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Research Report

Estrogen attenuates glutamate-induced cell death by inhibiting Ca^{2+} influx through L-type voltage-gated Ca^{2+} channels

Eric A. Sribnick[a], *Angelo M. Del Re*[a], *Swapan K. Ray*[b], *John J. Woodward*[a], *Naren L. Banik*[a,*]

[a]*Division of Neurology, Department of Neurosciences, Medical University of South Carolina, 96 Jonathan Lucas Street, Charleston, SC 29425, USA*

[b]*Department of Pathology, Microbiology, and Immunology, University of South Carolina School of Medicine, Columbia, SC 29209, USA*

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ABSTRACT

Estrogen-mediated neuroprotection is observed in neurodegenerative disease and neurotrauma models; however, determining a mechanism for these effects has been difficult. We propose that estrogen may limit cell death in the nervous system tissue by inhibiting increases in intracellular free Ca^{2+}. Here, we present data using VSC 4.1 cell line, a ventral spinal motoneuron and neuroblastoma hybrid cell line. Treatment with 1 mM glutamate for 24 h induced apoptosis. When cells were pre-treated with 100 nM 17β-estradiol (estrogen) for 1 h and then co-treated with glutamate, apoptotic death was significantly attenuated. Estrogen also prevented glutamate-mediated changes in resting membrane potential and membrane capacitance. Treatment with either 17α-estradiol or cell impermeable estrogen did not mimic the findings seen with estrogen. Glutamate treatment significantly increased both intracellular free Ca^{2+} and the activities of downstream proteases such as calpain and caspase-3. Estrogen attenuated both the increases in intracellular free Ca^{2+} and protease activities. In order to determine the pathway responsible for estrogen-mediated inhibition of these increases in intracellular free Ca^{2+}, cells were treated with several Ca^{2+} entry inhibitors, but only the L-type Ca^{2+} channel blocker nifedipine demonstrated cytoprotective effects comparable to estrogen. To expand these findings, cells were treated with the L-type Ca^{2+} channel agonist FPL 64176, which increased both cell death and intracellular free Ca^{2+}, and estrogen inhibited both effects. From these observations, we conclude that estrogen limits glutamate-induced cell death in VSC 4.1 cells through effects on L-type Ca^{2+} channels, inhibiting Ca^{2+} influx as well as activation of the pro-apoptotic proteases calpain and caspase-3.

1. Introduction

Glutamate has a pivotal role both in normal function of the central nervous system (CNS) and in the pathophysiology of neurodegenerative diseases (Michaelis, 1998). While this amino acid is a common excitatory neurotransmitter, excessive glutamate is also the endogenous mediator of excitotoxicity, a central cause of neuronal death following traumatic brain injury (TBI) (Globus et al., 1995), stroke (Benveniste et al., 1984), and spinal cord injury (SCI) (Yanase et al., 1995). After an

* *Corresponding author*. Fax: +1 843 792 8626.
E-mail address: baniknl@musc.edu (N.L. Banik).

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insult to the CNS, resulting cell death can be either apoptotic or necrotic in nature, and while necrosis within the lesion is thought to be unpreventable, prevention of apoptosis within the neighboring tissue is viewed as a promising drug target (Dumont et al., 2001). As excitotoxic cell death can be apoptotic in nature (Finiels et al., 1995), a compound that could prevent glutamate-induced apoptosis would be of great importance to pharmacotherapy for neurodegenerative diseases and neurotrauma.

While some inherent regeneration following CNS trauma is possible (Mitsumoto et al., 1998), preventing neuronal death before it occurs may more effectively limit loss of neurological function. In the hours following SCI, the only current recommended pharmacotherapy is methylprednisolone, but some studies, which note drug efficacy, also outline possible side effects (Bracken et al., 1984). Furthermore, the clinical research supporting the use of methylprednisolone has also generated some controversy (Hurlbert, 2000). As estrogen has been shown to prevent neurodegeneration in ischemia (Dubal et al., 2001), TBI (Roof and Hall, 2000), and SCI (Sribnick et al., 2006b), we sought to examine how estrogen may prevent glutamate-induced apoptosis in a spinal motoneuron cell line. While the complex composition of the spinal cord and the multiple cell death pathways that are initiated following SCI cannot be replicated in cell culture studies, there are several advantages in examining motoneuron death *in vitro*. One cell type can be isolated, and the impact of a single mediator of cell death can be examined. As several possible mechanisms have been proposed for estrogen-mediated neuroprotection, research using cells in culture may allow more sensitive examination of possible mechanisms for estrogen-mediated neuroprotection. In order to further study the effects of estrogen on spinal motoneurons, we have chosen the VSC 4.1 cell line, a hybrid cell line formed by the fusion of embryonic rat ventral spinal cord motoneurons with N18TG2 mouse neuroblastoma cells (Smith et al., 1994).

