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

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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.

Keywords

Apoptosis; Calpain; Ca^{2+} influx; Caspase-3; Glutamate; Motoneurons; Nifedipine

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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 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 $[\text{Ca}^{2+}]$ ($\text{ic}[\text{Ca}^{2+}]$). Metabotropic glutamate receptors can also raise $\text{ic}[\text{Ca}^{2+}]$ 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 $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) (Berman and Murray, 2000).

Rises in $\text{ic}[\text{Ca}^{2+}]$ 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 $\text{ic}[\text{Ca}^{2+}]$ 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 $\text{ic}[\text{Ca}^{2+}]$ 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 kD spectrin breakdown product (SBDP) while caspase-3-specific cleavage generates a 120 kD 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, 30 h with 100 nM estrogen, 24 h with 1 mM glutamate, and 1 h pretreatment with estrogen followed by 24 h cotreatment with glutamate. There was no significant difference between control cells and cells treated with estrogen ($P=0.17$). As compared to control, a 30% decrease in cell viability was noted in cells treated with glutamate alone ($P<0.0001$), and a 10% decrease was noted in cells treated with estrogen and glutamate ($P=0.045$). Treatment with estrogen did cause a significant 20% increase in cell viability, as compared to glutamate alone ($P=0.0014$), indicating that pretreatment with 100 nM estrogen could attenuate glutamate-induced cell death in the VSC 4.1 cells.

As the MTT assay does not distinguish between necrosis and apoptosis, we used other methods to determine the nature of death in VSC4.1 cells following the treatments (Fig. 2). The TUNEL assay was used to examine cell death-associated DNA fragmentation (Fig. 2A) and Wright staining was used to examine apoptotic cell morphology (Fig. 2C). When compared with control, cells treated with estrogen had no significant changes in either the number of cells exhibiting DNA fragmentation ($P=0.36$, Fig. 2B) or apoptotic morphology ($P=0.97$, Fig. 2D). Treatment with glutamate alone was associated with a 13-fold increase in apoptotic morphology ($P=0.0002$) and DNA fragmentation ($P \leq 0.0001$). Treatment with estrogen plus glutamate caused a 6-fold increase in apoptotic morphology ($P=0.032$) and a 7-fold increase in DNA fragmentation ($P=0.0002$), compared with control. Thus, compared with glutamate treated cells, cells pretreated with estrogen showed an approximately 50% decrease in both the number of cells exhibiting apoptotic morphology ($P=0.0073$) and the number of cells exhibiting DNA fragmentation ($P=0.00021$).

2.2. Electrophysiological recording in cells following treatments

In order to examine cell functionality, whole-cell voltage clamping and single cell recording were performed (Fig. 3). Resting membrane potential (RMP) was determined (Fig. 3A), and control cells were recorded as having an RMP of -48.7 mV and a membrane capacitance of 126.6 pF. There were no significant differences between RMP in control cells and in cells treated with either 100 nM estrogen ($P=0.56$) or cells treated with estrogen and 1 mM glutamate ($P=0.36$). Glutamate alone caused a significant 15 mV loss in RMP, compared with either control cells or cells treated with estrogen plus glutamate ($P \leq 0.0001$ for both). Membrane

capacitance was recorded as an indicator of cell size (Fig. 3B), and capacitance in control cells was not significantly different from either estrogen alone ($P=0.92$) or estrogen plus glutamate ($P=0.28$). Cells treated with glutamate alone demonstrated a highly significant 63% decrease in membrane capacitance ($P\leq 0.0001$), indicating shrinkage of the cells due to apoptosis. Cells treated with glutamate plus estrogen demonstrated a significant 2-fold increase in membrane capacitance, as compared to cells treated with glutamate alone ($P=0.0011$).

