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Soy phytoestrogens are neuroprotective against stroke-like injury *in vitro*

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Abstract

Diets high in soy are neuroprotective in experimental stroke. This protective effect is hypothesized to be mediated by phytoestrogens contained in soy, because some of these compounds have neuroprotective effects in *in vitro* models of cell death. We tested the ability of the soy phytoestrogens genistein, daidzein, and the daidzein metabolite equol to protect primary cortical neurons from ischemic-like injury *in vitro* at doses typical of circulating concentrations in human populations (0.1–1 μ M). All three phytoestrogens inhibited LDH release from cells exposed to glutamate toxicity or the calcium-ATPase inhibitor, thapsigargin. In cells exposed to hypoxia or oxygen-glucose deprivation (OGD), pretreatment with the phytoestrogens inhibited cell death in an estrogen receptor (ER) dependent manner. Although OGD results in multiple modes of cell death, examination of α -spectrin cleavage and caspase-3 activation revealed that the phytoestrogens were able to inhibit apoptotic cell death in this model. In addition, blockade of phosphoinositide 3-kinase prevented the protective effects of genistein and daidzein, and blockade of mitogen-activated protein kinase prevented genistein-dependent neuroprotection. These results suggest that pretreatment with dietary levels of soy phytoestrogens can mimic neuroprotective effects observed with estrogen and appear to use the same ER-kinase pathways to inhibit apoptotic cell death.

Introduction

In vivo and *in vitro* studies have demonstrated powerful neuroprotective effects of estrogens against a variety of insults. In rodents, estrogen reduces injury caused by focal cerebral ischemia (Gibson et al., 2006), global ischemia (Jover et al., 2002), cerebral trauma (Bramlett and Dietrich, 2001), β -amyloid (Aguado-Llera et al., 2006), and glutamate (Mendelowitsch et al., 2001) or MPTP toxicity (Callier et al., 2001, Morissette et al., 2007). *In vitro*, similar neuroprotective effects have been demonstrated in primary cortical (Singer et al., 1999, Honda et al., 2000, Sribnick et al., 2004), hippocampal (Nilsen and Brinton, 2002, Cimarosti et al., 2005, Chen et al., 2006) and mesencephalic cultures (Callier et al., 2001). Furthermore, these neuroprotective effects appear to utilize multiple mechanisms depending on the injury

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and cell type. These mechanisms include classical transcriptional signaling through estrogen receptors alpha and beta, activation of multiple kinases such as mitogen activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K), modulation of intracellular calcium levels, and anti inflammatory effects (for review see (Amantea et al., 2005). Unfortunately, these well-demonstrated neuroprotective effects of estrogen may not be applicable to postmenopausal women or men because of the potential deleterious side-effects of hormone replacement therapy, such as increased cancer and stroke risk (Wassertheil-Smoller et al., 2003, Brass, 2004).

Recent advances in pharmaceuticals have resulted in the development of selective estrogen receptor modulators (SERMs) that may provide many of the beneficial effects of estrogen without the deleterious side effects. However, many women now consider the use of complementary and alternative therapies, including the use of high soy diets or soy isoflavone supplements for menopausal symptoms (Wathen, 2006). It is increasingly clear that physiologically attainable doses of isoflavones, which can behave as phytoestrogens, can mimic some of the neuroprotective effects of estrogens. For example, a high soy diet reduces stroke injury in female and male rats (Schreihöfer et al., 2005, Burguete et al., 2006) and the soy isoflavone genistein is neuroprotective in a mouse cerebral ischemia model (Trieu and Uckun, 1999). *In vitro* some soy isoflavones can protect primary neurons from glutamate toxicity (Zhao et al., 2002), thapsigargin-induced apoptosis (Linford and Dorsa, 2002), and β -amyloid toxicity (Zeng et al., 2004). However, the mechanisms underlying protection from ischemic injury remains unclear. In this study we hypothesized that soy phytoestrogens would be neuroprotective in an *in vitro* model of ischemic stroke and that the mechanism would involve estrogen receptor activation.

