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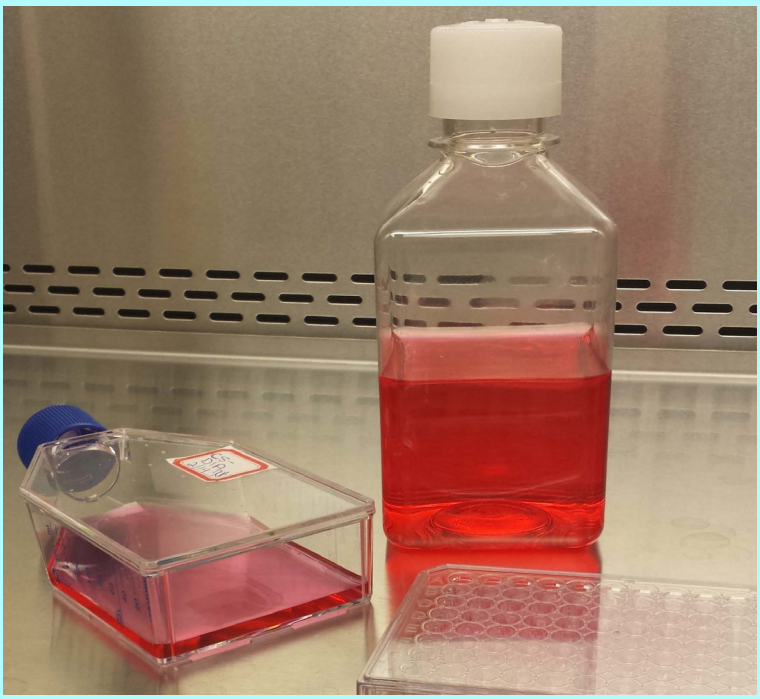

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Cover Photograph: Sample media and disposable vessels used in cultured astrocyte studies

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Protective Effects of Conjugated Equine Estrogens and 17-β Estradiol on Oxidatively Stressed Astrocytes

Whitley E. Grimes[1] and Kathleen S. Hughes[1,*]

Abstract - Conjugated equine estrogens (CEEs), estradiol, and progestins are used in common hormone replacement prescriptions for menopausal and post-menopausal women. While studies have linked 17-β estradiol to cell viability, little is known about the effects of individual CEEs (equilin and equilenin) during oxidative stress. This study sought to examine the protective effects of pretreating astrocytes with varying concentrations of equilin, equilenin, or 17-β estradiol, on the impact of oxidative stress on cell viability. We hypothesized that astrocytes pretreated with either CEE would not be protected from oxidative stress induced by exposure to hydrogen peroxide (H_2O_2), whereas cells pretreated with 17-β estradiol would be. MTT assay results revealed that the viability of untreated cells exposed to 600 μM H_2O_2 for either 1 h or 24 h was significantly reduced compared to the viability of unstressed cells. The viability of astrocytes pretreated with 10 nM – 10 μM of either CEE (equilin or equilenin) for 30 min prior to being stressed by a 1-h exposure to H_2O_2 did not differ from the viability of unstressed cells, indicating that CEE pretreatment provided some level of protection from oxidative stress. However, neither the CEEs- nor 17-β estradiol-pretreatment consistently protected astrocytes exposed to H_2O_2 for 24 h. Future research should examine the effect of increased duration of pretreatments, the effects of pretreatments on neuronal-like cell lines, and other measures of oxidative stress.

