

## A Select Combination of Clinically Relevant Phytoestrogens Enhances Estrogen Receptor $\beta$ -Binding Selectivity and Neuroprotective Activities *in Vitro* and *in Vivo*

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We have previously shown that a number of naturally occurring phytoestrogens and derivatives were effective to induce some measures of neuroprotective responses but at a much lower magnitude than those induced by the female gonadal estrogen 17 $\beta$ -estradiol. In the present study, we sought to investigate whether a combination of select phytoestrogens could enhance neural responses without affecting the reproductive system. We performed a range of comparative analyses of the estrogen receptor (ER)  $\alpha/\beta$  binding profile, and *in vitro* to *in vivo* estrogenic activities in neural and uterine tissues induced by clinically relevant phytoestrogens: genistein, daidzein, equol, and IBSO03569, when used alone or in combination. Our analyses revealed that both the ER $\alpha/\beta$  binding profile and neural activities associated with individual phytoestrogens are modifiable when used in combination. Specifically, the combination of genistein plus daidzein plus equol resulted in the greatest binding selectivity for ER $\beta$  and an overall improved efficacy/safety profile when compared with single or other combined formulations, including: 1) an approximate 30% increase in ER $\beta$ -binding selectivity (83-fold over ER $\alpha$ ); 2) a greater effect on neuronal survival against toxic insults in primary neurons; 3) an enhanced activity in promoting neural proactive defense mechanisms against neurodegeneration, including mitochondrial function and  $\beta$ -amyloid degradation; and 4) no effect on uterine growth. These observations suggest that select phytoestrogens in combination have the therapeutic potential of an alternative approach to conventional estrogen therapy for long-term safe use to reduce the increased risk of cognitive decline and neurodegenerative disease associated with menopause in women. (*Endocrinology* 150: 770–783, 2009)

**F**indings of the Women's Health Initiative (1, 2) and the Women's Health Initiative Memory Study (3–6), that the overall risks outweigh the benefits associated with estrogen-containing hormone therapy (HT), raised serious concerns regarding the use of HT in women. A consensus has been reached that, among many factors, "timing" could be a significant regulator of the health impact of HT on postmenopausal women. HT was found to be beneficial in reducing the incidence of cardiac disease in women who initiated the therapy within 10 yr from the onset of menopause, however, it was detrimental when started at an advanced age (7, 8). Similarly, HT appears to have a "healthy cell bias" for neural action (9, 10), suggesting its potential as a preventive rather than treatment strategy for cognitive decline and

neurodegenerative disease, such as Alzheimer's (AD), in postmenopausal women (11, 12). Despite the health benefits associated with the timely use of HT, long-term administration of HT has been associated with an increased risk of breast cancer (13). In addition, stroke remains a serious risk, regardless of time from the onset of menopause, for women receiving either estrogen-alone or combined therapy (8, 14, 15). In recent years, substantial research has focused on the development of alternative approaches that can mimic estrogenic beneficial impact on women's health while not eliciting the adverse effects seen with HT (16).

Plant-derived phytoestrogens, a weaker form than mammalian estrogens, produce estrogenic/antiestrogenic effects depending on the status of endogenous estrogens and the distribution of

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Abbreviations: AD, Alzheimer's disease; BW, body weight; COX, cytochrome c oxidase; D, daidzein; E, equol; 17 $\beta$ -E2, 17 $\beta$ -estradiol; ER, estrogen receptor; G, genistein; HT, hormone therapy; I, IBSO03569; IDE, insulin-degrading enzyme; LDH, lactate dehydrogenase; MIB, mitochondrial isolation buffer; NBM, Neurobasal medium; NEP, neprilysin; RCR, respiratory control ratio.

two subtypes of estrogen receptor (ER), ER $\alpha$  and ER $\beta$ , in specific tissues (17). It is suggested that the low incidence of a number of sex hormone-related disorders, such as menopausal hot flashes (18) and breast cancer (19, 20) in Asian women and prostate cancer in Asian men (21), compared with the incidence in Westerners, is in part attributable to the higher intake of phytoestrogen-rich soy foods in Asians (22, 23). The high intake of soy-derived phytoestrogens has also been linked to the low prevalence rate of AD in Asia compared with the Western world (17). However, this association lacks confirmation from randomized and controlled human studies, which have been found to be inconsistent. The disparity in outcomes across studies could be caused by variations in the constitutive composition of pharmacological preparations of soy extracts (17).

