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Role of Nitric Oxide in Activity-Dependent Neuronal Survival

Hye-Young Yun §, Tae Woon Kim, Kwang Jin Baek, Nyoun Soo Kwon, Hee Sung Lee,
Department of Biochemistry, Chung-Ang University, College of Medicine, Seoul 156-756, Korea.

§ Correspondence :
Hye-Young Yun, Ph D., Department of Biochemistry, Chung-Ang University, College of Medicine, 221 Heuksuk-dong, Dongjak-koo, Seoul 156-756, Korea. (Tel: (02) 820-5684, Fax: (02) 817-9866, E-mail: hyyun@cau.ac.kr)
윤혜영, 중앙대학교 의과대학 생화학교실, 서울시 동작구 흑석동 221 (우)156-756

Neural activity evoked by sensory stimuli and synaptic neurotransmission plays a critical role in the long-term neuronal changes such as development, survival, post-injury repair and memory (reviewed in Shatz, 1990). Calcium influx through N-methyl-D-aspartate (NMDA)-type glutamate receptors or voltage-sensitive calcium channels (VSCC) transduces activity-evoked signals to the nucleus and regulates gene expressions that are ultimately important for long-term neuronal changes. The molecular mechanisms by which synaptic activity-evoked calcium influx transmits signal to the nucleus are poorly understood.

Calcium-dependent activation of ras protein is thought to be a major signaling pathway in the long-lasting neuronal changes (Rosen *et al.*, 1994; reviewed in Finkbeiner *et al.*, 1996). Previous studies suggest the existence of multiple pathways that may be important in calcium-mediated activation of ras including Src, Ras-GRF, PYK2 and EGF receptor (Rusanescu *et al.*, 1995; Farnsworth *et al.*, 1995; Lev *et al.*, 1995; Rosen *et al.*, 1996). Despite the identification of these mediators of calcium-dependent ras activation, none of them have been directly demonstrated to mediate calcium dependent ras activation in neurons (Finkbeiner *et al.*, 1996). We proposed that nitric oxide (NO) is a key mediator in neurons for activation of the ras signaling pathway by NMDA receptor stimulation and that NO is a key link between NMDA-mediated changes in cytoplasmic calcium and activity-dependent long-term changes in neuronal responses (Yun *et al.*, 1997).

Our recent work using cerebral cortical neurons have shown that pharmacological inhibition or gene knockout of neuronal NO synthase (nNOS) completely blocked ras

activation by calcium influx through NMDA receptor stimulation (Yun *et al.*, 1998a; 1998b). Increases in intracellular calcium by NMDA receptor stimulation activated ras via calcium-dependent activation of nNOS and NO generation. NO directly activated ras GTPase activity in a guanylyl cyclase/cGMP-independent manner. Ras activation by NO in neurons may occur most likely through redox sensitive nitrosylation of Cys118 as shown previously by *in vitro* and tumor cell studies (Lander *et al.*, 1995; 1997). Our finding established that NO is a key neuronal mediator for the calcium signaling to ras.

A major issue derived from NO's role in linking cytoplasmic calcium and ras is that whether this signaling mechanism is critical for the activity-dependent long-term neuronal changes such as survival. Membrane depolarization by KCl promotes neuronal survival after serum or neurotrophic factor withdrawal and calcium influx through VSCC accounts for this effect (Scott, *et al.*, 1970; Gallo *et al.* 1987; Koike *et al.*, 1991). Futhermore, in cortical neurons calcium influx via VSCC induces brain-derived neurotrophic factor (BDNF) expression and secreted BDNF promotes neuronal survival (Ghosh *et al.*, 1994). Considerable progress has been made regarding *cis*- and *trans*-acting elements for calcium-dependent immediately early genes and BDNF gene expression (Shieh *et al.*, 1998; Tao *et al.*, 1998; Bito *et al.*, 1997). However, little is known about how calcium influx transduces survival signal to nucleus to regulate gene expression. We proposed that NO is an essential mediator of activity-dependent neuronal survival and is a key link between neural activity and long-lasting neuronal changes.

To test this hypothesis, we used PC12 cells neuronally differentiated by nerve growth factor (NGF). Upon NGF treatment, NOS expression (mostly calcium-dependent isoforms) starts to increase before cells cease to proliferate and maintains its high expression level in the fully differentiated state (Peunova *et al.*, 1995). Withdrawal of NGF leads to apoptosis of PC12 neurons. We found that pretreatment (10 min) of depolarizing concentration (50-60 mM) of KCl promoted survival of these cells after NGF withdrawal and this effect was completley blocked by pretreatment of NOS inhibitor, L-NAME. Effect of NOS inhibitor was specific since the effect was reversed by excess concentration of NOS substrate, L-arginine and inactive isomer D-NAME had no effect. Survival-promoting effect of KCl was blocked by EGTA and nifedipine, indicating that influx of extracellular calcium through L-type VSCC was critical for the depolarization-promoted survival. NO mediates depolarization-promoted survival via guanylyl cyclase/cGMP-independent mechanism since the effect of KCl was not blocked by guanylyl cyclase inhibitor, ODQ and

cell-permeable cGMP analog 8-Br-cGMP had little effect on survival of NGF-deprived cells. Addition of low micromolar concentrations of DETA-NO, an NO donor, mimicked the survival-promoting effect of KCl. Depolarization-promoted survival was reduced by actinomycin D or cycloheximide, suggesting the requirement of *de novo* gene expression. Treatment of cell-permeable ras inhibitor FPTI III abolished survival-promoting effect of KCl. Thus, these results suggest that calcium/NO/ras signaling pathway mediates membrane depolarization-promoted survival of PC12 neurons.

NO has been implicated in short-term changes in neuronal plasticity. Most of NO's physiologic actions in the nervous system are attributed to activation of guanylyl cyclase and increases in intracellular cGMP levels, or through interactions with the superoxide anion to mediate neurotoxicity. Our studies indicate that not only may nitric oxide be involved in short term changes in synaptic plasticity, but that it may be involved in long-term changes which involve regulation of gene transcription through ras signaling pathway. This mechanism may be particularly important during neuronal development and post-injury repair in which nNOS and ras are expressed at high levels (Aoki *et al.*, 1993; Bredt *et al.*, 1994; Finkbeiner *et al.*, 1996; Roskams *et al.*, 1994). Since NO is widespread messenger molecule in the central and peripheral nervous systems, NO-mediated activity-dependent neuronal survival and other long-lasting neuronal events may provide insights into a variety of use-dependent neuronal plasticity such as developmental plasticity, learning and memory, post-injury regeneration and rehabilitation.

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