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ESTROGEN PREVENTS GLUTAMATE-INDUCED APOPTOSIS IN C6 GLIOMA CELLS BY A RECEPTOR-MEDIATED MECHANISM

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Abstract—Estrogen-mediated neuroprotection is well established; however, no single mechanism of action for this effect has yet been established. As glial cells are integral for both the intact and injured nervous system, we hypothesized that estrogen-mediated neuroprotection may partly be attributed to attenuation of glial cell apoptosis, allowing them to protect neurons following injury. To assess the protective effects of estrogen on glia, C6 rat glioma cells were treated for 24 h with 500 μM glutamate. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and apoptosis was confirmed by cell morphology and DNA fragmentation. Pretreatment with 10 nM 17β-estradiol (estrogen) increased cell viability and attenuated apoptosis. Treatment with the stereoisomer 17α-estradiol, or estrogen plus estrogen receptor antagonist ICI 182,780, was significantly less effective, indicating that cytoprotection was receptor-mediated. Estrogen treatment upregulated expression of estrogen receptor α. Cell impermeable bovine serum albumin-conjugated estrogen was also protective, indicating activation of estrogen receptors on the cell membrane. Intracellular free [Ca^{2+}] was increased after glutamate treatment. This increase was attenuated in cells pretreated with estrogen. Glutamate increased the activity of pro-apoptotic proteases, such as calpain and caspase-3, and these protease activities were significantly attenuated by estrogen. The mechanism by which estrogen decreased intracellular Ca^{2+} was examined by assaying cell viability after using inhibitors that either blocked extracellular Ca^{2+} influx or prevented the release of intracellular Ca^{2+} stores. While several inhibitors increased cell viability in glutamate-treated cells, none were as protective as estrogen, and estrogen co-treatment significantly increased cell viability. These findings indicate that estrogen-mediated cytoprotection may be related to effects on Ca^{2+} entry but that these effects are not limited to any one of these Ca^{2+} entry points alone.

Key words: calcium, calpain, caspase, estrogen receptor, glia.

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; APV, DL-2-amino-5-phosphonopentanoic acid; BSA, bovine serum albumin; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DAN, dantrolene; DMSO, dimethyl sulfoxide; E2-BSA, estrogen conjugated with bovine serum albumin; ER, estrogen receptor; estrogen, 17β-estradiol; FBS, fetal bovine serum; IC [Ca^{2+}], intracellular calcium; ICI, ICI 182,780; IP_3, 1,4,5-inositol triphosphate; KBR, KB-R7943; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NCX, Na^+/Ca^{2+} exchanger; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; SBDP, spectrin breakdown product; TUNEL, terminal deoxynucleotidyl transferase dUTP-mediated nicked end labeling; VGCC, voltage-gated Ca^{2+} channels; 2-APB, 2-aminoethoxydiphenylborane.

Estrogen-mediated neuroprotection has been noted *in vitro* in neurons and neuronal cell lines under a variety of stress conditions including oxidants (Behl et al., 1997), amyloid-β (Fitzpatrick et al., 2002), serum withdrawal (Bishop and Simpkins, 1994; Gollapudi and Oblinger, 1999), and glutamate (Singer et al., 1999). In animal models, treatment with estrogen has been associated with improvements in traumatic brain injury (Roof and Hall, 2000) and ischemia (Simpkins et al., 1997; Dubal et al., 2001), and gender differences in humans have been noted in clinical studies examining CNS trauma (Bayir et al., 2004). Estrogen levels may directly alter lesion size in multiple sclerosis (Bansil et al., 1999). Some investigators have noted a reduced risk for Alzheimer's disease in patients on estrogen replacement therapy (Kawas et al., 1997); although, other studies have not noted a difference (Shumaker et al., 2003). A single mechanism for estrogen-mediated neuroprotection has not yet been established; however, several possibilities exist (Sribnick et al., 2003). Estrogen is a potent anti-oxidant due to its phenolic ring structure (Behl et al., 1997). Estrogen also has anti-inflammatory (Bruce-Keller et al., 2000) and anti-apoptotic (Sur et al., 2003) effects, which may contribute to attenuating damage following neurotoxic insult. Both estrogen receptor (ER) dependent (Wilson et al., 2002) and independent (Behl et al., 1997) mechanisms of neuroprotection have been reported. Two recent discoveries add complexity: the discovery of ERβ (Mosselman et al., 1996) and the finding that ER may be localized to the cell membrane (Razandi et al., 1999).