Basic science research determining the impact of phytoestrogens on brain functions has been based primarily upon exposure to either single phytoestrogens or soy diets containing the natural forms of soy intake seen in Asians. We have previously observed that a number of clinically relevant phytoestrogens, when used alone, only generated a small magnitude of neuroprotective response when compared with neurons exposed to the endogenous estrogen 17 $\beta$ -estradiol (17 $\beta$ -E2), which could be directly related to their weak binding to ERs (24). In the present study, we sought to test the hypothesis that the limited activity in the neural system associated with individual phytoestrogens could be modified when used in combination. In particular, combined use of rationally selected phytoestrogens that bind preferentially to ER $\beta$  over ER $\alpha$  could enhance neural responses without affecting the reproductive system. The strategy to selectively target ER $\beta$  is built upon recent advancements in understanding the roles of ER $\alpha$  and ER $\beta$  in conveying estrogen actions in the brain and periphery. First, ER $\beta$  is indicated to play a crucial role in mediating estrogenic activities to sustain neural defense against neurodegeneration (25, 26) and promotion of neural synaptic plasticity, learning, and memory function (27–29). In addition to its cognitive role, ER $\beta$  has also been suggested as a novel therapeutic target for the development of therapies for a range of pathological conditions mediated by ER $\beta$ , including menopausal vasomotor symptoms (30) and other brain disorders such as anxiety (31) and depressive behavior (32). Second, in view of the possibility that simultaneous activation of both ER $\alpha$  and ER $\beta$  by their cognate agonists may diminish their overall efficacy (26), a combined formulation composed solely of ER $\beta$  agonists could reduce the antagonistic interactions among ER $\alpha$  and ER $\beta$ -selective constituents in a random mixture. Third, selective activation of ER $\beta$  avoids ER $\alpha$ -mediated sexual modulation and proliferative responses known to cause elevated risk for reproductive cancers associated with the use of the conventional HT in women (33).

## Materials and Methods

### Chemicals

17 $\beta$ -E2 was purchased from Steraloids (Newport, RI). Genistein (G), daidzein (D), and equol (E) were purchased from Indofine Chemical (Hillsborough, NJ). IBSO03569 (I) was purchased from InterBioScreen (Moscow, Russia). The sources of other materials are indicated in the experimental methods described below.

### ER $\alpha$ / $\beta$ competitive binding

The ER $\alpha$ / $\beta$  binding profile of the test compounds or combinations was determined with a fluorescent polarization competitive binding assay (Invitrogen Corp., Carlsbad, CA), as previously described (34). Test compounds or combinations (composed of equivalent molar of individual phytoestrogens included) were serially diluted to a 2 $\times$  concentration in assay buffer (20  $\mu$ M to 200 pM). A total of 40  $\mu$ l assay buffer mixed with 2 $\times$  test compounds or combinations was added to a 384-well non-binding surface microplate, followed by addition of 40  $\mu$ l preincubated 2 $\times$  complex of ER $\alpha$  (30 nM) or ER $\beta$  (60 nM) and a fluorescent estrogen ligand EL Red (2 nM) for a final volume of 80  $\mu$ l. After a 6-h incubation, the polarization values were measured using a GENios Pro microplate reader (Tecan, San Jose, CA) at excitation/emission 535/590 nm and plotted against the logarithm of the concentrations of the test compounds or combinations. IC<sub>50</sub> values were determined from the plot by a non-linear least-squares analysis using GraphPad Prism version 4.03 (GraphPad Software Inc., San Diego, CA).

### Animals

The use of animals was approved by the Institutional Animal Care and Use Committee at the University of Southern California (Protocol No. 10780). Embryonic d 18 fetuses derived from time-pregnant Sprague Dawley rats (Harlan, Indianapolis, IN) were used to obtain primary neuronal cultures for *in vitro* experiments. Ovariectomized young adult female Sprague Dawley rats (14–16 wk old, weighing 270–290 g; Harlan) were used for *in vivo* experiments. Animals were housed under controlled conditions of temperature (22 C), humidity, and light (14 h light, 10 h dark) with water and food available *ad libitum*.

### Primary neuronal culture

Hippocampal neuronal cultures were prepared as previously described (26). Briefly, hippocampi derived from embryonic d 18 rat fetuses were treated with 0.02% trypsin in Hanks' balanced salt solution (137 mM NaCl, 5.4 mM KCl, 0.4 mM KH<sub>2</sub>PO<sub>4</sub>, 0.34 mM Na<sub>2</sub>HPO<sub>4</sub> · 7H<sub>2</sub>O, 10.0 mM glucose, and 10.0 mM HEPES) at 37 C for 5 min and dissociated by passage through fire-polished constricted Pasteur pipettes. Neurons were grown in Neurobasal medium (NBM) (Invitrogen) supplemented with B27, 5 U/ml penicillin, 5  $\mu$ g/ml streptomycin, 0.5 mM glutamine, and 25  $\mu$ M glutamate at 37 C in humidified 5% CO<sub>2</sub> atmosphere for the first 3 d and NBM without glutamate afterwards.