Experimental procedures

Primary Cortical Culture

Cortical neurons were cultured from Long Evans rats as previously described (Redmond, et al. 2002). Briefly, embryonic day 18 cortices were digested with papain (Worthington), dissociated, and plated onto polylysine and laminin coated wells in 24 well plates at 1.86×10^5 cells/cm². Cultures were maintained in Neurobasal media supplemented with 2% B27, 1mM glutamine and penicillin/streptomycin (Invitrogen). Cultures were maintained at 37°C in 5% CO₂ and media was changed at 4 and 7 days *in vitro* (DIV). Estrogen receptor (ER) expression in embryonic cortical neurons was determined by real time RT-PCR, immunoblotting, and immunohistochemistry as described below. For mRNA comparison, pooled adult rat cortical, hippocampal, and hypothalamic tissue was used. For immunoblotting, rat uterus and mouse α T3 pituitary cells were used.

Apoptotic challenges

In all experiments control and challenged wells underwent the same number of washes and medium changes. Cortical cells were used at 7 DIV. Cells were pretreated with estrogens or phytoestrogens for 24 hours in a fresh change of NB/B27, and treatments were present throughout the experiment. Phytoestrogens (genistein, daidzein, and equol) were obtained from LC Laboratories (Woburn, MA). Compounds were all dissolved and diluted in 95% ethanol to maintain a constant ethanol concentration in all experiments. The following day, one of the following challenges was applied and cell viability was assessed at 24 and 48 hours. For excitotoxicity, cells were challenged with 300 μ M glutamate in Hanks Buffered Salt Solution (HBSS) for 10 minutes and then replaced with conditioned medium. Thapsigargin (50 nM) was used to induce apoptosis by increasing intracellular calcium levels. Cells were treated with thapsigargin in NB/B27 for 48h in the presence of treatments. Mitochondrial respiration was inhibited with KCN (1 mM) treatment. Cells were exposed to KCN in NB/B27 for 2 hours and

then replaced with conditioned medium. The addition of 2 mM 2-deoxy-D-glucose to KCN treatment was also examined as a model for ischemia.

Hypoxia was induced by placing uncovered plates in a prewarmed and humidified chamber (Billups-Rothenberg) that was subsequently flushed with 95% N₂/5% CO₂ for 15 minutes. An oxygen sensor placed in the chamber confirmed oxygen levels of less than 0.6%. The sealed chamber was then placed in a standard incubator for 18 hours. Plates were then removed from the chamber and allowed to re-oxygenate in the tissue culture incubator for 6 hours before assessment.

Oxygen-glucose deprivation (OGD) was achieved using the same hypoxic chamber. After 24 hours of pretreatment in 500 µl of NB/B27, cells were washed twice with HBSS and then placed in glucose-free HBSS (300 µl) containing treatments and subjected to 2 or 5 hours of hypoxia, as described above. Following hypoxia, cells were allowed to reoxygenate in fresh Neurobasal medium containing 0.5 mM glutamine, but no B27 supplement. Control wells were subjected to the same washes but were maintained in HBSS+ 25 mM glucose under normoxic conditions during the OGD period.

Mechanism of action

The mechanism of phytoestrogen dependent neuroprotection was assessed with specific inhibitors of potential protective signaling molecules. Dependence on ER signaling was assessed with pretreatment (-30 min) with the ER α / β antagonist ICI182,780 (Tocris Cookson Inc., Ellisville, MO). Dependence on PI3K activated pathways was determined by pretreatment with the PI3K inhibitor LY294002 (Tocris Cookson), and dependence on MAPK activated pathways was determined by pretreatment with the specific MAPK kinase inhibitor PD98059 (Tocris Cookson).

Assessment of cell death

Cell death was determined 24 and 48 hours after challenge by the release of lactate dehydrogenase (LDH), a cytoplasmic enzyme released from cells with compromised cell membranes, and a marker of both apoptosis and necrosis. LDH release into the culture medium was detected using a colorimetric reaction read at an absorbance at 490 nm on a Tecan Genios plate reader using a commercial kit (Takara). Maximal LDH release was determined by treating control wells for 60 minutes with 1% triton-X 100 to lyse all cells.

Apoptotic cell death was examined by in situ detection of caspase-3 activity using a fluorogenic substrate (FAD-VMK-FITC) from Promega. Active caspase-3 cleaves the substrate, resulting in green fluorescence. Images from treated wells were captured on an Olympus IX50 inverted microscope with an Optronics Magnifier SP digital camera. Cleavage of α -spectrin and caspase-3 were also determined by immunoblotting, as described below. Calcein AM (Molecular Probes), a viability marker that is hydrolyzed by intracellular esterases in living cells, was used in some cultures to confirm results from apoptotic assays.

