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BRAIN RESEARCH

Research Report

17β-estradiol prevents reduction of retinal phosphorylated 14-3-3 zeta protein levels following a neurotoxic insult☆

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ABSTRACT

Previous studies demonstrated the substantial protective role of 17β-estradiol (E2) in several types of neuron, although its mechanism of action remains to be elucidated. In this study, we found that the levels of 14-3-3 zeta mRNA and phosphorylated and total 14-3-3 zeta proteins were significantly decreased in the rat retina after intravitreal injection of N-methyl-D-aspartate (NMDA). 17β-E2 implantation significantly inhibited NMDA-induced decreases in phosphorylated but not in total 14-3-3 zeta protein levels in the retina. There was a decrease in both phosphorylated and total 14-3-3 protein levels in RGC-5 cells, a retinal ganglion cell line, after glutamate and buthionine sulfoximine (BSO) exposure, and 17β-E2 treatment significantly inhibited only the decrease in phosphorylated but not in total 14-3-3 zeta protein levels. The cell viability assay showed substantial cell death after glutamate and BSO exposure and that 17β-E2 treatment significantly protects against this cell death. 17β-E2 treatment also significantly increased the level of phosphorylated 14-3-3 protein in RGC-5 cells without other treatments. These results suggest that a decrease in 14-3-3 zeta expression may be associated with retinal neurotoxicity induced by NMDA or the combination of glutamate and BSO. The regulation of 14-3-3 zeta phosphorylation is one possible mechanism of the protective effect of 17β-E2 in the retina.

1. Introduction

17β-Estradiol (E2) is a steroid hormone synthesized enzymatically from cholesterol and testosterone and has been reported to have crucial neuroprotective effects against some disease models or injuries in the central nervous system (Morissette et al., 2008; Numakawa et al., 2007; Yi and Simpkins, 2008). The protective effects of estrogen may be associated with the reduction of oxidative stress, regulation of transcription factors, and inhibition of inflammatory cytokines such as tumor necrosis factor (Behl et al., 1997; Liao et al., 2002; Sawada and Shimohama, 2000). In the eye, the protective effect of 17β-E2 against glutamate cytotoxicity has been demonstrated in a retinal ganglion cell line (RGC-5) without involvement of the classical estrogen receptors (Kumar et al., 2005). We previously demonstrated that pretreatment with 17β-E2 in a silastic implant, which

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allows maintenance of a high level of 17β-E2 in serum, exerts substantial protective effects on RGC survival after the intravitreal injection of N-methyl-D-aspartate (NMDA) (Hayashi et al., 2007). This 17β-E2-mediated protection is involved in the extracellular signal-regulated kinase (ERK) signaling pathway (Hayashi et al., 2007). However, the detail mechanisms of E2-mediated protection remain to be elucidated.

14-3-3 proteins exist in several types of neuron (Baxter et al., 2002; Watanabe et al., 1993) and are important mediators of anti-apoptotic signals (Masters and Fu, 2001). They are required for a presynaptic form of long-term potentiation in the cerebellum (Simsek-Duran et al., 2004). Among their seven isoforms (β: beta, γ: gamma, ε: epsilon, ς: zeta, η: eta, τ: tau, σ: sigma), zeta has been demonstrated to be a putative modulator of neurite growth dependent on L1, a cell adhesion molecule, in hippocampal neurons (Ramser et al., 2010). In addition, a recent study has demonstrated that depletion of 14-3-3 zeta levels using siRNA induces the expression of endoplasmic reticulum stress proteins, and that 14-3-3 zeta siRNA increases vulnerability to kainite-induced neuronal cell death (Murphy et al., 2008). Although high levels of 14-3-3 zeta have been found in the retina (Ivanov et al., 2006; Roseboom et al., 1994), its role in retinal cell survival or death remains to be elucidated.

Reports on 14-3-3 zeta focused on its phosphorylation mediated by several kinases, including protein kinases A and D, Akt, mitogen-activated protein kinase-activated kinase 2 (MK2), and sphingosine-dependent protein kinase 1 (Hamaguchi et al., 2003; Megidish et al., 1995, 1998; Powell et al., 2002, 2003). Phosphorylated 14-3-3 binds to multiple protein ligands, and phosphorylation-dependent binding with 14-3-3 modulates the subcellular localization, phosphorylationstate, and molecular interactions of many target proteins, thereby implicating 14-3-3 proteins as key regulators in diverse intracellular signal transduction pathways (Masters and Fu, 2001; Muslin et al., 1996; Yaffe et al., 1997). Although 17β-E2 alters the interaction with pBad and 14-3-3 binding in middle cerebral artery occlusion, it is not clear which specific kinases and molecules mediate phosphorylation under specific circumstances, nor are the biological consequences clear (Won et al., 2006).