Introduction

Hormone replacement therapy (HRT) is an approved method of treatment for menopausal and post-menopausal women, and clinical trials reveal both detrimental and beneficial effects of HRT (Howard, B.V. and J.E. Rossouw. 2013, Prentice, R.L. 2014). While hormone replacement therapy can decrease some of the negative side effects of menopause, long-term HRT is associated with dementia, Alzheimer's disease, and an increased risk of uterine cancer (Rousseau 2010). Estrogens are primarily female sex hormones that bind to estrogen receptors (ER) in the cell. Much of the research on estrogen signaling focuses on its traditional pathway of binding to a receptor such as the estrogen receptor alpha in the cytoplasm, translocating to the nucleus, and affecting transcription (McDevitt, M.A et. al 2008). Estrogens are also able to elicit faster effects by binding to transmembrane G protein-coupled receptors and activating signaling pathways (Weatherman 2006). Previous research identified that estrogen pretreatment for only 30 min protected cells from oxidative stress, which points to non-nuclear signaling (Yu et al. 2004).

There are three estrogen classes that naturally occur in women: estrone, estradiol, and estriol. Estradiol levels in the body drop from 15–350 pg/mL in premenopausal

Department of Biology, Columbus State University, Columbus, GA 31907. *Corresponding author – hughes_kathleen1@columbusstate.edu, 706-568-2325
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women to less than 10 pg/mL in postmenopausal women. Estrone levels in the body drop from 17–200 pg/mL for premenopausal women to 7–40 pg/mL in postmenopausal women. Estriol levels in premenopausal women are 8–11 pg/ml (Rotti et al. 1975).

Estradiol, specifically 17-β estradiol, plays a key role in the protection of neurological faculties and cardiac functions (Ito et al. 2006, Zhao et al. 2006). Seventeen-β estradiol is thought to contribute to cell protection through transcriptional regulation, though evidence points to action through transmembrane G protein-coupled receptors as well. Evidence shows that 17-β estradiol is also known to protect against oxidative stress, especially oxidative stress-induced neuronal cell apoptosis in relation to Alzheimer's disease (Behl et al. 1997).

Prescribed medications for hormone replacement therapy can include multiple types of estrogens such as estradiol, estrone, the conjugated equine estrogens (CEEs) equilin, and equilenin, as well as progestins. Despite the seemingly positive benefits of endogenous 17-β estradiol, the effect can be deleterious when combined with CEEs. Conjugated equine estrogens are taken from the urine of pregnant mares and are not found in humans. Two types available for study are equilin and equilenin. CEEs have been shown to minimize the side-effects of menopause such as hot flashes, night sweats, and mood swings (Brunner, R.L. et al. 2010). Unlike 17-β estradiol, they do not protect against neuronal intracellular ATP loss when delivered at 300–1000 pg/ml in vitro (Zhao et al. 2006). However, when several CEEs are administered together, the neuroprotective benefits such as protection from stroke and a lessened chance for Alzheimer's are measurable (Zhao et al. 2006). Equine estrogens have been linked to detrimental effects in women. Humans lack enzymes to process and metabolize equine estrogens properly (Hendrix et al. 2006). Since CEEs are slow to metabolize, they remain in the system for a longer period of time. The Women's Health Initiative through the National Institutes of Health found that postmenopausal women taking CEEs had a higher risk for ischemic stroke and an increased risk for developing dementia (Hendrix et al. 2006 and Shumaker et al. 2004). Shumaker et al. (2004) noted that the risks might be attenuated if the CEEs are administered immediately after a woman completes her menopausal cycle as opposed to administering the CEEs months to years later. Overall, equine estrogens are effective in alleviating menopausal symptoms, but may increase the likelihood of other health-related problems in some women.

The potential neurological risks associated with treatment with CEEs demand further understanding of their effects at the cellular level. While endogenous estrogens have often been linked to neuroprotection, it is essential to examine the role of human and equine estrogens in the various cell types found in the brain. Studying the role of both CEEs and endogenous estrogens *in vitro* will help elucidate their overall effects on the brain. Might discrepancies in estrogen protection stem from non-uniform effects depending on the cell type? Astrocytes play a key role in protecting and supporting neurons by performing specific tasks. First, they provide a balanced chemical environment appropriate for the neuron that allows action potentials to occur (Purves et al. 2012). Second, they are involved in the uptake of neurotransmitters like glutamate, regulation of synaptic signaling, and maintain-