Real-time RT-PCR

Total RNA was collected and isolated using a commercial kit (Qiagen RNEasy). RNA was reverse transcribed with oligo-deoxy-thymidine primers using Omniscript (Qiagen). Real time PCR was performed using ER α , ER β and GPR30 predesigned Taq-Man assays from Applied Biosystems. GAPDH was amplified as an endogenous control gene. RT-PCR was performed two independent times from independent samples. Relative message levels were normalized to expression in the adult rat cerebral cortex.

Immunoblotting

Total protein was collected in MPER lysis buffer (Pierce) containing a protease inhibitor cocktail (HALT, Pierce) from cortical cells grown in 35 mm plates. Subcellular fractionation of the cytoplasm and nucleus was achieved with the NPER lysis kit (Pierce). Protein concentrations were determined in duplicate using the Pierce BCA protein assay reagent and bovine serum albumin to generate standard curves. Absorbance at 595 nm was detected on a Tecan Genios plate reader. Proteins were aliquoted and frozen at -80°C to avoid multiple freeze-thaw cycles. Equivalent amounts of protein were separated by SDS-polyacrylamide gels electrophoresis using Pierce Precise precast gels. Proteins were transferred to nitrocellulose (BioRad) and detected with the following antibodies: ER α (LabVision Ab15, 1:500); ER β (Upstate 06-029, 4 $\mu\text{g}/\text{ml}$); α -spectrin (Chemicon MAB1622, 1:1000); active caspase-3 (Cell Signaling Technologies 9661, 1:1000); β -actin (Sigma AC15, 1:1000). Secondary detection was achieved with LiCor infrared-labeled antibodies (1:10,000) and the LiCor Odyssey infrared scanner. Expression levels were determined using Odyssey software and normalized to blot background levels and β -actin.

Immunocytochemistry

For immunocytochemical detection of ERs, cells grown in chamber slides (Falcon) were washed with PBS and fixed for 20 minutes at room temperature with 4% formaldehyde. Cells were permeabilized with 0.2% triton X-100 in PBS for 10 min at 4°C . Non-specific binding was blocked with 5% BSA and 1% horse serum in PBS for 1 h at 4°C , and cells were incubated with primary antibodies overnight at 4°C . ER α was detected with Upstate ER α (C1355, 1:5,000) and ER β was detected with Upstate ER β (06-029, 1:100). Secondary detection was accomplished with Cy3 conjugated donkey anti-rabbit antibody (Jackson Immunologicals, 1:500), and nuclei were counterstained with DAPI (Pierce). The slides were coverslipped with Vectashield (Vector Laboratories), and images were captured with an Optronics Magnifier SP digital camera on an Olympus BX60 microscope with appropriate fluorescent filters.

Statistics

All data was collected from at least 4 independent experiments. Data are all presented as normalized to vehicle controls, but statistical analyses were performed on raw data. Differences among groups were detected by analysis of variance for repeated measures and pair-wise comparisons to vehicle control wells were made with Dunnett's test using InStat software (GraphPad). Data presented in Figure 5 and 6 did not fit a normal distribution and was analyzed by non-parametric methods using Friedman's test followed by Dunn's post-hoc comparisons. In all cases a P value of <0.05 was considered significant.

Results

Because the reported expression of ER expression in primary cortical cultures has been inconsistent across the literature, we first examined the expression of ER in primary cortical neurons used in our experiments. After 7 DIV, approximately 95% of cells were neurons, as detected by MAP2 and GFAP immunostaining (not shown). Real time RT-PCR revealed expression of ER α levels 9-fold greater than the adult rat cortex in primary cortical neurons under our culture conditions (Figure 1). ER β mRNA levels were 40% of those in the adult cortex, and GPR30 levels were only 11% of cortical levels (Figure 1). For comparison, hypothalamic levels of ER mRNA are 20-50 times higher than in cortex (Figure 1). Immunoblotting revealed ER α protein in the cultured cortical neurons was primarily nuclear (Figure 1B) and ER β was primarily cytoplasmic in our cells (Figure 1B), an observation confirmed by immunostaining (Figure 1C). These analyses were performed twice with similar results, but are not intended to be quantitative.