The purpose of the present study was to examine whether changes occur in 14-3-3 zeta expression in retinal neurotoxicity in an in vivo and in vitro system and to investigate the effects of 17β-E2 on 14-3-3 zeta expression and its phosphorylation in both systems.

2. Results

2.1. *Changes in 14-3-3 zeta expression after NMDA injection in the retina*

To examine the expression of 14-3-3 zeta, we performed the real-time PCR using retinal samples. Real-time PCR showed that there was a substantial decrease in 14-3-3 zeta mRNA in rat retinal samples 24 h after NMDA injection compared with that after PBS injection (Fig. 1A). The products from completed real-time PCR runs were confirmed to be the specific amplification of 14-3-3 zeta cDNA showing the corresponding specific band upon agarose gel electrophoresis with ethidium bromide staining and UV transillumination (Fig. 1B). Immunohistochemical study showed abundant total 14-3-3 zeta immunoreactivity in the RGC layer in PBS-treated eyes (Fig. 1C). NMDA induced a decrease in total 14-3-3 zeta immunoreactivity in the RGC layer 24 h after injection (Fig. 1C).

2.2. *Effects of 17β-E2 on NMDA-induced changes in phosphorylated and total 14-3-3 zeta protein in the retina*

Next, we examined the changes in phosphorylated (at serine 58 of 14-3-3 zeta protein) and total 14-3-3 zeta protein in retina. Western blot analysis showed that NMDA induced a significant decrease in phosphorylated 14-3-3 zeta protein levels in the retina 24 h after injection (Fig. 2A). 17β-E2 implantation significantly prevented the decreases in phosphorylated 14-3-3 zeta protein levels induced by NMDA (Fig. 2A). On the other hand, total 14-3-3 zeta protein levels were significantly decreased 24 h after NMDA injection (Fig. 2B). NMDA affected the phosphorylation much more than the synthesis of 14-3-3 zeta in the retina (62.98% of phosphorylated 14-3-3 zeta reduction and 33.13% of total 14-3-3 zeta reduction). However, 17β-E2 implantation did not alter this decrease in total 14-3-3 zeta protein levels (Fig. 2B).

2.3. *Effects of 17β-E2 on cell viability in glutamate and buthionine sulfoximine-induced RGC-5 cell death*

To examine the effect of 17β-E2 in vitro, we conducted the cell viability assay using RGC-5 cells. The combination of glutamate and buthionine sulfoximine (BSO) resulted in substantial RGC-5 cell death (Fig. 3B) compared with the control (Fig. 3A), in agreement with the findings of our previous studies (Munemasa et al., 2008). Pretreatment of RGC-5 cells with 17β-E2 at concentration of 10 μM resulted in better-preserved cells (Fig. 3C). The results of the water-soluble tetrazolium salt (WST) assay showed that the combination of glutamate and BSO caused approximately 60% loss of cell viability, and this decreased cell viability was significantly prevented by pretreatment with 17β-E2 (Fig. 3D). According to our data and those from a previous study, 17β-E2 showed protective effects against not only glutamate but also glutamate and BSO treatment, which has greater oxidative impact, compared with glutamate alone (Kumar et al., 2005).

2.4. *Changes in phosphorylated and total 14-3-3 zeta protein in RGC-5 cells treated with glutamate and BSO with or without 17β-E2*

Changes in phosphorylated (at serine 58 of 14-3-3 zeta protein) and total 14-3-3 zeta protein were evaluated in RGC-5 cells. Western blot analysis showed that the combination of glutamate and BSO induced a significant decrease in phosphorylated 14-3-3 zeta protein levels in RGC-5 cells 24 h after exposure (Fig. 4A). Pretreatment with 17β-E2 significantly prevented the decreases in phosphorylated 14-3-3 zeta protein levels induced by glutamate and BSO (Fig. 4A). Although the combination of glutamate and BSO induced a significant decrease in total 14-3-3 zeta protein levels in RGC-5 cells 24 h