ing neuronal excitability (Koehler et al. 2006). Third, astrocytes play a key role in facilitating communication between neural cells through gap junctions (Volterra et al. 2005). When it comes to neuroprotection, the role of astrocytes is complicated. On the positive end, evidence suggests that Alzheimer's disease and dementia are associated with damaged astrocytes, resulting in an environment unsuitable for neuronal survival (Chen et al. 2003). In addition, they promote viability of neural cells by removing toxins from extracellular fluid. However, they can also heighten neuronal apoptosis by triggering an inflammatory response (Zhang and Jiang 2015). Research using cultured astrocytes in cell viability studies could further our understanding of specific cellular roles.

The endogenous estrogen in humans, 17-β estradiol, has been shown to prevent neuronal cell death (Guo et al. 2012). Relatively little is known, however, about (i) the role of CEEs in cell viability, and (ii) how estrogens influence the viability of astrocytes under oxidative stress. We sought to determine the degree to which *in vitro*-pretreatment of astrocytes with various concentrations of equilin, equilenin, or 17-β estradiol affected cell viability and protected cells from oxidative stress.

Methods

Cells

The astrocytes (C8-D1A, American Type Cell Culture) used in this study were originally cultured from *Mus musculus* (mouse) cerebellum. They were incubated at 37°C and 5% CO_2. The cells were initially grown in T-75 flasks with 10mL Dulbecco's Modified Eagle's Medium (American Type Cell Culture)

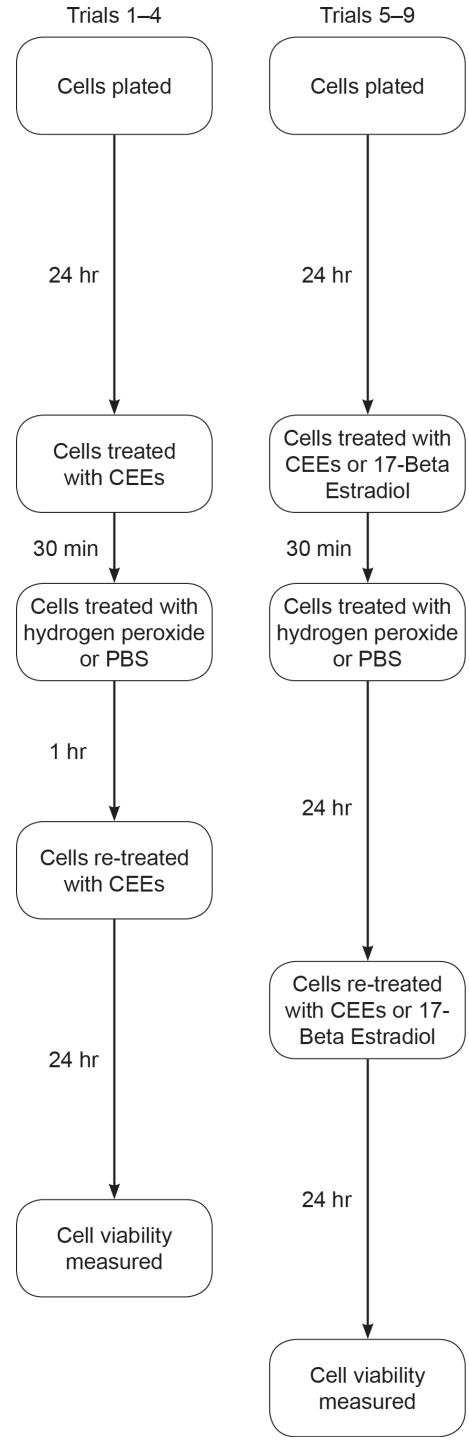

Figure 1. Timeline for steps and duration between them for Trials 1–4 and for Trials 5–9.