2.3. Treatment with 17 α -estradiol

As treatment with nM doses of estrogen was sufficient to prevent glutamate-induced cell death, a role for estrogen receptors (ERs) was examined by treating the cells with the less estrogenic 17 α -estradiol and then examining cell viability using the MTT assay (Fig. 4). Two concentrations of estradiol were used in these experiments (100 nM and 1 μ M) and treatment with neither 1 μ M 17 α -estradiol nor 1 μ M 17 β -estradiol caused a significant change in cell viability, compared with control ($P=0.05$ and 0.147 , respectively). Compared with control, glutamate alone caused a significant 25% decrease in cell viability ($P\leq 0.0001$), as did glutamate plus either dose of 17 α -estradiol ($P\leq 0.0001$). Glutamate plus either 100 nM or 1 μ M 17 β -estradiol caused less than a 15% decrease in cell viability, compared with control ($P=0.0027$ and 0.00052 , respectively). In comparison with glutamate alone, there was no significant difference in cell viability in cells treated with either 100 nM or 1 μ M 17 α -estradiol ($P=0.50$ and 0.78 , respectively); however, concentrations of both 100 nM and 1 μ M 17 β -estradiol did significantly increase cell viability ($P=0.0002$ and 0.0011 , respectively). Significant differences were also seen when comparing cells treated with 17 α -estradiol plus glutamate with those treated with 17 β -estradiol plus glutamate. In cells treated with glutamate, both the 100 nM and 1 μ M doses of 17 β -estradiol were associated with significantly lower levels of cell death than equal concentrations of 17 α -estradiol ($P=0.0012$ and 0.0023 , respectively). While these data suggested that the cytoprotective effects of estrogen were ER-mediated, there were no significant differences in protein levels of either ER α or ER β when samples from all 4 treatment groups were analyzed by Western blotting (data not shown).

2.4. Ca²⁺ levels in cells treated with glutamate and estrogen

As previous studies in our laboratory have indicated that both physiologic (Sribnick et al. 2006a) and supraphysiologic (Sur et al., 2003) concentrations of estrogen may alter post-traumatic ic[Ca²⁺], the Ca²⁺ sensitive dye fura-2 was used to measure ic[Ca²⁺] levels in treated cells (Fig. 5). Basal ic[Ca²⁺] in control VSC 4.1 cells was 81.9 nM, and treatment with 100 nM estrogen caused no significant changes in ic[Ca²⁺] ($P=0.36$). Compared with control, treatment with 1 mM glutamate caused a significant 2-fold increase in ic[Ca²⁺]. Cells treated with estrogen and glutamate demonstrated levels of ic[Ca²⁺] that were significantly higher than control ($P=0.030$) but were also significantly lower than in cells treated with glutamate alone ($P=0.021$).

2.5. Calpain and caspase-3 activities following treatments

Because of the finding that estrogen prevented glutamate-induced increases in ic[Ca²⁺], activities of the Ca²⁺-sensitive calpain and the downstream protease caspase-3 were examined by Western blotting (Fig. 6). The calpain-specific 145 kD SBDP (Fig. 6A) and the caspase-3-specific 120 kD SBDP (Fig. 6B) were determined. Compared with control, treatment with estrogen was not associated with a significant change in either the calpain activity ($P=0.94$) or the caspase-3 activity ($P=0.16$). Treatment with glutamate alone caused a 46% increase in both calpain activity and caspase-3 activity ($P=0.0004$ and 0.0026 , respectively), compared with control. When cells were treated with estrogen and glutamate, neither the calpain activity nor the caspase-3 activity ($P=0.49$ and 0.28 , respectively) was significantly different from control.

Compared with cells treated with glutamate alone, cells treated with glutamate and estrogen demonstrated significantly less calpain activity ($P=0.0016$) and caspase-3 activity ($P=0.021$).

2.6. Using Ca^{2+} antagonists and agonists to elucidate a mechanism of action for estrogen

As estrogen was shown to attenuate both $\text{ic}[\text{Ca}^{2+}]$ and calpain activity, a role for estrogen in blocking increases in $\text{ic}[\text{Ca}^{2+}]$ was examined using Ca^{2+} entry inhibitors. Cells were treated with glutamate and non-lethal, saturating doses of Ca^{2+} entry inhibitors in order to determine the pathway for Ca^{2+} entry following glutamate treatment (Fig. 7). Inhibitors used (at concentrations less than IC_{50} values) to prevent the influx of extracellular Ca^{2+} included APV for NMDA-R, CNQX for AMPA-R, KBR for NCX, and nifedipine for L-type VGCC. The possibility that Ca^{2+} might be released from intracellular stores was also examined using 2-APB to block IP3-mediated Ca^{2+} release and DAN to inhibit the ryanodine receptor. Treatment with 1 mM glutamate significantly decreased 42% cell viability ($P \leq 0.0001$). Treatment with glutamate plus 100 nM estrogen significantly attenuated cell death ($P \leq 0.0001$), compared with glutamate alone. Compared with glutamate alone, treatment with glutamate and either 1 μM APB or 10 μM DAN did not cause a significant change in cell viability ($P=0.45$ and 0.39 , respectively). A significant attenuation in cell death was seen when cells were treated with glutamate and 5 μM APV ($P=0.0006$), 1 μM CNQX (0.0024), or 1 μM KBR (0.012); however, in each case, a significantly higher cell viability was noted following treatment with glutamate and 100 nM estrogen. Only treatment with 100 nM nifedipine was as effective as 100 nM estrogen in limiting glutamate-induced cell death ($P=0.60$).