Estradiol is neuroprotective in primary cortical neurons faced with several different apoptotic challenges. Similarly, various phytoestrogens can be neuroprotective. We compared the neuroprotective efficacy of physiological concentrations of 17 β -estradiol (E2) to concentrations of the soy isoflavones genistein and daidzein (0.1–1 μ M) at levels observed in the plasma of individuals on a high soy diet (Setchell, 2001). In addition, we examined the neuroprotective potential of the daidzein metabolite equol, which makes up the majority of circulating isoflavone in rodents. In agreement with published reports, E2 significantly reduced neuronal death in response to glutamate and thapsigargin (Figure 2). Similarly, pretreatment with the soy isoflavones genistein and daidzein also reduced LDH release under these conditions (Figure 2). Equol (1 μ M) had no significant effect (Figure 2). Anoxia induced with KCN or oxygen-glucose deprivation induced by KCN combined with 2-deoxy-D-glucose led to LDH release that was not significantly reduced by E2 or phytoestrogen treatment (data not shown). Exposure of cortical cells to 18 hours of hypoxia in the presence of treatments led to increased LDH release (Figure 3). Pretreatment for 24 hours with 1 nM E2 or 0.1 and 1 μ M soy isoflavones reduced LDH release in response to hypoxia (Figure 3). The ER antagonist ICI182,780 partially reversed the neuroprotective effects of genistein and daidzein, but not equol (Figure 3).

Although glutamate, thapsigargin, and hypoxia in serum-free conditions mimic many of the characteristics of *in vivo* cerebral ischemia, glucose deprivation is also an important *in vivo* consequence of ischemia. Oxygen-glucose deprivation (OGD) for 2 hours leads to both apoptotic and necrotic cell death (Jones et al., 2004). Twenty-four hours of pretreatment with 10 nM E2, or 1 μ M, but not 0.1 μ M, genistein, daidzein, or equol significantly reduced LDH release 48 hours after OGD (Figure 3). Pretreatment with the ER antagonist ICI182,780 prevented the reduction of LDH, suggesting an ER-mediated effect (Figure 3). Cell staining with the viability marker calcein AM confirmed the reduction in cell viability in OGD cells and protection by phytoestrogens (Figure 3). Examination of α -spectrin cleavage revealed that OGD slightly, but significantly, increased the caspase-dependent 120 kDa fragment, but not the 150 kDa or calpain-dependent 145 kDa fragment (Figure 4). Phytoestrogens and estradiol treatment prevented accumulation of the 120 kDa fragment, but this effect was small (Figure 4). Similarly, phytoestrogens prevented the OGD-induced increase in caspase-3 cleavage (Figure 5). In situ staining for caspase activation confirmed results of immunoblotting (Figure 5).

The neuroprotective effects of estrogen in primary cortical cultures have been attributed, in part, to their ability to activate the PI3 kinase (Honda et al., 2000, Honda et al., 2001, Wilson et al., 2002) and MAP kinase pathways (Singer et al., 1999, Mize et al., 2003). To determine whether the protective effects during OGD are mediated by these pathways, we pretreated cells with the specific PI3 kinase inhibitor LY294008 or the MEK inhibitor PD98059 just prior to estrogen or isoflavone treatment and OGD. Inhibition of both MEK and PI3K prevented genistein-induced protection from OGD (Figure 6) whereas only PI3K inhibition prevented daidzein-induced neuroprotection (Figure 6). Under our experimental conditions, the DMSO vehicle alone increased basal LDH release and prevented equol-dependent neuroprotection (Figure 6). Nevertheless, cotreatment with LY294008 increased LDH release in equol-treated cells exposed to OGD (Figure 6).

Discussion

Estradiol and estrogen-like compounds are powerful neuroprotective agents against numerous *in vivo* and *in vitro* apoptotic stimuli including stroke (Hurn and Brass, 2003, McCullough and Hurn, 2003, Alonso de Lecinana and Egido, 2006, Gibson et al., 2006). Diets high in soy leads to significant neuroprotection from focal cerebral ischemia in the rat (Schreihöfer et al., 2005, Burguete et al., 2006). In addition, a major soy phytoestrogen, the isoflavone genistein,

can reduce cerebral ischemic damage in mice (Trieu and Uckun, 1999). However, the precise mechanisms underlying these protective effects are unclear. In the present study, we show that dietary levels of the isoflavones genistein, daidzein, and the daidzein metabolite equol, can reduce cell death in cultured embryonic cortical cells exposed to challenges that mimic many of the events in cerebral ischemia including glutamate excitotoxicity, calcium mobilization, hypoxia, and oxygen-glucose deprivation (OGD). Estrogen receptors play a critical role in the protective effects of phytoestrogens as an ER antagonist blocked neuroprotection. In addition, activation of kinase survival pathways appears to play a role in protection by both genistein and daidzein, with the PI3K pathway appearing most important.