Table 1. Treatment design for Trials 1–4. We pretreated astrocytes with conjugated equine estrogen(s) for 30 min followed by a 1-h exposure to 600 µM hydrogen peroxide or PBS (control = unstressed cells). Then we changed the media and re-treated cells with the original estrogen(s). Rows list estrogen treatment and columns list estrogen concentration.

Treatment	Conjugated equine estrogen concentration				
Equilin PBS	Control (no estrogen)	10 nM	100 nM	1 µM	10 µM
Equilenin PBS	Control (no estrogen)	10 nM	100 nM	1 µM	10 µM
Equilin + Equilenin PBS	Control (no estrogen)	10 nM	100 nM	1 µM	10 µM
Equilin H_2O_2	Control (no estrogen)	10 nM	100 nM	1 µM	10 µM
Equilenin H_2O_2	Control (no estrogen)	10 nM	100 nM	1 µM	10 µM
Equilin + Equilenin H_2O_2	Control (no estrogen)	10 nM	100 nM	1 µM	10 µM

supplemented with 10% fetal bovine serum and 1% streptomycin ampicillin. The medium was changed every two days. Once 80% confluent, the cells were subcultured using trypsin-EDTA. For each trial, the cells were plated at a concentration of $1x10^6$ cells/mL in a 96 well plate (200 µL media per well), n = 4. Treatments began the following day (Figure 1).

Astrocyte Treatment

We had to determine the optimal conditions for measuring the effect of estrogen pretreatment on the reduction in cell viability due to oxidative stress. To induce oxidative stress, we exposed half of the astrocyte cultures to 600 µM hydrogen peroxide (H_2O_2) for either 1 h (Trials 1-4) or 24 h (Trials 5-9) and exposed the other half to PBS as a control. Following exposure to H_2O_2 or PBS, we compared cell viability as measured by an MTT assay (see below). In trials 1–4, we applied 3 types of CEE treatments (equilin, equilenin, and equilin plus equilenin) at 5 concentrations of estrogen (0 nM (control), 10 nM, 100 nM, 1 µM, 10 µM) (Table 1). We pretreated the astrocyte cultures for 30 min with one of the estrogen treatments before exposing them to H_2O_2. After the 1-hour exposure to H_2O_2, we changed the media and re-treated cells with their estrogen treatment (Table 1). We then incubated the astrocyte cultures for 24 h before measuring cell viability. Figure 1 summarizes the treatment timeline. Because Trials 1–4 showed that pretreatment with equine estrogens afforded astrocytes some protective benefit during a 1-h period of oxidative stress, we wanted to know whether the protection continued during a longer stress period. Therefore, in Trials 5–9, we exposed astrocytes to 600 µM H_2O_2 for 24 h after the 30-min pretreatment with estrogen. In addition, we added 17-β estradiol to the estrogen treatment groups to examine whether the endogenous estrogen was protective as well. We used the same estrogen concentrations as in Trials 1–4 (0 nM, 10 nM, 100 nM, 1 µM, 10 µM). Table 2 summarizes the treatments used for these trials; Figure 1 summarizes the treatment timeline.

Cell Viability Assay

We measured cell viability using the MTT In Vitro Toxicology Assay Kit (TOX-1, Sigma Aldrich, St. Louis, MO). Following pretreatment with estrogen,

Table 2. Treatment design for Trials 5–9. We pretreated astrocytes with conjugated equine estrogen(s) for 30 min followed by a 24-h exposure to 600 µM hydrogen peroxide or PBS (control = unstressed cells). Then we changed the media and re-treated cells with the original estrogen(s). Rows list estrogen treatment whereas columns list estrogen concentration.

