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A Novel, Synergistic Interaction Between 17 β-Estradiol and Glutathione in the Protection of Neurons against β-Amyloid 25–35-Induced Toxicity *In Vitro*

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ABSTRACT

The present studies were undertaken to investigate the possibility of an interaction between 17 β-estradiol (E2) and glutathione in protecting cells against the presence of β-amyloid 25–35 (βAP 25–35). We demonstrate that when evaluated individually, supraphysiological concentrations of either E2 (200 nM) or of reduced glutathione (GSH; 325 μM) can protect SK-N-SH human neuroblastoma cells from βAP 25–35 (20 μM) toxicity. This dose of βAP 25–35 was chosen based on the LD_{50} (28.9 μM) obtained in our earlier work. However, in the presence of 3.25 μM GSH, the neuroprotective EC_{50} of E2 was shifted from 126 ± 89 nM to 0.033 ± 0.031 nM, approximately 4000-fold. Similarly, in primary rat cortical neurons, the addition of GSH (3.25 μM) increased the potency of E2 against βAP 25–35 (10 μM) toxicity, as evidenced by a shift in the EC_{50} values of E2 from 68 ± 79 nM in the absence of GSH to 4 ± 6 nM in its presence. The synergy between E2 and GSH was not antagonized by the addition of the estrogen receptor antagonist, ICI 182,780. Other thiol-containing compounds did not interact synergistically with E2, nor were any synergistic interactions observed between E2 and ascorbic acid or α-tocopherol. Based on these data, we propose an estrogen-receptor independent synergistic interaction between glutathione and E2 that dramatically increases the neuroprotective potency of the steroid and may provide insight for the development of new treatment strategies for neurodegenerative diseases.

An increasing body of evidence indicates that oxidative damage plays a key role in the pathological events occurring in AD (Good *et al*., 1996; Yan *et al*., 1996), with use of antioxidants [i.e., any substance that under physiological conditions significantly delays or inhibits oxidation when present in low concentrations compared with those of an oxidizable substrate (Halliwell and Gutteridge, 1989)] emerging as a possibly useful therapy. Substances that fit this description and that are currently used in clinical trials include vitamin E and estrogens. A role for estrogens in the prevention of AD has been implicated by a retrospective epidemiological study, showing a dose- and duration-dependent relationship of ERT with a reduction in the incidence of AD (Paganini-Hill and Henderson, 1994). Recent studies revealed that ERT delays the onset of AD symptoms regardless of the ethnic origin of the subjects (Tang *et al*., 1996b). As such, there is much to be gained in elucidating the mechanism by which ERT alters the course of AD.

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In some cases of familial AD, genetic mutations result in the increased production of βAP (Selkoe, 1997), a 39–43 amino acid peptide which upon aggregation (Pike *et al*., 1991) and contact with the plasma membrane (Mattson *et al*., 1993) is toxic to neurons in culture. The toxic portion of this peptide seems to be an 11-amino acid sequence (βAP 25–35) (Yankner *et al*., 1989), and increased amounts of H_2O_2 (Behl *et al*., 1994) and lipid peroxides (Behl *et al*., 1992; Goodman *et al*., 1996; Gridley *et al*., 1997; Keller *et al*., 1997) result from a βAP 25–35 challenge. We have observed (Green *et al*., 1996) that this fragment rapidly aggregates, causing cell death with a dose-dependence and time-course similar to that reported for in primary neurons and B12 cells (Behl *et al*., 1994). This provides an *in vitro* model system for studying the mechanism by which estrogens ameliorate βAP-induced toxicity on a variety of cell types.

Estrogens have long been recognized as antioxidants in a variety of *in vitro* and *in vivo* models, and evidence is emerging to suggest that their antioxidant activity is involved in their neuroprotective capacity. Structure-activity relationship studies revealed that a phenolic A ring (Behl *et al*., 1997; Green *et al*., 1997b) and at least three rings of the steroid

ABBREVIATIONS: AD, Alzheimer disease; βAP, β-amyloid peptide; DMEM, Dulbecco's modified Eagle's medium; E2, 17 β-estradiol; ERT, estrogen replacement therapy, GSH, reduced glutathione; ICI, ICI 182,780; RPMI-1640, Roswell Park Memorial Institute-1640 Medium; PDHS, plasma-derived horse serum; ANOVA, analysis of variance.

structure (Green *et al*., 1997b) are required for the molecule to demonstrate neuroprotection. Additionally, estrogens reduce the cellular toxicity of βAP and other oxidative insults (Behl *et al*., 1995; Goodman *et al*., 1996; Green *et al*., 1996) and we have recently demonstrated that this correlates to a decrease in the amount of generated lipid peroxidation (Gridley *et al*., 1997). The mechanism by which estrogens act as an antioxidant at higher concentrations may directly relate to their ability to scavenge free radicals (Mooradian, 1993). We hypothesized that estrogens at physiologically relevant concentrations may be participating in a cycle whereby estrogens are regenerated by other endogenous antioxidants. Because reparation of lipid membranes relies on the glutathione peroxidase enzyme system (Meister and Anderson, 1983), and low concentrations of estrogen can reduce lipid peroxidation (Gridley *et al*., 1997), we targeted glutathione as a likely candidate for this interaction. The present studies were undertaken to investigate the possibility of an interaction between E2 and glutathione in protecting cells against the presence of βAP 25–35.