The complexity of *in vivo* studies makes elucidation of mechanisms of estrogen neuroprotection during cerebral ischemia difficult to study. Even with the availability of selective ligands and knockout mice controversy remains regarding the necessity for specific ERs in the protective effects of E2 (Dubal et al., 2000, Sampei et al., 2000, Carswell et al., 2004). Furthermore, the ability of estrogen to modulate cerebral blood flow (Pelligrino et al., 1998, Krause et al., 2006), inflammatory responses (Suzuki et al., 2007), blood brain barrier permeability (Bake and Sohrabji, 2004, O'Donnell et al., 2006), and glial cell function (Mahesh et al., 2006) makes dissecting out mechanisms difficult. Thus, *in vitro* models of injury have been employed as surrogates for the *in vivo* condition. Using embryonic cortical cells enriched for neurons we demonstrate that low micromolar concentrations of soy phytoestrogens can be directly neuroprotective against many of the insults associated with ischemic injury. However, the OGD model results in both apoptosis and necrosis (Jones et al., 2004) and although our results demonstrate inhibition of caspase pathways, it is likely that necrotic mechanisms are influenced as well.

Previous investigators have demonstrated neuroprotective effects of genistein against glutamate (Singer et al., 1999, Sribnick et al., 2004) and thapsigargin-induced (Linford and Dorsa, 2002) apoptosis in embryonic cortical cells, and we report similar effects here with E2 and genistein. In addition we observed protective effects of daidzein. However, we saw no significant protection against chemical anoxia with KCN, chemical anoxia and hypoglycemia (KCN + 2-deoxy-D-glucose). These results contrast with those shown in organotypic cortical explant cultures treated for 1-7 days with E2, in which KCN/2-DG induced apoptosis was reduced 24-72 hours after challenge via an ER-dependent mechanism (Wilson et al., 2000). Although we observed a trend for neuroprotection by E2 and genistein after 48 hours, this effect failed to reach significance (data not shown).

In contrast to results with chemical ischemia, E2 and soy phytoestrogens all reduced LDH release in response to hypoxia. A protective effect of E2 has previously been shown in primary hippocampal neurons exposed to 15 hours of hypoxia, and this effect was attributed to non-genomic effects since it could be mimicked with E2-BSA and was not blocked with ICI182,780 (Heyer et al., 2005). We did observe diminution of the protective effects of genistein and daidzein with ICI182,780 suggesting the involvement of ERs. However, ICI182,780 did not alter the ability of equol to inhibit hypoxia-induced cell death. Thus, like estrogens, multiple mechanisms may be employed by different phytoestrogens.

Similar to hypoxia, neuronal death induced by OGD was significantly inhibited by E2, genistein, daidzein, and equol in an ER- and dose-dependent manner. However, although caspase activation and caspase-dependent α -spectrin cleavage were reduced by phytoestrogens, caspase activation was not extensive. Examination of caspase activation in cells confirmed immunoblotting. However, the total number of live cells was also reduced suggesting non-apoptotic cell death was also present.

Although soy phytoestrogens are often thought of as ER β selective ligands, the doses used in the present experiment (0.1 and 1 μ M) can readily activate ER α -dependent transcription in neurons (Schreihöfer, 2005). The relative expression levels of ERs in our cells further supports a role for ER α -dependent neuroprotection. Although other investigators have implicated ERs in the neuroprotective effects of genistein (Zeng et al., 2004, Kajta et al., 2007), the ER subtype has not been determined. Experiments in primary cortical neurons by Linford and Dorsa, support a role for ER β in genistein-dependent neuroprotection against thapsigargin-induced apoptosis (Linford and Dorsa, 2002). Sawada et al. (Sawada et al., 2000) also attributed the neuroprotective effects of genistein to ER β in nigrostriatal dopamine neurons. However, it is clear that either ER α or ER β can mediate E2-dependent neuroprotection in neuronal cell lines (Gollapudi and Oblinger, 1999, Fitzpatrick et al., 2002). Furthermore, some neuroprotective effects of estrogen-like compounds appear to be independent of their ability to bind ERs (Prokai and Simpkins, 2007). Although our results support a role for ER α , other mechanisms cannot be ruled out.

