY294002, 10 μM bisindolylmaleimide 1 (BIS-1) or 5 μM U-73122 [40–45].

2.2. Immunoblot analysis

PC12 cells were lysed in 250 μL of Triton X lysis buffer (final concentration: 50 mM TrisHCl, pH 8.5, 150 mM NaCl and 1% TritonX-100) and 50 μg of total protein was boiled in Laemmli sample buffer (final concentration: 62.5 mM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate (w/v), and 0.01% bromophenol blue) for 3 min. Immunoblotting was carried out as described previously [39–44]. Membranes were incubated with 1:400 rabbit apoE antibody (Santa Cruz Biotechnology Inc., Dallas, TX, USA) in 5% non-fat milk in tris-buffered saline with 0.05% Tween-20 (TBS-T) overnight at 4 °C. The following day, bands were detected using luminol (Santa Cruz Biotechnology Inc.) and the Fluorochem 9900 imaging system (Alpha Innotech, Santa Clara, CA, USA) [44]. Membranes were stripped [44], exposed to 1:200 goat ribosomal protein 29 (RPL29) antibody in 1% non-fat milk in TBS-T and the bands quantified as described previously [39–44].

2.3. ApoE promoter construct and luciferase assay

PC12 cell DNA was extracted and PCR reactions carried out as previously described [39]. A 500 bp fragment of the apoE promoter with Mlu and BglII restrictions sites was amplified with the following primers: forward 5′-CGTACACGCGTGGTAGTATCAATCTTTAGCG-3′ and reverse 5′-ACGTAC AGATCT TGCGGAAGGAAAGCTGTC-3′. The purified PCR product was ligated into the pGL3 basic vector [39], purified by polyethylene glycol precipitation [45] and the identity of the apoE promoter confirmed by sequencing.

PC12 cells were co-transfected with the apoE-pGL3 basic plasmid and βSV plasmid encoding β-galactosidase using Transfectin™ (Bio-Rad Laboratories, Mississauga, ON, Canada) and following NGF treatment, cells were lysed and luciferase and β-galactosidase activity were measured and quantified as previously described [39,41,42].

2.4. Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). Data were assessed for normality and homogeneity of variance and statistical analysis was carried out using a two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons *t*-test to assess the effects of treatment and time on apoE protein levels and a one-way ANOVA followed by the Newman-Keuls multiple comparisons test to determine which groups were significantly different in promoter analysis experiments.

3. Results

3.1. Effect of NGF on apoE expression

A representative western blot depicting levels of *apoE* (upper panel) and RPL29 (lower panel) in control and NGF treated PC12 cells harvested at 24, 48 and 72 h is shown in Fig. 1A. Densitometric analysis (Fig. 1B) revealed an increase in relative apoE protein levels in cells treated with NGF for 72 h ($^{*}p < 0.05$).

ApoE transcription was assessed in PC12 cells transfected with pGL3 basic vector containing a 500 bp apoE promoter fragment. As seen in Fig. 1C, there was a 3-fold increase in relative luciferase activity in cells treated with NGF for 24 h compared to control cells ($^{**}p < 0.01$). This increase in luciferase activity was significantly higher than that measured in NGF-treated cells transfected with the pGL3 basic vector alone ($+p < 0.05$).

3.2. Effect of NOS and TrkA signaling pathway inhibitors on apoE promoter activation

The role of NO was assessed in control and NGF-treated cells pretreated with 2 mM S-MIU or 20 mM L-NAME (Fig. 2A). There was no difference in luciferase activity between the control and S-MIU treated groups. NGF increased luciferase activity relative to control in the presence and absence of S-MIU ($^{**}p < 0.01$) but levels were lower in the presence of S-MIU when compared to treatment with NGF alone ($+p < 0.05$). In contrast, L-NAME significantly increased luciferase activity relative to control ($^{**}p < 0.01$) and in the presence of NGF, this increase was higher than that observed following treatment with either NGF ($+p < 0.05$) or L-NAME ($^{*}p < 0.05$) alone.

The roles of the individual NGF-TrkA signaling pathways was assessed with 50 μM U0126, 10 μM LY294002, 5 μM U73122 or 10 μM BIS-1. As seen in Fig. 2B–D, treatment of PC12 cells with