Materials and Methods

Materials. Lyophilized βAP 25–35 (1 mg; Bachem, Torrance, CA) was initially dissolved in 200 μl of double deionized H_2O and with the addition of 800 μl of PBS, rapid aggregation was observed. E2 (Steraloids, Wilton, NH) was initially dissolved at 10 mg/ml in absolute ethanol (Fisher Scientific, Orlando, FL) and diluted in cell culture media to obtain the necessary concentrations. ICI (Zeneca, Chesire, England) was dissolved in absolute ethanol and spiked into individual cell culture wells to obtain the 200 nM concentrations. α-Tocopherol acetate was initially dissolved in 200 μl of absolute ethanol and diluted in cell culture media to the appropriate concentrations. Lipoic acid (thiotic acid), taurine (2-aminoethanoic acid), and ascorbic acid were initially dissolved in cell culture media and used at the concentrations indicated. Unless otherwise noted, materials were obtained from Sigma Chemical Corp (St. Louis, MO).

SK-N-SH neuroblastoma cell culture. SK-N-SH neuroblastoma cells were obtained from American Type Culture Collection (Rockville, MD). Cell cultures were grown to confluency in RPMI-1640 media (Fisher Scientific, Pittsburgh, PA) supplemented with 10% charcoal/dextran-treated FBS, (Hyclone, Logan, UT), 100 units/ml of penicillin G and 100 mg/ml of streptomycin (Sigma) in monolayers in Corning 150-cm^2 flasks (Fisher Scientific) at 37° under 5% CO_2, 95% air. Media was changed three times weekly. Cells were observed with a phase contrast microscope (Nikon Diaphot-300; Nikon, Tokyo, Japan).

SK-N-SH cells used in the following experiments were in passes 4 to 12. The growth media was initially decanted and the cells were rinsed with 0.02% EDTA for 30 min at 37. Cells were then counted on a Neubauer hemacytometer (Fisher Scientific) and resuspended in appropriate media. Studies were initiated by plating 1×10^6 cells per well in 24-well plates, allowing attachment in regular media and then decanting that media and replacing with the appropriate treatment after 4 hr. Cells were cultured in DMEM or RPMI-1640 without GSH (Life Technologies, Grand Island, NY), supplemented with 10% FBS and antibiotics, with absolute ethanol as a vehicle control, or supplemented with the addition of βAP 25–35 (20 μM), E2 (0.002–200 nM), GSH (0.0325–325 μM), α-tocopherol acetate (50 μM), ascorbic acid (100 μM), lipoic acid (10 μM), taurine (5 mM), ICI (200 nM), or a combination as indicated. The 20-μM concentration of βAP was selected as we have shown that it is near the LD_{50} for this peptide (Green *et al*., 1996). Selection of antioxidant concentrations were made on the basis of preliminary dose-response evaluations used to identify the maximal concentration at which neuroprotection was not obtained (data not shown).

SK-N-SH cell viability was determined utilizing the trypan blue exclusion method (Black and Berenbaum, 1964). After 72 hr of incubation, treatment media was decanted and cells were lifted by incubating with 0.2 ml 0.02% EDTA for 30 min at 37°. Cells were suspended by repeated pipetting. Aliquots (100 μl) from each cell suspension were incubated with 100 μl aliquots of 0.4% trypan blue stain for 5 min at room temperature. All suspensions were counted on a Neubauer hemacytometer within 10 min of addition of trypan blue stain. Two independent counts of live and dead cells were made for each aliquot.