In order to further evaluate the role of L-type VGCC in VSC 4.1 cells treated with glutamate and the effect of estrogen on L-type VGCC, cells were treated with 100 nM FPL, an L-type VGCC agonist (Fig. 8). Using the MTT assay (Fig. 8A), treatment with 100 nM FPL caused a 40% decrease in cell viability, compared with control ($P \leq 0.0001$). Treatment with 100 nM estrogen and FPL also caused a significant decrease in cell viability, compared with control ($P \leq 0.0001$). However, cell viability in cells treated with estrogen plus FPL was significantly higher than viability in cells treated with FPL alone ($P=0.0064$).

To further examine the effect of estrogen on L-type VGCC, $\text{ic}[\text{Ca}^{2+}]$ was also examined (Fig. 8B). Basal $\text{ic}[\text{Ca}^{2+}]$ in the VSC 4.1 cells was measured at 106.2 nM, and there was no significant change in cells treated with estrogen alone ($P=0.71$). Treatment with FPL caused a 5-fold increase in $\text{ic}[\text{Ca}^{2+}]$ ($P \leq 0.0001$). Levels of $\text{ic}[\text{Ca}^{2+}]$ in cells treated with FPL and estrogen was not significantly different from control ($P=0.17$) but was significantly less than $\text{ic}[\text{Ca}^{2+}]$ in cells treated with FPL alone ($P=0.0002$).

3. Discussion

There is an increasing recognition that steroid hormones have a role in normal CNS function as well as in preventing cell death in neurotrauma or neurodegenerative disease. This work has involved estrogen to a large extent; however, recent discoveries such as a second ER (i.e. ER β) (Kuiper et al., 1996), the putative membrane ER (Towle and Sze, 1983), and rapid effects of estrogen on cell signaling pathways (Singer et al., 1999) have further complicated these studies. Previous work in our laboratory indicated that estrogen-mediated cytoprotection may involve attenuation of post-traumatic Ca^{2+} influx and downstream calpain activation (Sur et al., 2003; Sribnick et al., 2004; Sribnick et al., 2006a). Furthermore, estrogen has been shown to protect the CNS tissue following SCI (Sribnick et al., 2006b), so we examined the effects of estrogen on a spinal motoneuron hybrid cell line in order to elucidate a possible mechanism of action for this protection. Treatment with 1 mM glutamate for 24 h induced significant levels of cell death in VSC 4.1 cells (Fig. 1), and apoptosis was confirmed by assaying DNA fragmentation and examining cell morphology (Fig. 2). Beyond simply preventing cell death,

estrogen also prevented glutamate-induced changes in RMP and membrane capacitance (Fig. 3).

While higher levels of glutamate have been used to induce cell death in this cell line (La Bella et al., 1996), our focus was on inducing apoptosis, so a lower effective dose was used. As glutamate-induced cell death may be related to the generation of reactive oxygen species (Sattler et al., 1999), one possible explanation for the present results is that the natural anti-oxidant effects of estrogen inhibited oxidative stress. Indeed, the phenolic ring structure of estrogen is thought to be responsible for the strong anti-oxidant properties of this hormone (Green et al., 2000). Nonetheless, we do not think that our findings are merely due to anti-oxidant effects of estrogen. First, dose response studies have shown that anti-oxidant effects of estrogen are generally seen with at least low μM concentrations (Moosmann and Behl, 1999). As estrogen-induced protein expression occurs 2 h following hormone treatment (Barnea and Gorski, 1970), the 1 h pre-incubation that was required for cytoprotection suggested a requirement for *de novo* protein synthesis. Further evidence to indicate that the present observations are ER mediated is that equal concentrations of the less estrogenic 17α -estradiol fail to replicate findings seen with 17β -estradiol (Fig. 4). While 17α -estradiol has been shown to induce cytoprotection in spinal motoneurons, these effects are not ER-mediated and required μM concentrations of hormone (Nakamizo et al., 2000). Estrogen treatment has been noted in some studies to enhance ER expression (Ihionkhan et al., 2002; Sribnick et al., 2006a) and in others to enhance ER removal by promoting ubiquitination and proteasome-mediated degradation (Lee et al., 2002). In the present study, estrogen-mediated changes in ER expression at the translational level were not noted (data not shown).