Treatment	Estrogen Concentration				
Equilin PBS	Control (no estrogen)	10 nM	100 nM	1 µM	10 µM
Equilenin PBS	Control (no estrogen)	10 nM	100 nM	1 µM	10 µM
Equilin + Equilenin PBS	Control (no estrogen)	10 nM	100 nM	1 µM	10 µM
17-β Estradiol PBS	Control (no estrogen)	10 nM	100 nM	1 µM	10 µM
Equilin H_2O_2	Control (no estrogen)	10 nM	100 nM	1 µM	10 µM
Equilenin H_2O_2	Control (no estrogen)	10 nM	100 nM	1 µM	10 µM
Equilin + Equilenin H_2O_2	Control (no estrogen)	10 nM	100 nM	1 µM	10 µM
17-β Estradiol H_2O_2	Control (no estrogen)	10 nM	100 nM	1 µM	10 µM

exposure to oxidative stress, re-treatment with estrogen and 24-h incubation, we added 10 µL of MTT reagent to each well. Then, we incubated the astrocytes for 4 h at 37°C. Afterwards, we added 100 µL of the MTT solubilization solution to each well, placed the well plate into the Bio-Rad Model 680 microplate reader (Hercules, CA), and recorded the absorbance at 570 nm.

Statistical Analysis

The independent variables were duration of oxidative stress and estrogen treatment (type and concentration). The dependent variable was cell viability as measured by the MTT assay. Four trials were analyzed in the first set of experiments applying 1-h exposures of oxidative stress, n = 4 per treatment. Five trials were analyzed in the second set of experiments applying 24-h exposures of oxidative stress, n = 5 per treatment. Statistical significance among the test groups was evaluated using a two-way ANOVA followed by Tukey's post hoc test. A p-value ≤ 0.05 indicated statistical significance. Reported values are mean ± SD.

Results

Figure 2 shows the average cell viability (absorbance) values from 4 trials of astrocyte cultures exposed to either hydrogen peroxide (H_2O_2) or PBS (control) for 1 h following pretreatment with a CEE treatment: equilin, equilenin, or equilin plus equilenin. Without pretreatment with a CEE, 1-h exposure to H_2O_2 significantly reduced the cell viability of astrocyte cultures compared to that of control, unstressed cultures (0.10 ± 0.03 versus 0.19 ± 0.07 absorbance units, p = 0.05, Figure 2). With equilin, 1-h oxidative stress didn't reduce the viability of astrocyte cultures pretreated with 10 nM, 100 nM, 1µM or 10 µM equilin prior to oxidative stress relative to those of unstressed astrocytes Figure 2A). With equilenin, viability of stressed astrocytes pretreated with 10 nM equilenin was lower than that of unstressed cultures (0.11 ± 0.04 versus 0.21 ± 0.05, $p < 0.05$). However, when cells were pretreated with equilenin at any of the higher concentrations (100 nM, 1 µM, and 10 µM), viability of stressed and unstressed cultures did not differ significantly

(Figure 2B). Pretreatment with equilin plus equilenin produced the same pattern as pretreatment with equilenin alone; cell viability after 1 h of oxidative stress was not significantly different from that of unstressed astrocytes when they were pretreated with the 100 nM, 1 µM, or 10 µM concentrations (Figure 2C).

We performed an additional 5 trials to determine whether increasing the duration of the oxidative stress, from a 1-h to 24-h exposure to H_2O_2, would change the results documented above, and added another estrogen treatment group, 17-β estradiol. For cells not treated with an estrogen, 24-hour exposure to H_2O_2 significantly reduced the cell viability of cultures compared to that of unstressed cultures (0.07 ± 0.01 versus 0.12 ± 0.02, $p \leq 0.05$, Figure 3). It also reduced the cell viability of stressed astrocyte cultures pretreated with any of the lowest 3 concentrations of 17-β estradiol (10 nM: 0.08±0.02 versus 0.12 ±0.03, 100 nM: 0.07±0.01 versus 0.13±0.02, 1 µM: 0.08±0.03 versus 0.12 ±0.02). However, pretreating cells with 10 µM 17-β estradiol protected cells exposed to 24-h oxidative stress so that the viability of stressed and unstressed astrocytes did not differ significantly. At all 4 concentration levels of pretreatment with equilenin, 24-h exposure to H_2O_2 reduced the cell viability of stressed cultures compared to unstressed cultures (10 nM: 0.07±0.006 versus 0.12±0.02, 100 nM: 0.07±0.02 versus 0.12±0.03, 1 µM: 0.07±0.02 versus 0.11±0.03, 10 µM: 0.070±0.02 versus 0.11±0.02, Figure 3B). Cultures pretreated with any of the