Primary rat cortical cultures. Primary neuronal cultures were prepared according to methods described elsewhere (Chandler *et al*., 1993). Briefly, Female Sprague-Dawley rats (Charles River Farms, Wilmington, MA) were housed and bred in our animal facility. Primary cortical neurons were prepared from 1-day-old rat pups as follows: brain tissue was removed from rat pups and placed in isotonic salt solution containing 100 units of penicillin G, 100 μg of streptomycin and 0.25 μg of amphotericin B (Fungizone; Life Technologies) per ml (pH 7.4). After removal of blood vessels and pia mater, the tissue was sectioned into approximately 2-mm chunks, suspended in 25 ml of 0.25% trypsin (weight/volume) in isotonic salt solution (pH 7.4), and placed in a shaking water bath for 10 min at 37° to dissociate the cells. The dissociated cell suspension was then removed and combined with 10 ml of DMEM containing 10% PDHS (Central Biomedia, Irwin, MO) and the undissociated chunks were mixed with 160 μg of DNase 1 and triturated until the cells dissociated. The cell suspensions were then combined, centrifuged at $1000 \times g$ for 10 min, and the resulting cell pellet washed with 50 ml of DMEM with 10% PDHS and plated on precoated poly-L-lysine 35-mm culture dishes at a density of 4×10^6 cells per dish and incubated in a humidified incubator containing 95% air and 5% CO_2 at 37°. On day 3, cells were treated with β-cytosine arabinoside (10 μM) for 48 hr and media was then aspirated and replaced with DMEM containing 10% PDHS and incubated for an additional 5 days before being used in experiments. At this time, cultures contain approximately 90% neurons and 10% astroglia. These appeared as many phase-contrast bright cells with characteristic neuronal morphology overlaying a number of flat phase dark cells that had typical astroglial morphology.

Treatments were made directly to primary cultures on culture day 10, maintaining a constant volume added regardless of treatment. Cultures were supplemented with the following treatments: Absolute ethanol and PBS as vehicle controls, βAP 25–35 (10 μM), E2 (0.02 nM-2 μM), GSH (3.25 μM), or combinations as indicated. The 10 μM concentration of βAP 25–35 was selected following preliminary studies aimed at causing a 40–60% cell death in 24 hr. Once treatments were added, primary cultures were incubated for an additional 24 hr and viability determined using the Live/Dead viability/cytotoxicity kit (Molecular Probes, Eugene, OR) according to manufacturer's instructions. Basically, the calcein AM (5 μM) and ethidium homodimer (5 μM) dyes were made fresh before use, and 300 μl were used to cover the bottom of the culture dish. Live cells were distinguished by the presence of intracellular esterase activity, which cleaves the calcein AM dye, producing a bright green fluorescence when excited. Ethidium homodimer enters cells with damaged membranes, and upon binding to nucleic acids, produces a red fluorescence. Both dyes are excited at 485 nm, and cultures plates were viewed with a fluorescent microscope (Nikon Diaphot-300). Three random fields were photographed, and the average number of live cells per field was determined by counting the number of bright green cells.

Statistics. The significant treatment effects on cell viability were determined using ANOVA followed by Scheffé's *post hoc* test, with significance determined at $p < 0.05$. For dose-response evaluations, EC_{50} values were calculated by randomly assigning cell counts at the indicated doses to generate 3–5 lines per treatment and determining the average value for those lines. Mann-Whitney rank sum nonparametric analyses were used on EC_{50} values because the variances for

the standard deviation were not equivalent. Comparisons between dose response relationships were calculated using two-way ANOVA to determine the significance of GSH or E2 presence or absence.

Results

The neuroprotective capability of physiologically relevant concentrations of E2 relies on the presence of glutathione in the cell culture milieu (Fig. 1). Using DMEM media, which lacks GSH in the cell culture recipe, and RPMI-1640 manufactured specifically without GSH, we demonstrated that the addition of βAP 25–35 reduced the number of viable cells by 41% and 47%, respectively, in these culture media. Concomitant treatment with E2 (2 nM) had no effect, which contrasted with our earlier work that showed these same concentrations of E2 to be neuroprotective in RPMI-1640 media (Green *et al*., 1996). Based on our hypothesis that GSH may play a role in this system, we supplemented GSH (3.25 μM) to the cell culture milieu. The addition of GSH was not neuroprotective alone; when added with low concentrations of E2 (2 nM), however, it increased the number of viable cells by 88% and 47% in DMEM and RPMI-1640 lacking GSH, respectively.

To further evaluate the interaction noted between E2 and GSH, we assessed the neuroprotective capacity of GSH (Fig. 2). We noted that in the absence of estrogen, high concentrations of GSH were necessary to achieve neuroprotection, with a significant reduction (50%) in βAP 25–35-induced toxicity at the 325 μM dose. By contrast, in the presence of E2 (2 nM), the neuroprotective dose of GSH was reduced to 0.325 μM. Two-way ANOVA revealed a highly significant effect of E2 on the GSH induced neuroprotection (F, 41.4; $p < 0.001$). The neuroprotective EC_{50} value generated for GSH without E2 in the media was 82.6 ± 60 μM, contrasted by 0.04 ± 0.02 μM when E2 was present, an approximate 2000-fold increase in GSH potency in the presence of E2. Using this information, we analyzed the ability of E2 to increase the number of viable cells exposed to βAP 25–35 (Fig. 3). In the absence of GSH, high concentrations of E2 (200 nM) were necessary for significant neuroprotection (Fig. 3). However, in the presence of a nonprotective dose of GSH (3.25 μM), the neuroprotective dose of E2 shifted from 200 nM to 0.2 nM (Fig. 3). Again, two-way ANOVA revealed a highly significant effect of the presence of GSH on the neuroprotective effect of E2 (F, 44.33; $p < 0.001$). The calculated EC_{50} values likewise shift to the left, from 126 ± 87 nM in the absence of GSH to 0.033 ± 0.031 nM in the presence of GSH, a ~4000-fold increase in the potency of E2 in the presence of GSH.