There are several potential mechanisms involved in glutamate-induced cell death. Activation of N-methyl-D-aspartate (NMDA) receptors (Ahmed et al., 2002) and certain α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors (Bennett et al., 1996) readily allows calcium (Ca^{2+}) influx into the cell, raising intracellular [Ca^{2+}] (ic[Ca^{2+}]). Metabotropic glutamate receptors can also raise ic[Ca^{2+}] via the IP_3-mediated release of Ca^{2+} from the endoplasmic reticulum (Nash et al., 2001). Release of Ca^{2+} from intracellular stores can also occur with stimulation of ryanodine receptors (Favero et al., 1995). Other potential pathways for Ca^{2+} entry include activation of the voltage-gated Ca^{2+} channels (VGCCs) (Cano-Abad et al., 2001) or reversal of the Na^+/Ca^{2+} exchanger (NCX) (Berman and Murray, 2000).

Rises in ic[Ca^{2+}] can lead to several events, including upregulation of mitochondrial activity, mitochondrial dysfunction (Duchen, 2000), activation of phospholipases (Dhillon et al., 1999), and protease activation (Sur et al., 2003). One of the proteins activated by post-traumatic elevations in ic[Ca^{2+}] is the Ca^{2+}-activated neutral protease calpain. There are two major ubiquitous forms: μ-calpain (EC 3.4.22.52) and m-calpain (EC 3.4.22.53), requiring μM and mM Ca^{2+} levels for activation, respectively (Ray and Banik, 2003). While calpain does play a role in normal cell function (Bhatt et al., 2002), robust increases in ic[Ca^{2+}] can upregulate calpain activity, and over activation of this protease has been implicated in both necrosis (Gores et al., 1998) and apoptosis (Ray et al., 1999). Calpain has been observed to activate calcineurin through direct cleavage (Wu et al., 2004) and by degrading its endogenous inhibitor Cain/Cabin1 (Kim et al., 2002). Calpain also upregulates pro-apoptotic Bax activity (Wood et al., 1998), which eventually leads to downstream activation of caspase-3, another pro-apoptotic cysteine protease (Chan and Mattson, 1999). While some observations indicate that calpain and caspase-3 act antagonistically (Lankiewicz et al., 2000), other studies indicate that these proteases can act synergistically (Blomgren et al., 2001). Calpastatin, the endogenous inhibitor of calpain, is a caspase-3 substrate (Neumar et al., 2003). Calpain and caspase-3 cleave the cytoskeletal protein α-spectrin at specific sites, and calpain-specific cleavage generates a 145 kDa spectrin breakdown product (SBDP) while caspase-3-specific cleavage generates a 120 kDa SBDP (Wang et al., 1998).

A role for estrogen as a neuroprotectant has been demonstrated both *in vitro* (Sribnick et al., 2004) and *in vivo* (Dubal et al., 2001) in a variety of disease and cell death models (Sribnick et al., 2003). Furthermore, several clinical studies have shown gender differences in response to neurotrauma (Groswasser et al., 1998; Bayir et al., 2004). While estrogen has been shown to attenuate increases in ic[Ca^{2+}] (Nilsen et al., 2002) and to protect cells from excitotoxicity (Singer et al., 1999), the mechanism for such actions of estrogen has been elusive.

2. Results

2.1. *Changes in cell viability in VSC 4.1 cells following treatments*

In order to examine cell viability in VSC 4.1 cells, the MTT assay was used (Fig. 1). The four treatment groups examined were: control, 25 h with 100 nM estrogen, 24 h with 1 mM

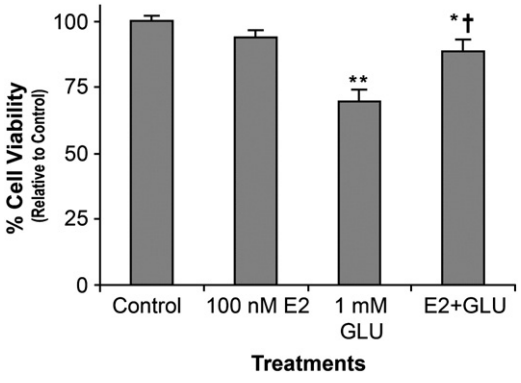

Fig. 1 – Determination of cell viability in VSC 4.1 cells. Treatment groups: control, 100 nM estrogen (E2) for 25 h, 1 mM glutamate (GLU) for 24 h, and 1 h pre-treatment with E2 followed by 24 h co-treatment with GLU (E2 + GLU). Cell viability was assessed using the MTT assay, and results were reported relative to control. Significant difference from control value was indicated by $^*P<0.05$ or $^{**}P<0.0001$, and significant difference between GLU alone and E2 + GLU was indicated by $^{\dagger}P<0.05$ ($n\geq6$).