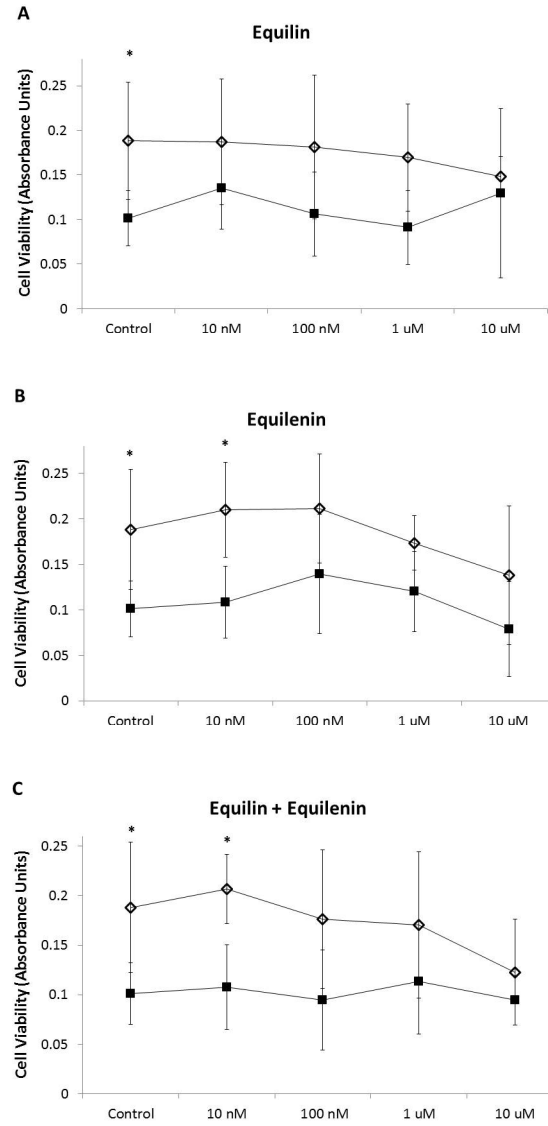

Figure 2. Astrocyte viability following a 1-h exposure to hydrogen peroxide. Astrocytes were treated with conjugated estrogens (10 nM, 100 nM, 1 µM, or 10 µM) for 30 min in vitro before being treated with either 600 µM H_2O_2 (stressed, ■) or PBS control (unstressed, ◇). Reported cell viability, mean ± SD. * $p \leq 0.05$

experimental concentrations of equilin had lower cell viability after the 24-h stress period compared to non-stressed cultures (10 nM: 0.09±0.02 versus 0.13±0.02, 1µM: 0.08±0.01 versus 0.13±0.02, and 10 µM: 0.07±0.003 versus 0.12±0.04, Figure 3C). However, viability of stressed and non-stressed astrocytes pretreated with 100 nM equilin did not differ significantly for cultures pretreated with a combination of equilin plus equilenin at all 4 experimental concentrations, 24-h oxidative stress resulted in lower viability compared to non-stressed astrocytes (10 nM: 0.07±0.02 versus 0.12±0.01, 100 nM: 0.08±0.02 versus 0.12±0.03, 1 µM: 0.07±0.02 versus 0.12±0.01, and 10 µM: 0.01±0.02 versus 0.12±0.02, Figure 3D).