To ensure that this synergy was not caused simply by cell origin or tumorigenicity, we performed similar experiments in rat primary cortical neurons. Again, the ability of E2 to protect neurons was evaluated in the presence and absence of GSH (3.25 μM) (Fig. 4). The addition of βAP 25–35 (10 μM) to

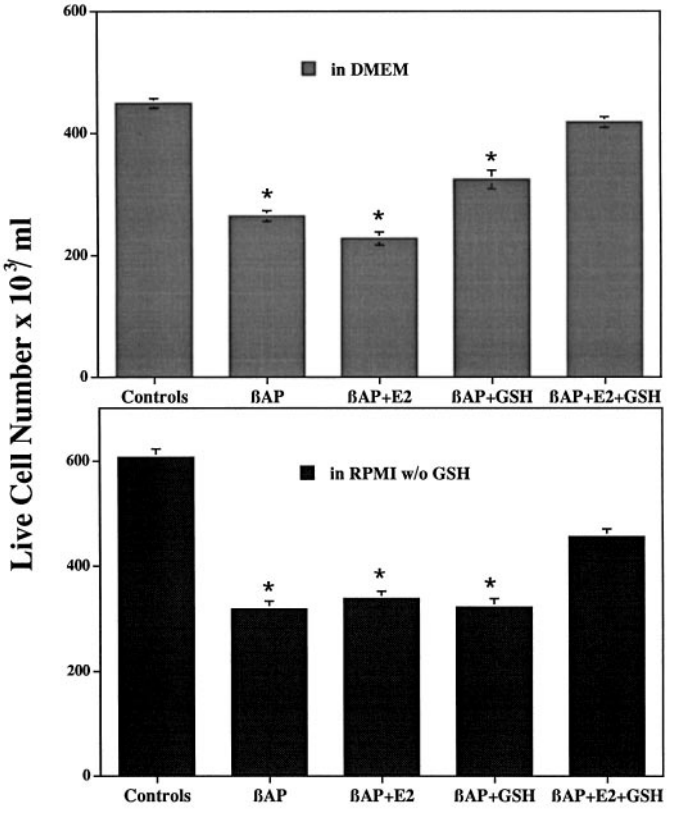

Fig. 1. Effects of 17-β estradiol (E2; 2 nM) and GSH (3.25 μM) on the toxicity induced by βAP 25–35 (20 μM) in different cell culture media that lack GSH in the cell culture recipe. Cells were plated at 10^6 cells/ml and were exposed to vehicle, βAP 25–35, E2, GSH, or a combination as indicated for 72 hr. *Controls*, cell numbers for the vehicle, GSH, E2, and GSH+E2 control groups (no βAP 25–35) pooled together after it was determined they were not statistically different from each other. Depicted are the mean values ± standard error for four or five wells/group. *, $p < 0.05$ versus controls determined by ANOVA followed by Scheffé's *post hoc* test.

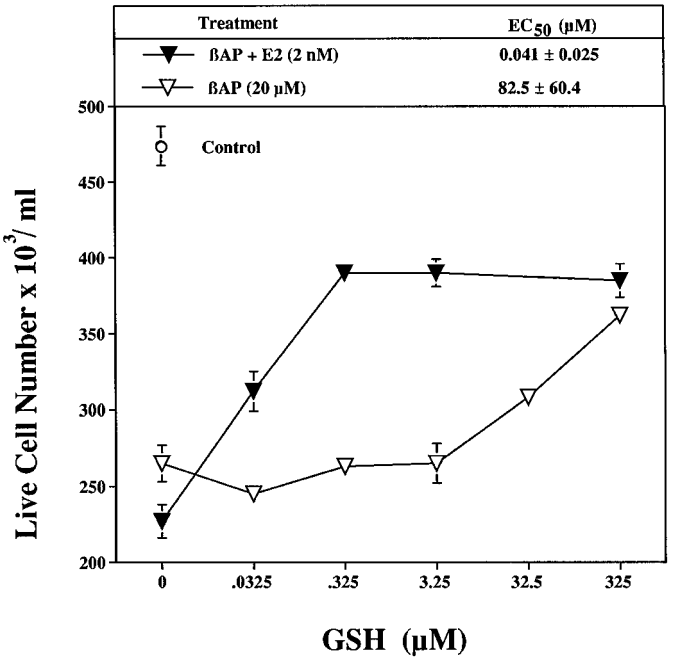

Fig. 2. Effects of GSH concentration on live SK-N-SH cell number subjected to a βAP 25–35 (20 μM) challenge in the presence and absence of a nonprotective dose of E2 (2 nM). Cells were plated at 10^6 cells/ml and exposed to treatments at the doses indicated. Controls represents cell numbers for the vehicle, GSH, E2, and GSH+E2 control groups (no βAP 25–35), pooled together after it was determined they were not statistically different from each other. Depicted are the mean values ± standard error for six wells per group. When error bars are absent, they are smaller than the symbol used to depict mean values. The effect of E2 on the response to GSH was highly significant (F, 41.48; $p < 0.001$). A comparison of the EC_{50} values for the different dose response curves using the Mann-Whitney rank sum test showed $p = 0.036$.