Activation of cell signaling by estrogen has also been suggested as a pathway responsible for cytoprotection. However, this is also an unlikely explanation for the present findings as estrogen-mediated CREB phosphorylation occurs within 15 min and MAPK activation within 30 min (Singer et al., 1999; Honda et al., 2001). Furthermore, MAPK activation may be related to stimulation of membrane-ER (Toran-Allerand et al., 2002), and stimulation of putative membrane ERs with BSA-E2 does not mimic the results that we have seen with unconjugated hormone (data not shown).

One possible explanation for estrogen-mediated cytoprotection may be attenuation of rises in $\text{ic}[\text{Ca}^{2+}]$ that can occur following trauma or exposure to toxin. Our laboratory (Sur et al., 2003; Sribnick et al., 2006a) and others (Goodman et al., 1996) have shown a correlation between cytoprotection and inhibition of increases in $\text{ic}[\text{Ca}^{2+}]$. In the present study attenuation of $\text{ic}[\text{Ca}^{2+}]$ (Fig. 5) was associated with significant decreases in both calpain and caspase-3 activities (Fig. 6). Such observations are also seen *in vivo*. In one study examining TBI in rats, a gender difference was noted: formation of the calpain-specific 145 kD SBDP occurred at a later time-point in females (Kupina et al., 2003). There are several examples in the literature of antagonistic effects between ER and calpain. While calpain activity upregulates calcineurin activity (Kim et al., 2002; Wu et al., 2004), treatment with estrogen has been observed to reduce calcineurin activity (Sharroff et al., 2002). Calpain activation may facilitate translocation of pro-apoptotic Bax to the mitochondrial membrane (Wood et al., 1998) while ER activation has been noted to upregulate anti-apoptotic Bcl-2 expression (Alkayed et al., 2001). Finally, while ER degradation is primarily proteasome-mediated (Lee et al., 2002), ER is a noted calpain substrate (Murayama et al., 1984), and estrogen treatment has been shown to attenuate both calpain activity (Tiidus et al., 2001; Sur et al., 2003; Sribnick et al., 2006a) and protein levels (Sribnick et al., 2004). ER activation may also down regulate caspase-3 activity. Treatment with either estrogen (Linford and Dorsa, 2002) or phytoestrogens (Wang et al., 2001) *in vitro* has been shown to inhibit caspase-3 activity, and estrogen withdrawal in chicks leads to an upregulation of caspase-3 activation and activity (Monroe et al., 2002).

In order to further understand the mechanism of estrogen-mediated cytoprotection in VSC 4.1 cells, the pathway for glutamate-induced increases in $ic[Ca^{2+}]$ was examined. The possibility that Ca^{2+} increases were from an extracellular source was examined by treating cells with inhibitors for NMDA-R, AMPA-R, NCX reverse-mode, and L-type VGCC (Fig. 7). Several effects have been attributed to estrogen treatment, including direct inhibition of NMDA-R activation (Weaver et al., 1997), direct inhibition of AMPA-R activation (Xue and Hay, 2003), direct inhibition of L-type VGCC (Chaban et al. 2003), and down regulation of channel subunits (Ba et al. 2004). As $ic[Ca^{2+}]$ can also be affected by release from intracellular stores, inhibitors for IP₃-R and ryanodine receptor were also used. In previous studies, estrogen has been observed to prevent thapsigargin-induced apoptosis (Linford and Dorsa, 2002) and to inhibit the release of Ca^{2+} from intracellular stores (Morales et al., 2003). Only treatment with nifedipine demonstrated results similar to those seen with estrogen treatment (Fig. 7).