Discussion

In all of the trials, cell viability of untreated astrocytes exposed to oxidative stress was lower than that of the unstressed control groups, indicating that 1-h and 24-h exposure to 600 µM H_2O_2 reduced cell viability. Overall, astrocytes pretreated with CEEs did not differ in viability after 1-h oxidative stress compared to their respective unstressed treatment groups. Specifically, viability of 1-h stressed astrocytes treated with any of the treatment concentrations (10 nM, 100 nM, 1 µM and 10 µM) of equilin did not differ

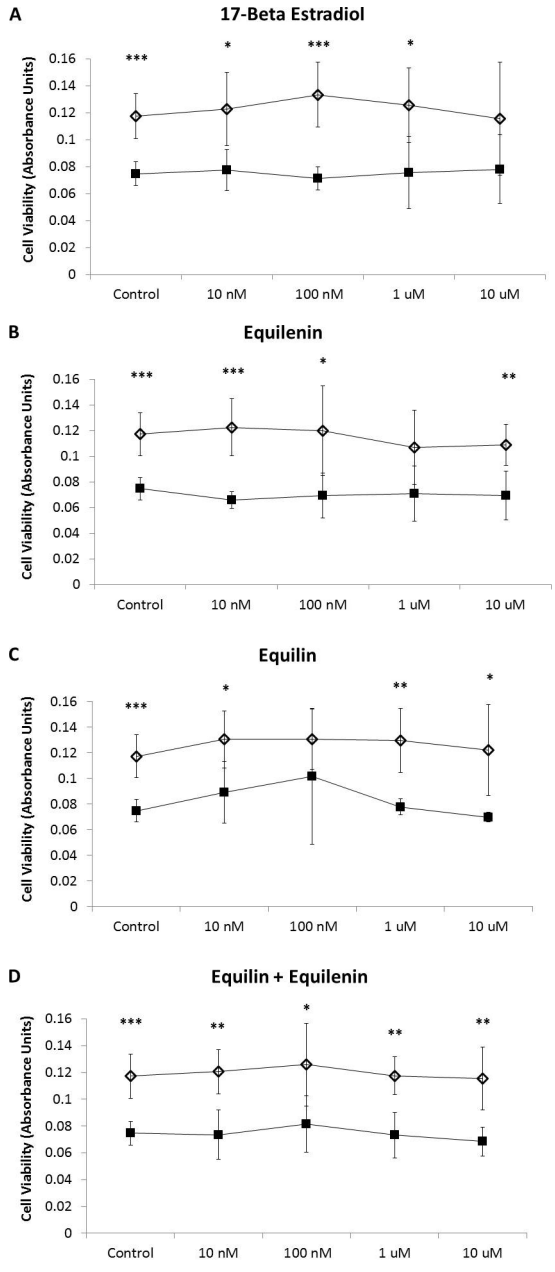

Figure 3. Astrocyte viability following a 24-h exposure to hydrogen peroxide. Astrocytes were treated with conjugated estrogens or 17-β estradiol (10 nM, 100 nM, 1 µM, 10 µM) for 30 min in vitro before being exposed to either 600 µM H_2O_2 (stressed, ■) or PBS control (unstressed, ◇) for 24 h. Reported cell viability, mean ± SD. * p≤0.05, **p≤0.01, ***p≤0.001

significantly from the viability of unstressed astrocytes. The same result was seen with equilenin (except at the lowest concentration of 10 nM). Equilin-plus-equilenin-treated astrocytes were not significantly different compared to either control or within concentrations. Taken together, the results indicate that pretreatment with a CEE may offer protection from short-term hydrogen peroxide-mediated cell death in astrocytes. This finding parallels protection associated with 17-β estradiol pretreatment (Yu et al. 2004).