primary cortical neurons resulted in a 39% to 40% reduction in the average number of viable cells per field in the absence and presence of GSH, respectively (Fig. 4). When increasing concentrations of E2 were evaluated against βAP 25–35, 200 nM E2 was the lowest concentration found to be protective (Fig. 4), which is in full agreement with our SK-N-SH cell line studies (Fig. 3). With the addition of GSH (3.25 μM), all concentrations of E2 of 2 nM or higher were neuroprotective (Fig. 4). Evaluation of EC_{50} values demonstrated similar changes in potency, from 68.1 ± 79 nM in the absence of GSH, to 4.3 ± 5.9 nM in the presence of GSH. Likewise, evaluation of the effect of GSH on the neuroprotective effect of E2 in rat primary cultures using two-way ANOVA demonstrated a significant effect (F, 8.53; $p < 0.005$).

We examined the specificity of this estrogen-antioxidant interaction for the GSH system by evaluating protection of SK-N-SH cells from βAP 25–35-induced toxicity. This was achieved by addition of a concentration of E2 (20 nM) that was at or near the protective threshold in the presence or absence of nonprotective concentrations of other well known antioxidants (Table 1). In four separate experiments, the reduction in viable cells ranged from 35% to 68% when subjected to a βAP 25–35 (20 μM) challenge. The 20 nM dose of E2 alone was either nonprotective or slightly protective (Table 1). No significant effect was observed on βAP 25–35-induced toxicity with the addition ascorbic acid (100 μM), α-tocopherol acetate (50 μM), taurine (5 mM), or lipoic acid (10 μM) alone or in combination with E2 (Table 1). However, the addition of GSH (3.25 μM) or the oxidized form of glutathione (1.5 μM) was enough to significantly reduce the cytotoxicity of βAP 25–35 when 20 nM E2 was present (Table 1).

The importance of an estrogen receptor in this interaction was determined by using ICI as an antiestrogen (Fig. 5). Again, βAP 25–35 (20 μM) was added to SK-N-SH cells in the presence and absence of E2 (2 nM), GSH (3.25 μM), and/or ICI (200 nM). βAP 25–35 reduced viable cells after 72 hr of exposure by 54% when compared with vehicle controls. Using concentrations of E2 with GSH that were not protective when administered alone but were neuroprotective when added together, the addition of ICI in 100-fold excess of the E2 concentration did not significantly alter the protective effects of E2 and GSH in combination. Indeed, ICI addition alone exerted neuroprotective activity.

Discussion

It is the purpose of this article to report a novel synergistic interaction between E2 and glutathione for neuroprotection. This interaction is independent of species origin and the tumorigenicity of the cells, as we have demonstrated protection in a human neuroblastoma cell line, in rat primary cortical neurons (present report), and the HT-22 transformed mouse hippocampal cell line (Green *et al.*, 1998). Furthermore, this effect seems to be independent of the type of cytotoxic insult used, as we have observed the same synergy with both serum deprivation and zinc toxicity (Gridley KE,