To further establish a correlation between estrogen and L-type VGCC, VSC 4.1 cells were treated with the L-type VGCC agonist FPL (Fig. 8). In previous studies, estrogen treatment prevented $[Ca^{2+}]$ increases mediated by another L-type VGCC agonist Bay K 8644 (Kurata et al., 2001). In the present study, estrogen significantly attenuated FPL-induced increases in $ic[Ca^{2+}]$ and cell death (Fig. 8). While other studies (Kim et al., 2000; Kurata et al., 2001; Chaban et al., 2003) have found that large concentrations of estrogen are needed to effect rapid changes in the electrophysiological properties of the cell, this study utilized a nM dose of estrogen. These differences might be due to the levels of toxin used: a previous study indicated that while estrogen prevented necrosis when used in μ M doses with a short preincubation, prevention of apoptosis required only nM doses but a longer period of preincubation (Harms et al., 2001).

In conclusion, we propose on the basis of our results that estrogen treatment attenuates glutamate-induced apoptosis in VSC 4.1 cells by modulating the L-type VGCC and inhibiting both Ca^{2+} influx and downstream activation of the pro-apoptotic proteases such as calpain and caspase-3. Future research will examine more precisely how estrogen may modify VGCC activity, and *in vivo* experiments will be performed to examine whether such hormone-related activities occur in SCI rats after estrogen treatment.

4. Experimental procedures

4.1. Cell culture

The VSC 4.1 cell line is a hybrid of neuroblastoma and spinal motoneuron and was obtained from Stanley H. Appel, M.D. (Baylor College of Medicine, Houston, TX, USA). As described previously (Smith et al., 1994), cells were grown in DMEM/F12 media with 2% defined bovine calf serum (Hyclone, Logan, UT, USA), 2% 50x Sato's medium (Bottenstein and Sato, 1979), and 1 % penicillin and streptomycin. Each cultureware was pre-coated with poly-L-ornithine (Sigma, St. Louis, MO, USA). Cells were incubated at 37°C with 5% CO₂ in a fully humidified incubator. Prior to experiments, cells were transferred from DMEM/F12 medium with 2% bovine calf serum to phenol red-free serum poor medium with 0.5% bovine calf serum, and cells were incubated in serum-poor medium for 24 h. Unless otherwise indicated, the 4 treatment groups were: control, treatment with 100 nM estrogen for 25 h, treatment with 1 mM L-glutamate (Sigma) for 24 h, and 1 h pretreatment with estrogen followed by co-treatment with glutamate. Cells were harvested immediately following treatment. Dose-response studies were conducted with glutamate to determine an apoptotic dose, and further dose-response curves were generated to determine the lowest protective dose of estrogen.

4.2. Reagents and antibodies

Stock solutions of 17 β -estradiol (Sigma) and 17 α -estradiol (Sigma) were made in the lowest volume of dimethyl sulfoxide (DMSO, Sigma) possible. Fresh glutamate (Sigma) solutions

were prepared on a daily basis, and glutamate was dissolved in phenol red-free DMEM/F12 media. Bovine serum albumin (BSA)-conjugated 17 β -estradiol [(1,3,5(10)-estratrien-3, 17 β -diol 17-hemi-succinate:BSA] (Steraloids, Newport, RI, USA) was filtered to remove any unconjugated estrogen, as previously reported (Stavis et al., 1999). Antibodies used were: polyclonal estrogen receptor α (ER α , C1355, Upstate, Lake Placid, NY, USA), polyclonal estrogen receptor β (ER β , H-150, Santa Cruz Biotech, Santa Cruz, CA, USA), β -actin (clone AC-15, Sigma), and α -spectrin (clone AA6, Affiniti, Exeter, UK). Other reagents used include KB-R7943 (KBR, Tocris), DL-2-amino-5-phosphonopentanoic acid (APV, Sigma), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, Sigma), nifedipine (Spectrum, Gardena, CA, USA), 2-aminoethoxydiphenylborane (2-APB, Tocris), dantrolene (DAN, Sigma), and FPL 64176 (FPL, Sigma).

4.3. MTT assay

Cells were distributed onto 96-well plates at 15,000 cells/well and were maintained for one day in phenol red-free DMEM/F12 media with 2% bovine calf serum. The following day, the media was replaced for 24 h with phenol red-free, low-serum DMEM/F12. Experiments occurred on the following day, and following experimental treatments, 50 μ g of MTT (Sigma), dissolved in phosphate buffered saline (PBS), was added to each well. Following 1 h incubation, plates were centrifuged at 1900 x g for 10 min, medium was then removed and replaced with DMSO. Following another 30 min incubation, plates were examined for the conversion of the tetrazolium salt to a purple formazan product by assessing absorbance at 570 nm using a microplate reader (ELx800, Bio-Tek, Winooski, VT, USA). Absorbance was reported as a percentage of control.