Among the mechanisms that contribute to estrogen-dependent neuroprotection is activation of intracellular kinase cascades including PI3K/AKT and MAPK. *In vitro* estrogen increases AKT phosphorylation and inhibits glutamate (Honda et al., 2000) and staurosporine-induced apoptosis in cortical neurons (Harms et al., 2001, Honda et al., 2001). In retinal neurons Akt also mediates estrogen-dependent neuroprotection from H₂O₂-induced apoptosis (Yu et al., 2004). AKT activation is also linked to estrogen-dependent neuroprotection in OGD exposed hippocampal organotypic cultures (Cimarosti et al., 2005) and chemical ischemia in cortical explants (Wilson et al., 2002).

In vivo PI3K inhibition prevent estrogen-dependent neuroprotection in 6-OHDA lesioned rats (Quesada et al., 2008), MPTP-treated mice (D'Astous et al., 2006), and global ischemia (Wang et al., 2006). However, PI3K/AKT also serves as an endogenous survival factor and inhibition of PI3K alone can increase cerebral ischemic injury (Noshita et al., 2001). Nevertheless, PI3K/AKT appear to play a role in neuroprotection by genistein and daidzein in response to OGD. This action in neurons is counter to the inhibition of AKT demonstrated with higher genistein doses in cancer cells (Sarkar and Li, 2002), and may represent the ability of estrogenic rather than tyrosine kinase inhibitor activity of these compounds.

In addition to a role for PI3K/AKT in phytoestrogen-dependent neuroprotection, inhibition of MAPK with PD98059 also prevented genistein inhibition of LDH release. High doses of genistein can induced apoptosis in primary cortical neurons, partially through MAPK activation, although only at doses greater than 10 μ M (Linford et al., 2001). In contrast, lower doses of genistein can inhibit glutamate-induced apoptosis in primary hippocampal neurons (Zhao et al., 2002) and thapsigargin-induced apoptosis in cortical neurons (Linford and Dorsa, 2002). However, these effects have not been shown to involve MAPK. In the present study, MAPK appears to be involved in genistein neuroprotection but not that seen with daidzein or equol.

Like estrogen, doses of isoflavones obtained by diet can be protective against a number of apoptotic challenges *in vitro*. The present study extends these observations to inhibition of several aspects of ischemic cell death, including glutamate, calcium, hypoxia, and OGD dependent cell death. At least part of this effect is mediated by an inhibition of caspase activity and depends on ER and PI3K, similar to estrogen-mediated neuroprotection. These results further support a role for soy isoflavones as neuroprotectants and possible alternatives to estrogen.

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Abbreviations

6-OHDA	6-hydroxydopamine
AKT	Akt serine-threonine kinase
B27	B-27 serum-free supplement
BCA	bicinchoninic acid
BSA	bovine serum albumin
C	celsius
Calcein AM	calcein acetoxymethylester
cm²	square centimeters
CO₂	carbon dioxide
DAPI	4',6-diamidino-2-phenylindole
DIV	days <i>in vitro</i>
DMSO	dimethyl sulfoxide
E2	17 β -estradiol
E2-BSA	17 β -estradiol conjugated to bovine serum albumin
ER	estrogen receptor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
GPR30	

	G-protein coupled receptor 30
H₂O₂	hydrogen peroxide
HBSS	Hank's buffered saline solution
KCN	potassium cyanide
kDa	kiloDalton
LDH	lactate dehydrogenase
MAP2	microtubule associated protein 2
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
MPTP	1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine
mRNA	messenger RNA
NB	neurobasal medium
OGD	oxygen-glucose deprivation
PBS	phosphate buffered saline
PI3K	phosphoinositide 3-kinase
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
SERM	selective estrogen receptor modulator

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Figure 1.

Estrogen receptor expression in cultured embryonic neurons. **A.** Steady state mRNA levels of ER α , ER β , and GPR30 in cultured embryonic cortical cells, adult rat cortex, hypothalamus, and hippocampus determined with real-time RT-PCR. **B.** Protein levels of ER α and ER β in cultured cortical whole cell (WC), cytoplasm (Cyt), and nuclear (Nuc) extracts compared to adult rat uterus (Ut) and mouse α T3 pituitary cells determined with immunoblotting. **C.** ER α and ER β staining and DAPI nuclear localization in cultured cortical cells.