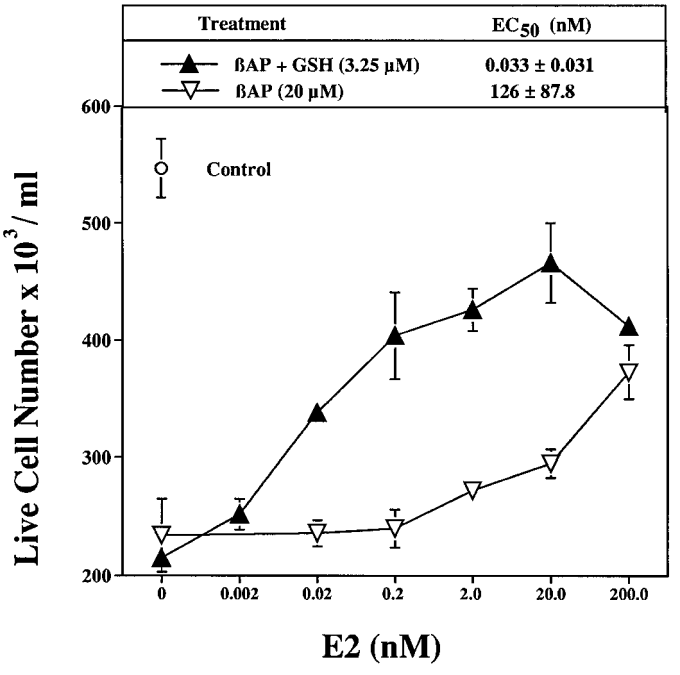

Fig. 3. Effects of E2 concentrations on live SK-N-SH cell number subjected to a βAP 25–35 (20 μM) challenge in the presence and absence of a nonprotective dose of GSH. Cells were plated at 10^6 cells/ml and exposed to treatments at the doses indicated. *Controls*, cell numbers for the vehicle, GSH, E2, and GSH+E2 control groups (no βAP 25–35), pooled together after it was determined they were not statistically different from each other. Depicted are the mean values ± SEM for four wells per group. When error bars are absent, they are smaller than the symbol used to depict mean values. The effect of GSH on the response to E2 was highly significant (F, 44.33; $p < 0.001$). A comparison of the EC_{50} values for the different dose response curves using the Mann-Whitney rank sum test showed a $p < 0.057$.

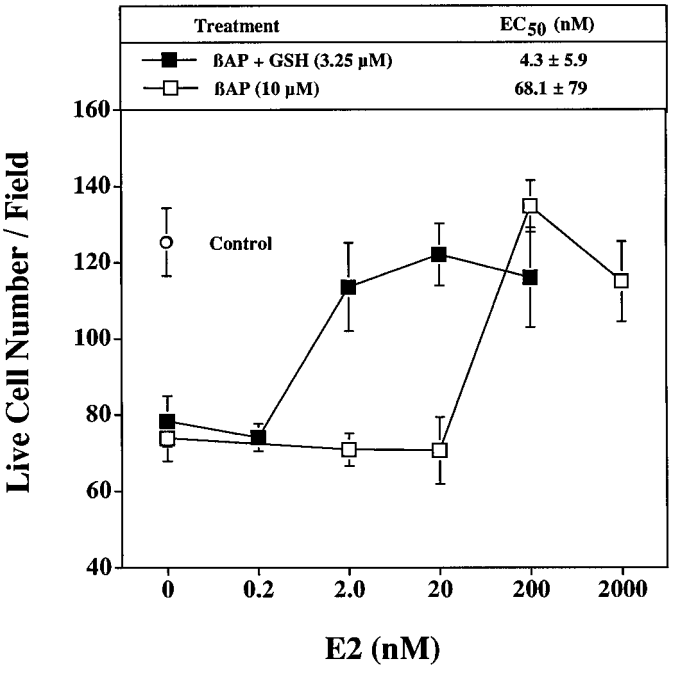

Fig. 4. Effects of concentrations of E2 on the average number of live rat primary cortical neurons per photographic field when subjected to a βAP 25–35 (10 μM) challenge in the presence and absence of a nonprotective dose of GSH. Cells were plated and exposed to treatments as previously indicated at the doses noted. *Controls*, cell numbers for the vehicle, GSH, E2, and GSH+E2 control groups (no βAP 25–35), pooled together after it was determined they were not statistically different from each other. Depicted are the mean values ± standard error for four to seven plates per group. The effect of GSH on the response to E2 was highly significant (F, 8.53; $p < 0.005$). A comparison of the EC_{50} values for the different dose response curves using the Mann-Whitney rank sum test showed $p < 0.057$.

Pariokar KS, Simpkins JW unpublished observations) as herein reported with βAP toxicity in SK-N-SH cells. This effect does not depend on the form of βAP used, as we have seen synergistic interactions against both βAP 1–40 and βAP 25–35 in HT-22 cells (Green *et al*., 1998). Although differences exist in the glutathione-induced shift in the neuroprotective potency of E2 in these cell types, these may directly relate to the different cell culture techniques used to assess viability. Furthermore, intracellular concentrations of glutathione may play a role as we have determined that primary rat cortical neurons have higher intracellular glutathione concentrations (172 ± 12 μM) than SK-N-SH cells (15 ± 2 μM). In either case, we can resolve discrepancies with regard to differences reported between our work (Green *et al*., 1996) and others (Behl *et al*., 1995; Goodman *et al*., 1996) for the neuroprotective concentrations of estrogens. In our previous work, we used a cell culture milieu containing GSH, whereas others used media that lacked GSH.