4.4. TUNEL assay

Following treatment of cells, the terminal deoxynucleotidyl transferase dUTP-mediated nick end labeling (TUNEL) assay was used to monitor cell death by examining DNA fragmentation, as we described recently (George et al., 2009). Briefly, cells were grown on 6-well cell culture plates (Corning, Corning, NY, USA) and treated as described above. Following experimental treatment, cells were washed with PBS and then sedimented onto microscopic slides. Residual PBS was then removed, and cells were fixed using 95% ethanol. A second fixation was performed using 4% methanol-free formaldehyde in PBS. Slides were again washed with PBS, and fragmented DNA was detected in apoptotic cells by adding fluorescein 12-dUTP to nick ends of DNA (Apoptosis Detection System, Promega, Madison, WI, USA). Slides were incubated for 1 h at 37 °C, and the reaction was terminated with 2x sodium chloride/sodium citrate (2xSSC buffer, Promega). The slides were washed in PBS and then visualized with a fluorescent light microscope at 400x, and green fluorescence correlated with DNA fragmentation. Experiments were done in triplicate, and the percentage of TUNEL-positive cells was determined.

4.5. Wright staining

Cells were grown on 6-well plates and harvested by scraping. Both floating and attached cells were obtained. Cells were washed twice in PBS and sedimented onto microscopic slides using cytobucket and an IEC Centra CL2 centrifuge (San Lorenzo, CA, USA) at 106 x g for 5 min. Once sedimented onto slides and air-dried, cells were then stained using a Wright stain protocol (Ray et al., 1999). At least 600 total cells were counted, and cells were only labeled apoptotic if they showed cell shrinkage and condensation of the chromatin and/or cell membrane blebbing. Cells exhibiting apoptotic morphology were reported as a percentage of total cells.

4.6. Electrophysiology

VSC 4.1 cells were grown on 35-mm cell culture dishes and were treated as described above. Culture dishes were then placed on the stage of an Olympus IX70 microscope (Olympus, Melville, NY, USA). Culture media was removed and replaced by a continuously perfused recording solution that contained (in mM): 135 NaCl, 5 KCl, 1.8 CaCl₂, 10 glucose, and buffered with 5 HEPES (pH adjusted to 7.2 with NaOH; osmolarity adjusted to 325 mOsm with sucrose). Standard whole-cell patch clamp techniques were employed for recording (Hamill et al., 1981). The pipette recording solution contained (in mM): 150 KCl, 2.5 NaCl, 4 Mg-ATP, 2 Na₂ATP, 0.3 NaGTP, 5 Na₂phosphocreatine, buffered with 10 HEPES (pH adjusted to 7.4 with NaOH; osmolarity adjusted to 310 mOsm with sucrose). Whole-cell patch clamp recordings were made using an Axopatch 200B amplifier equipped with a CV 203BU thermal cooled headstage (Axon Instruments, Union City, CA, USA). Experiments were performed at room temperature and viewed on a Macintosh computer running AxoGraph software. Patch electrodes were pulled from thin-walled borosilicate glass with inner filament (Warner, Hamden, CT, USA) to an open resistance of 3–5M. Cells were voltage clamped at –60 mV initially, then the resting membrane potential was directly read from the amplifier by setting the clamp control to current = 0 setting. Membrane capacitance was read from AxoGraph and also at the cell's resting membrane potential. Membrane capacitance was used to determine relative cell size as this value reflects the size of the neuron, indicating the total number of ion channels in the membrane (Levitan and Kaczmarek, 1997).