Although neurological insults are diverse in nature, there are common mechanisms of cell injury, and glutamate-toxicity plays an integral role in a variety of neurodegenerative diseases and neurotrauma (Dumont et al., 2001; Lankiewicz et al., 2000). While ongoing research is still trying to define the exact mechanism of glutamate-induced cell death, several pathways have been elucidated. Activation of glutamate receptors coupled to nitric oxide synthase leads to the production of nitric oxide, which can be converted to a potent oxidant, peroxynitrite (Sattler et al., 1999). Rises in intracellular calcium (IC [Ca^{2+}]) may also contribute to cell death. Ca^{2+} from both extracellular and intracellular sources accumulates in the cytosol following treatment with glutamate. Glutamate activates ionotropic *N*-methyl-D-aspartate (NMDA) (Michaelis, 1998) or α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors (Bennett et al., 1996) to permit Ca^{2+} influx. Activation of metabotropic glutamate receptors by glutamate leads to phospholipase C activation with downstream 1,4,5-inositol triphosphate (IP_3) pro-

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duction, releasing Ca^{2+} from IC Ca^{2+} stores (Michaelis, 1998). Other possible contributors of Ca^{2+} into the cytosol include: Ca^{2+} release from the endoplasmic reticulum following stimulation of the ryanodine receptors (Charles et al., 1993) and reversal of the Na^{+}/Ca^{2+} exchanger (NCX) (Czyz et al., 2002).

Rises in IC [Ca^{2+}] can trigger a number of cell death pathways. While initial rises in mitochondrial [Ca^{2+}] increase energy production, long-term [Ca^{2+}] accumulation in the mitochondria ultimately disrupts cellular metabolism (Brookes et al., 2004). Increased IC Ca^{2+} can also activate phospholipases and proteases, and one such protease is the Ca^{2+}-activated neutral protease calpain. While several tissue specific calpains exist, there are two ubiquitous forms, μ-calpain (EC 3.4.22.52) and m-calpain (EC 3.4.22.53), which require μM or mM [Ca^{2+}] for activation, respectively (Ray and Banik, 2003). While calpain is a vital protein (Zimmerman et al., 2000), calpain overactivation leads to its autocatalysis, cleavage of the endogenous inhibitor calpastatin, and either necrotic (Pang et al., 2003) or apoptotic (Ray et al., 1999) cell death. Calpain may activate pro-apoptotic Bax (Wood et al., 1998), leading to the downstream activation of the cysteine protease caspase-3. Caspase-3 ultimately activates caspase-3-activated DNase, which cleaves the genomic DNA into 180 bp fragments (Enari et al., 1998). While reports are conflicting as to whether calpain and caspase-3 act synergistically (Blomgren et al., 2001) or antagonistically (Lankiewicz et al., 2000), both proteases are involved in apoptosis. Since both cleave α-spectrin at two specific sites, their activities can be measured from the formation of spectrin breakdown products (SBDPs). The 120 kD SBDP is specific for caspase-3, and the 145 kD SBDP is specific for calpain (Wang et al., 1998).