Conversely, the longer 24-h exposure to H_2O_2 caused a significant decrease in cell viability compared to unstressed cells in most of the equilin-, equilenin-, and 17-β estradiol-treatment groups so the overall trend across estrogen concentrations was that the pretreatment with estrogens did not offer protection from cell death under a longer H_2O_2 exposure. Arguably, the longer, 24-h exposure to H_2O_2 overrode protection exhibited by cells pretreated with estrogen before their 1-h exposed to H_2O_2. Therefore, under continued oxidative stress induced by H_2O_2, cells will generally experience heightened apoptosis as evidenced in this experiment. The 10 μM 17-β estradiol, 100 nM equilin, and 1 μM equilenin pretreatments were the only treatment groups in which cell viability did not differ significantly in stressed and unstressed cells These results open the door to further examining this protection. Would astrocytes pretreated with CEEs have lower levels of oxidative stress markers compared to untreated cells? Is a specific CEE (alone or in combination) more protective than another when it comes to these oxidative stress markers? The mechanism of hydrogen peroxide's influence on astrocyte regulation is also an interesting topic for future study. For example, H_2O_2 exposure decreases the potentiation of neuronal activation via astrocytes (Safiulina et al. 2006). Would CEE treatment of primary neuronal cultures that contain astrocytes mimic the results from the current study?

Estradiol has been linked to neuroprotection and brain function, though the specific effects on astrocytes are not widely known (Dhandapani et al 2005). Seventeen-β estradiol has been shown to decrease calcium concentration changes in astrocytes (Rao 2006). Unfortunately, there are not many studies focused on the effects of CEEs on neurons and glial cells. While CEEs differ in structure, our results point to overall consistency in their effects on astrocyte viability in the presence of H_2O_2. It will be interesting to examine other CEE forms individually as well as in combination with endogenous estrogens.

The Women's Health Initiative through the American Heart Association studied the relationship between CEEs and the risk of ischemic and hemorrhagic stroke. They found that taking CEEs resulted in an increased risk for ischemic stroke (Hendrix et al. 2006). There was no evidence to link CEEs to hemorrhagic stroke. In a separate study, however, equilin and equilenin were found to be significantly neuroprotective with very little cell death observed (Zhao et al. 2006). That study also found that when CEEs were combined in a dosage, they were seemingly more effective than when a CEE was administered individually (Zhao et al. 2006). In this study, 10 nM – 10 μM equilin and equilenin, administered *in vitro* either separately or in combination, were found to protect astrocytes against short-term oxidative

stress (1 h). The astrocytes in this study were pretreated with CEE for only 30-min prior to H_2O_2 exposure. The rationale for this short exposure was to determine whether protection due to CEEs activating G protein-coupled receptor pathways was possible (as opposed to transcriptional regulation). For example, one study points to 17-β estradiol activating a phosphoinositide 3-kinase pathway in the rat retinal neurons (Yu et al. 2004). Since our results indicate protection with a 30-min pretreatment, we are encouraged to focus on estrogen signaling. Is a longer estrogen pretreatment linked to the same effects? Does estrogen receptor alpha and/or beta inhibition affect oxidative-stress protection? What are the measurable effects on downstream targets and transcriptional regulation?

These results encourage inquiry into brain cell variation. Future studies should explore the role of CEEs in neurons compared to astrocytes. While stable neuronal culture lines are not available, neuronal precursor lines as well as primary neuronal cultures containing astrocytes could be compared *in vitro*. The primary neuronal/astrocyte cultures will also help to further our knowledge of whether astrocytes have a helpful or harmful influence on neurons during oxidative stress. Another consideration is the species effect. The C8-D1A astrocyte cells that we used are cultured from the mouse cerebellum; astrocytes cultured from humans may have a different viability pattern following these treatments. It is imperative to determine whether these results are reproducible in human cells. Finally, an interesting direction would be to measure the actual level of oxidative stress as opposed to cell viability. Future studies should use functional assays to measure whether stress levels are affected by estrogen pretreatment. Overall, these results point to estrogen protection in astrocytes and indicate that short-term delivery of CEEs offers some protection from oxidative stress.

Acknowledgments

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