Mounting evidence supports the hypothesis that part of the neuroprotective activity of estrogens resides in their antioxidant capacity. Decreases in oxidative by-products are correlated with decreases in cellular toxicity in our previous studies (Gridley *et al*., 1997), as well as those done by others (Behl *et al*., 1997; Goodman *et al*., 1996). Because the actions of most antioxidants are multifaceted, we expect that estrogens may be working through several mechanisms to provide this neuroprotection. Estrogen can participate in the nonenzymatic reduction of free radicals (Mukai *et al*., 1990), further demonstrated in a cell-free system where peroxy-nitrite radicals were found to be reduced by estrogens (Mooradian, 1993) at the same concentration at which we see neuroprotection in the absence of glutathione. In addition, estrogens can participate in iron reduction (Ruiz-Larrea *et al*., 1995), which may be paramount to decreasing the production of free radicals. Multiple studies have demonstrated that estrogens decrease lipid peroxidation in a variety of model systems (Sugioka *et al*., 1987; Behl *et al*., 1995; Lacort *et al*., 1995; Goodman *et al*., 1996; Tang *et al*., 1996a; Behl *et al*., 1997; Gridley *et al*., 1997; Keller *et al*., 1997). Estradiol has also been shown to reduce oxidative impairment of membrane transporters for ions and glucose resulting from βAP 25–35 exposure (Keller *et al*., 1997). However, estrogens did not prevent impairment of membrane transport systems from toxic lipid peroxidation by-products (Keller *et al*., 1997) providing additional support for the idea that their antioxidant nature is the basis for their activity in neuroprotection.

Likewise, GSH exerts its antioxidant activity through several mechanisms (Meister and Anderson, 1983). GSH can scavenge free radicals via a nonenzymatic mechanism (Meister and Anderson, 1983). In our system, high concentrations of GSH (325 μM) were necessary to protect cells from the βAP insult (20 μM). This neuroprotective concentration is a much higher concentration of GSH than is present in extracellular fluids (Smith *et al*., 1996). Perhaps a more practical explanation involves the ability of GSH to act on intracellular peroxides via GSH peroxidases and GSH *S*-transferases (Meister and Anderson, 1983). This system functions in the defense against free radicals through the reduction of hydrogen peroxide. This action of GSH may be relevant to the βAP insult, because generation of increased amounts of H_2O_2 has been demonstrated in both βAP 25–35- and βAP 1–40-induced toxicity (Behl *et al*., 1994) and increases in the activity of glutathione peroxidase are correlated with increases in cell survivability for both peptides (Sagara *et al*., 1996).

The observed synergistic interaction between E2 and GSH seems to be mediated through an estrogen-receptor independent mechanism. Both the SK-N-SH human neuroblastoma and the HT-22 mouse hippocampal cell lines lack a functional estrogen receptor as determined by nuclear exchange assay (Green *et al*., 1998). Further, a variety of estratrienes that act only transiently at the estrogen receptor exhibit neuroprotection equivalent to that of E2 (Green *et al*., 1996; Green *et al*., 1997a, 1997b; Green *et al*., 1998). Additionally, we (Green *et al*., 1997b) and Behl (Behl *et al*., 1997) have demonstrated that an intact phenolic group is necessary for neuroprotection. The use of ICI as an antiestrogen is further support for this idea. The amount of ICI used, which in 100-fold excess of the E2 concentration satisfies the criteria required for demonstration of competitive inhibition, is at concentrations at which other phenolic A ring containing compounds demonstrate protection in the absence of glutathione (Green *et al*., 1998).

Given the phenolic nature of estrogens, they could activate the antioxidant response element/electrophilic response element (Montano and Katzenellenbogen, 1997). The antioxidant response element/electrophilic response element has been shown to be activated by phenolic antioxidants to increase phase II enzyme production, which includes GSH *S*-transferase (Jaiswal, 1994). Yet another possibility involves the conjugation of glutathione to estrogens (Jellnick *et al*., 1967). This action proceeds via glutathione *S*-transferases, where conjugation on the 4 position of the A ring of the steroid molecule provides bulky substituents (Elce and Harris, 1971), which has been shown *in vivo* to increase greatly the antioxidant potential (Miller *et al*., 1996). Given that 20–30% of the cellular mitochondrial pool resides in the mitochondria in some cell types (Smith *et al*., 1996), and estrogens stabilize mitochondrial function (Mattson *et al*., 1997), the estrogen-glutathione synergy could function to protect mitochondria against oxidative damage.

TABLE 1

Effects of E2, a variety of antioxidant treatments, and their combination on the βAP 25-35-induced toxicity in SK-N-SH live cell number after 72 hr of treatment.

	Ascorbic acid (100 μM)	α-Tocopherol (50 μM)	Taurine (5 mM)	Lipoic acid (10 μM)	GSH (3.25 μM)	Oxidized glutathione (1.5 μM)
Control	365 ± 9	641 ± 25	628 ± 13	628 ± 13	657 ± 30	657 ± 30
βAP	118 ± 9[a]	366 ± 24[a]	408 ± 10[a]	408 ± 10[a]	331 ± 17[a]	331 ± 17[a]
βAP + E2	144 ± 9[a]	407 ± 21[a]	394 ± 3[a]	393 ± 3[a]	357 ± 19[a]	357 ± 19[a]
βAP + Treatment	124 ± 6[a]	448 ± 12[a]	402 ± 9[a]	406 ± 7[a]	369 ± 7[a]	350 ± 29[a]
βAP + E2 + treatment	188 ± 11[a]	456 ± 14[a]	439 ± 11[a]	416 ± 10[a]	534 ± 16[b]	513 ± 7[a,b]

[a] $p < 0.05$ versus vehicle-treated controls.
[b] $p < 0.05$ versus βAP-treated controls.