4.7. Western blot analysis

We analyzed the levels of specific proteins in the cells using the Western blotting, as we described previously (Karmakar et al., 2007; Das et al., 2008). Briefly, VSC 4.1 cells were grown in T-75 flasks, treated as described above, and harvested following treatment. Cells were resuspended and homogenized in homogenizing buffer [50 mM Tris (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, and 5 mM EGTA]. Cells were homogenized by sonication (Micro Ultrasonic Cell Disrupter, Kontes, Vineland, NJ, USA), and protein concentration was determined using Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL, USA) with spectrophotometric measurement at 595 nm (Spectronic, Rochester, NY, USA). Equal volume sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 5 mM β-mercaptoethanol, 10% glycerol) was added to each sample, and samples were then boiled for 5 min. Prior to gel electrophoresis, samples were diluted to 1 mg/ml protein with equal volume sample and homogenizing buffer, and 30 μl of each sample was loaded onto gels. For assaying ERα and ERβ, 4–20% gradient gels (Bio-Rad, Hercules, CA, USA) were used and were electrophoresed at 200 V for 30 min. Electrophoresis for α-spectrin was performed using 5% gels run for 4 h at 50 V. Protein was then transferred from the gel to a nylon membrane (Millipore, Bedford, MA, USA) in an electrophoresis transfer apparatus (Genie, Idea Scientific, Minneapolis, MN, USA). Membranes were blocked for 1 h in 5% powdered nonfat milk dissolved in a Tris/Tween solution (20 mM Tris-HCl, pH 7.6, 0.1% Tween-20 in saline). Primary antibody was diluted in blocking buffer and incubated with the membrane overnight. Membranes were then incubated in secondary antibody (ICN Biomedicals, Aurora, OH, USA) at a 1:2000 dilution for 1 h. Between steps, membranes were washed three times in Tris/Tween solution. Membranes were incubated with ECL Western blotting detection reagents (Amersham Pharmacia, Buckinghamshire, UK) and exposed to X-OMAT AR films (Eastman Kodak, Rochester, NY, USA). Films were scanned on a UMAX PowerLook 1000 Scanner (UMAX Technologies, Fremont, CA, USA) using Photoshop software (Adobe Systems, Seattle, WA, USA), and optical density of each band was determined using Quantity One software (Bio-Rad). Calpain and caspase-3 activities were measured by monitoring the formation of SBDPs: the 145 kD SBDP being specific for calpain and the 120 kD SBDP being specific for caspase-3 (Wang et al., 1998).

4.8. Determination of ic[Ca²⁺] using fura-2

After the treatments, the levels of ic[Ca²⁺] were determined in VSC4.1 cells using the fluorescence Ca²⁺ indicator fura-2 as we described in a previous report (Das et al., 2005), which was a modification of the original method (Grynkiewicz et al., 1985) for determination of ic [Ca²⁺]. VSC 4.1 cells were grown in phenol red-free RPMI DMEM/F12 for 72 h. Cells were then removed by trituration, resuspended in medium, and incubated at 37 °C for 2 h with gentle shaking. After incubation, cells were washed twice and resuspended in Locke's buffer [(mM): 154 NaCl, 5.6 KCl, 3.4 NaHCO₃, 1.2 MgCl₂, 5.6 glucose, 5 HEPES (pH 7.4), and 2.3 CaCl₂] (Grynkiewicz et al., 1985). Fura-2 (Molecular Probes, Eugene, OR, USA) was dissolved in DMSO, and cells were incubated in 5 μM fura-2 at 37 °C for 30 min. Cells were then washed three times and resuspended in cold Locke's buffer. [Ca²⁺] concentration was calculated using the equation $[Ca^{2+}] = K_d(R - R_{min}) / (R_{max} - R)$. Spectrophotometric analysis of the fluorescence ratio (R) was done using an SLM 8000 fluorometer at 340 nm and 380 nm wavelengths (Thermospectronic, Rochester, NY, USA). Maximal (R_{max}) and minimal (R_{min}) ratios were determined using 250 μM digitonin (Fisher Scientific, Two Rivers, WI, USA) and 500 mM EGTA (Sigma), respectively.

4.9. Statistical analysis

Results were analyzed using JMP In software (SAS Institute, Cary, NC, USA). Results were compared using one-way analysis of variance (ANOVA) with Fisher's protected least significant difference post-hoc test at a 95% confidence interval. Data were presented as mean ± standard error of the mean (SEM) of separate experiments ($n \geq 3$). Difference between two treatments was considered significant at $P \leq 0.05$.

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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 are 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 † $P < 0.05$ ($n \geq 6$).

Fig. 2.