The role of astrocytes following CNS injury is largely unclear. Reactive astrocytes form a glial scar and can prevent regrowth after trauma (Okada et al., 2004); however, a protective role for astrocytes has also been noted. Astrocytes, co-cultured with neurons, have been shown to inhibit neuronal death (Desagher et al., 1996), and glial-derived neurotrophic factor, secreted from astrocytes (Bresjanac and Antauer, 2000), has been shown to be neuroprotective *in vivo* (Cheng et al., 2002; Faulkner et al., 2004). If estrogen can protect astrocytes from the post-traumatic chemical milieu, then CNS degeneration may be decreased and the opportunity for regeneration increased.

In the current study, we examined the effects of 17β-estradiol (estrogen) pre-treatment on C6 glioma cells exposed to the levels of glutamate suitable for apoptosis. Treatment with 500 μM glutamate for 24 h was shown to be cytotoxic following examining the cells by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, Wright staining, and DNA fragmentation. Pretreatment with 10 nM estrogen for 2 h, followed by cotreatment with glutamate, was shown to significantly attenuate apoptosis. Estrogen-mediated protection of cells was shown to be receptor-mediated, and treatment with estrogen significantly increased ERα protein content. Activation of ER at the cell membrane, shown using conjugated estrogen, was sufficient for cytoprotection. Treatment with estrogen significantly decreased intracellular Ca^{2+} and attenuated the activation of proteases calpain and caspase-3. While several inhibitors aimed at preventing increases in IC Ca^{2+} were also cytoprotective, all showed significantly less protection than estrogen. Furthermore, in each case, cytoprotection was augmented when the cells were co-incubated with the inhibitor plus estrogen, indicating that estrogen-mediated cytoprotection in C6 cells was not dependent on any one of the Ca^{2+} transport mechanisms investigated but was ER-dependent.

EXPERIMENTAL PROCEDURES

Cell culture

C6 cells were grown in RPMI 1640 with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. Cells were incubated at 37 °C with 5% CO_2 in a fully humidified incubator. Prior to experiments, cells were transferred from medium with 10% FBS to 0.5% FBS, a serum-poor medium, and cells were incubated in serum-poor medium for 24 h. Unless otherwise indicated, the four treatment groups were: control, treatment with 10 nM estrogen for 26 h, treatment with 500 μM L-glutamate (Sigma, St. Louis, MO, USA) for 24 h, and 2 h pretreatment with estrogen followed by cotreatment 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.

Reagents and antibodies

Stock solutions of ICI 182,780 (ICI; Tocris, Ellisville, MO, USA), estrogen (Sigma), and 17α-estradiol (Sigma) were made in the lowest volume of dimethyl sulfoxide (DMSO, Sigma) possible. The final concentration of DMSO was 3 μM, which was found to be nontoxic (data not shown). Stock solutions of glutamate were made daily, and glutamate was dissolved in RPMI media. Bovine serum albumen (BSA)-conjugated estrogen [(1,3,5(10)-estratrien-3, 17β-diol-17-hemi-succinate:BSA] (Steraloids, Newport, RI, USA) was filtered to remove any unconjugated estrogen, as previously reported (Stevis et al., 1999). Antibodies used were: ERα (C1355, Upstate, Lake Placid, NY, USA), ERβ (H-150 Santa Cruz, Santa Cruz, CA, USA), β-actin (clone AC-15, Sigma), caspase-3 (clone 5F6.H7, Oncogene, La Jolla, CA, USA), 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), and dantrolene (DAN, Sigma).

MTT assay

Cells were distributed onto 96 well plates at 10,000 cells/well and maintained for one day in RPMI media with 10% FBS. The following day, the medium was replaced for 24 h with RPMI media with 0.5% FBS. Experiments occurred on the following day, and following experimental treatments, 10 μg of MTT (Sigma), dissolved in phosphate-buffered saline (PBS), was added to each well. Following 1 h incubation, plates were centrifuged at 1900×*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 (Purnanam and Boustany, 1999) by assessing absorbance at 570 nm using a microplate reader (ELx800, Bio-Tek, Winooski, VT, USA). Absorbance was reported as a percentage of control,