Figure 2.

Estrogen and phytoestrogens reduce LDH release in glutamate or thapsigargin treated cortical cells. A. LDH release in cortical cells pretreated 24 h with estradiol (E2, 10 nM), genistein (Gen, 1 μ M), daidzein (Daid, 1 μ M), or equol (1 μ M) and subjected to 10 min of 300 mM glutamate followed by 48 hours of recovery. Values are normalized to the glutamate only condition (Veh) and represent mean $\pm$ SE of 5 independent experiments ($F_{4,16}=3.856$, $P=0.223$). B. LDH release in cortical cells pretreated as in (A) and subjected to 48 hours of 50 nM thapsigargin. Values are normalized to the thapsigargin only condition (Veh) and represent mean $\pm$ SE of 5 independent experiments ($F_{4,16}=8.512$, $P=0.0007$). Asterisks (*) represent significant difference ($P<0.05$) from vehicle control.

Figure 3.

Estrogen and phytoestrogens reduce LDH release in cortical cells exposed to hypoxia (Hypox) or OGD. A. LDH release in cortical cells pretreated 24 h with estradiol (E2, 10 nM), or 0.1 (open bar) or 1 μ M (hatched bar) genistein, daidzein, or equol and subjected to 18 hours of hypoxia and 6 hours of reoxygenation. Filled bars represent 1 μ M phytoestrogen treatments + 1 μ M ICI182,780. Values are normalized to the vehicle only condition (Vehicle) and represent mean $\pm$ SE of 5-7 independent experiments. Asterisks (*) represent significant difference ($P < 0.05$) from vehicle control ($F_{10,40} = 2.082$, $P = 0.0495$). B. LDH release in cortical cells pretreated as in (A) and subjected to 90 minutes of hypoxia in glucose-free HBSS and 48 hours of reoxygenation in conditioned medium. Values are normalized to the vehicle only condition (Vehicle) and represent mean $\pm$ SE of 5-7 independent experiments. Asterisks (*) represent significant difference ($P < 0.05$) from vehicle control ($F_{10,40} = 2.486$, $P = 0.0266$). C. Representative photomicrographs of cortical cells stained with the viability marker calcein AM 48 hours after OGD.

Figure 4.

Cleavage of α -spectrin in cortical cells exposed to OGD. A. Representative western blot showing α -spectrin cleavage 48 hours after 2 hour OGD exposure in the absence or presence of 1 μ M isoflavones or 10 nM estradiol. Quantification of western blot band density for p120 (B), p145 (C), and p150 (D) kDa fragments of α -spectrin. Values represent means of 5-7 independent experiments $\pm$ SE. Optical density of each fragment was normalized to the full-length α -spectrin and β -actin. Asterisk (*) represents significant difference ($P < 0.05$) from vehicle (non-OGD) control ($F_{5,20} = 3.566$, $P = 0.0182$ for B).

Figure 5.

Activation of caspase-3 in OGD exposed cells. A. Representative western blot showing p17 caspase-3 cleavage product in 1 μ M phytoestrogen treated cells exposed to OGD. B. Quantification of western blot band density for p17 kDa fragment of caspase 3. Values represent means of 5 independent experiments $\pm$ SE. Optical density of p17 was normalized to β -actin. Asterisk (*) represents significant difference ($P < 0.05$) from vehicle (non-OGD) control (Friedman Statistic 11.328, $P = 0.0231$, Dunn post-hoc $P < 0.05$). C. Representative photomicrographs from of cortical cells stained with the *in situ* caspase 3 marker VAD-FMK-FITC 48 hours after OGD.

Figure 6.

Role of PI3K and MAPK in phytoestrogen neuroprotection. LDH release in cortical cells pretreated 24 h with 1 μ M genistein (Gen), daidzein (Daid), or equol alone or + PD98059 (50 μ M, solid bars) or LY294002 (10 μ M, hatched bars) and subjected to 2 hours of OGD and 48 hours recovery. Values are normalized to the vehicle OGD only condition (OGD) and represent mean $\pm$ SE of 6 independent experiments (Friedman Statistic 9, $P=0.0218$, Dunn post-hoc $P<0.05$). Asterisks (*) represent significant difference ($P<0.05$) from vehicle control.