Determination of apoptotic DNA fragmentation and cell morphology after the treatments of VSC4.1 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). To examine cells for apoptotic changes, DNA fragmentation was assessed using the TUNEL assay (A) and then quantification of TUNEL+ cells was performed (B). Apoptotic cell morphology was assessed using the Wright staining (C) and then quantification of cells exhibiting apoptotic morphology was performed (D). Significant difference from control values was indicated by * $P < 0.05$ or ** $P < 0.0001$, and significant difference between GLU alone and E2 + GLU was indicated by † $P < 0.05$ ($n \geq 3$).

Fig. 3. Resting membrane potential and membrane capacitance in VSC4.1 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). Cells were targeted to assay whole-cell recording for resting membrane potential (A) and membrane capacitance (B). Significant difference from control values was indicated by ** $P < 0.0001$, and significant difference between GLU alone and E2 + GLU was indicated by †† $P < 0.0001$ ($n \geq 16$).

Fig. 4.

Determination of cell viability following the treatments of VSC4.1 cells. Treatment groups: control, 1 μM 17β-estradiol (17β-E2) for 25 h, 1 μM 17α-estradiol (17α-E2) for 25 h, 1 mM glutamate (GLU) for 24 h, and pre-treatment for 1 h with 100 nM or 1 μM 17β-E2 or 17α-E2 followed by 24 co-treatment with 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 ** $P < 0.0001$. Significant difference between GLU alone and (17β-E2 + GLU) or (17α-E2 + GLU) was indicated by † $P < 0.05$, and significant difference between (17β-E2 + GLU) and (17α-E2 + GLU) was indicated by ‡ $P < 0.05$ ($n \geq 6$).

Fig. 5.

Determination of $\text{ic}[\text{Ca}^{2+}]$ in VSC4.1 cells following treatments. Fura-2 was used to measure $\text{ic}[\text{Ca}^{2+}]$ in VSC4.1 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). 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 † $P < 0.05$ ($n \geq 4$).

Fig. 6.

Western blotting for determining calpain and caspase-3 activities in the formation of SBDPs. Calpain and caspase-3 activities were measured in the formation of the calpain-specific 145 kD SBDP and the caspase-3-specific 120 kD SBDP (A). Treatment groups: control, 100 nM estrogen (E2) for 25 h, 1 mM glutamate (GLU) for 24 h, and 16 h pre-treatment with E2 followed by 24 h co-treatment with GLU (E2 + GLU). Densitometry (B, C) was performed to report results (B and C) as OD relative to control. Significant difference from control values was indicated by * $P < 0.05$, and significant difference between GLU alone and E2 + GLU was indicated by † $P < 0.05$ ($n \geq 4$).

Fig. 7.

Treatment of VSC4.1 cells with Ca^{2+} entry inhibitors. Cell viability in VSC 4.1 cells was examined using the MTT assay following treatments with different agents. Control cells were compared with cells treated with glutamate (GLU) alone for 24 h. Other treatment groups were 1 h pre-treatment with 100 nM E2, treatment with a Ca^{2+} entry inhibitor, and then co-treatment with GLU for 24 h. Inhibitors examined included: 5 μM APV, 1 μM CNQX, 1 μM KBR, 100 nM nifedipine, 1 μM 2-APB, and 10 μM DAN. Cell viability was reported relative to control. Significant difference from control value was noted in all treatment groups. Significant difference between control and GLU alone was indicated by $**P < 0.0001$. Significant difference between GLU alone and an inhibitor + GLU $\dagger P < 0.05$ or $\dagger\dagger P < 0.0001$, and significant difference between E2 + GLU and other inhibitor + GLU was indicated by $\ddagger P < 0.05$ or $\ddagger\dagger P < 0.0001$ ($n \geq 12$).

Fig. 8.

Cell viability and $\text{ic}[\text{Ca}^{2+}]$ in VSC4.1 cells treated with L-type VGCC agonist. Following treatment with the L-type VGCC agonist FPL, cell viability was examined using the MTT assay (A), and $\text{ic}[\text{Ca}^{2+}]$ was examined using fura-2 (B). Treatment groups: control, 100 nM estrogen (E2) for 25 h, 100 nM FPL for 24 h, and 1 h pre-treatment with E2 followed by 24 h co-treatment with FPL (E2 + FPL). Cell viability was reported relative to control. Significant difference from control values was indicated by $**P < 0.0001$, and significant difference between FPL alone and E2 + FPL was indicated by $\dagger P < 0.05$ ($n \geq 4$).