Lipophilic estrogens that partition to the plasma membrane should associate their phenolic A rings with the charged hydrophilic head groups of the membrane phospholipids. Conjugation of estradiol with bovine serum albumin at the 17- (Green *et al*., 1997b) or 6- carbon positions (Green *et al*., 1998), which prevents the appropriate orientation of the molecule into the plasma membrane, blocks the neuroprotective action of estradiol. Based upon the observation that βAP aggregates extracellularly and causes membrane lipid peroxidation (Behl *et al*., 1995; Goodman *et al*., 1996; Gridley *et al*., 1997), we predict the hydroxyl hydrogen of estradiol is donated to prevent the cascade of membrane lipid peroxidation. Additionally, the enhanced potency of estrogens may result from its ability to donate hydrogen ions from several positions on the A ring (Jellnick and Bradlow, 1990). A relatively stable oxidized form of estradiol could result from this hydrogen ion donation and glutathione peroxidase could regenerate the reduced form of estrogen. This would operate by using GSH as a substrate for donation of the hydrogen group back to estrogen, and thus explain the synergy between the two molecules.

The specificity of estrogen's interaction for this glutathione system is supported by two lines of evidence. First, there are no apparent interactions noted between estrogen and the other thiols tested, lipoic acid or taurine, or any other antioxidants, including ascorbic acid or α-tocopherol. It is interesting to note that although α-tocopherol is a powerful antioxidant in its own right, estrogen has been argued to be even more powerful. Sugioka *et al*. (Sugioka *et al*., 1987) postulate that this may be because of the ability of the tocopheroxyl radical to regenerate estrogen. We have not observed any such apparent interactions in our system. Second, the ability of oxidized glutathione to work in this system supports the argument that estrogens may be interacting with the glutathione peroxidase/reductase process. *In vivo* evidence supports this idea, in that oral contraceptive use has been correlated with an increase in glutathione peroxidase activity (Capel *et al*., 1981; Massafra *et al*., 1993).

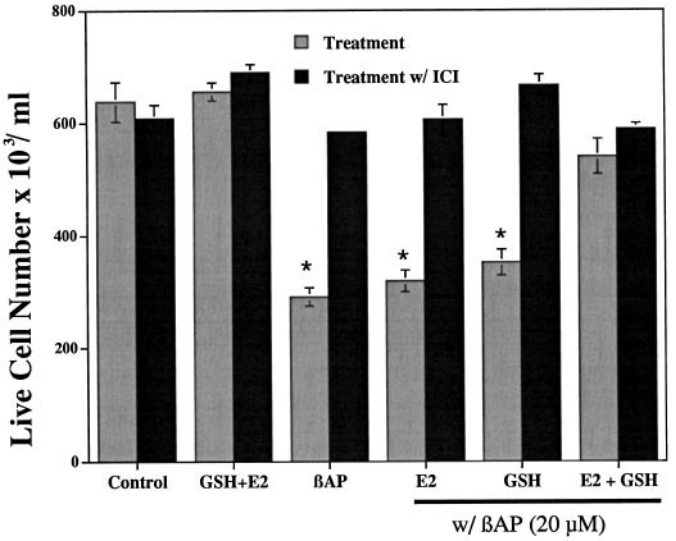

Fig. 5. Effects of the antiestrogen ICI (200 nM) on 20 μM βAP 25–35-induced toxicity in SK-N-SH cells using doses of E2 (2 nM) and GSH (3.25 μM), which alone are not protective but will exhibit protection when used in combination. Cells were plated at 10^6 cells/ml and were exposed to vehicle, βAP 25–35, E2, GSH, or a combination as indicated for 72 hr in the presence and absence of ICI. Depicted are the mean values ± standard error for three wells/group. *, $p < 0.05$ versus controls determined by ANOVA followed by Scheffé's *post hoc* test.

Finally, the identification of this synergistic interaction between estrogens and glutathione in neuroprotection has implications for the area of drug development for neurodegenerative diseases. Our observation that the potency of E2 was markedly affected by physiological concentrations of GSH in the cell culture milieu indicates that careful consideration for antioxidant defenses must accompany experimental design when assessing antioxidant drugs *in vitro*. Additionally, our data indicate that estrogen therapy can be improved by agents that enhance intracellular or extracellular glutathione concentrations.

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