### Glutamate exposure and neuronal viability

Hippocampal neuronal cultures grown on 96-well culture plates for 7 d *in vitro* were pretreated with vehicle alone, test compounds, or combinations for 48 h, followed by exposure to 200  $\mu$ M glutamate for 5 min in HEPES-buffered saline solution containing: 100 mM NaCl, 2.0 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.0 mM MgSO<sub>4</sub>, 1.0 mM NaH<sub>2</sub>PO<sub>4</sub>, 4.2 mM NaHCO<sub>3</sub>, 10.0 mM glucose, and 12.5 mM HEPES. After glutamate exposure, cultures were washed with HEPES-buffered saline solution and replaced with fresh NBM containing the test compounds or combinations. Cultures were returned to the culture incubator and allowed to incubate for an additional 24 h before neuronal viability analyses by a colorimetric measurement of lactate dehydrogenase (LDH) release in the media and/or a fluorometric measurement of live-cell calcein acetoxyethyl ester staining as previously described (26), on the following day.

### A $\beta$ <sub>1–42</sub> exposure and neuronal viability

Fibrillar A $\beta$ <sub>1–42</sub> (American Peptide, Sunnyvale, CA) was prepared as previously described (35). Hippocampal neuronal cultures grown on 96-well culture plates for 7 d *in vitro* were pretreated with vehicle alone, test compounds, or combinations for 48 h, followed by exposure to freshly prepared 3  $\mu$ M A $\beta$ <sub>1–42</sub> in NBM in the presence of vehicle alone, test compounds, or combinations at 37 C for 3 d before neuronal viability analyses using a multiplex cytotoxicity assay (Promega Corp., Madison, WI), which contains two fluorogenic peptide substrates allowing a simultaneous measurement of live and dead-cell protease activities. GF-

AFC is a cell-permeant live-cell protease substrate and cleaved to generate a fluorescent signal proportional to the number of live cells in the cultures. Bis-AAF-R110 is a cell-impermeant dead-cell protease substrate and released from cells that have lost membrane integrity, therefore, it serves as a marker of cytotoxicity. Briefly, after  $AB_{1-42}$  exposure, 80  $\mu$ l culture medium was kept in each well of the culture plate, and 80  $\mu$ l of the assay buffer mixed with two substrates was added to incubate at 37 C for 45–60 min. The fluorescence intensities were measured on a SpectraMax dual-wavelength-scanning microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA) at excitation/emission filter combinations, 400/505 nm for live-cell fluorescence and 485/520 nm for dead-cell fluorescence.

### Animal treatment, tissue collection, and uterine weight

Ovariectomized young adult female rats were placed on a phytoestrogen-reduced diet, TD.96155 (Harlan Teklad, Madison, WI) during a 2-wk recovery from the surgery before the treatment. Test compounds or combinations (composed of equivalent weight of individual phytoestrogens included) were first dissolved in analytically pure dimethylsulfoxide and then diluted in corn oil (50  $\mu$ l dimethylsulfoxide in 950  $\mu$ l corn oil) to the working concentrations at 100  $\mu$ g/ml for 17 $\beta$ -E2 and 10 mg/ml for phytoestrogens. Rats were treated once daily for 2 d with a sc injection of vehicle alone, 17 $\beta$ -E2 [70  $\mu$ g/kg body weight (BW)], G (6 mg/kg BW), or phytoestrogen combinations (6 mg/kg BW). After the second injection, rats fasted for 24 h before being killed and brain dissection. Hippocampal and cortical tissues were collected from one hemisphere. The remaining brain tissues minus cerebellum, pineal gland, and brainstem were used for mitochondrial isolations. Uteri were excised, trimmed of fat and connective tissue, and a wet weight was recorded. The uteri were then air-dried for 1 wk and then at 70 C overnight, and the dry weight was recorded.

### Forebrain mitochondrial isolation

Rat forebrain tissues were homogenized in mitochondrial isolation buffer (MIB) containing 320 mM sucrose, 10 mM Tris-HCl, and 1 mM EDTA (pH 7.4)-KOH, with freshly added 0.5 mg/ml-MIB of BSA and 10  $\mu$ l/ml-MIB of protease inhibitor cocktail right before use, at 4 C. Homogenates were centrifuged at 1330  $\times$  g for 5 min. Pellets were rehomogenized and centrifuged. The two postnuclear supernatants were combined and centrifuged at 21,200  $\times$  g for 10 min. The resulting crude mitochondrial pellets were resuspended in 15% Percoll (Sigma, St. Louis, MO) and layered over a 23%/40% discontinuous Percoll gradient, and centrifuged at 31,000  $\times$  g for 10 min. The fraction accumulating at the 23%/40% interface was collected and washed with 10 ml MIB by centrifugation at 16,700  $\times$  g for 13 min. The pellets were then transferred to 1.5-ml Eppendorf tubes (VWR, Brisbane, CA) and centrifuged at 6600  $\times$  g for 8 min. The purified mitochondrial pellets were resuspended in MIB to an approximate concentration of 5 mg/ml. The purity and integrity of isolated mitochondria were confirmed as previously described (36). The isolated mitochondrial samples were used immediately for respiratory activity measurements or stored at  $-70$  C for enzymatic assays.

### Mitochondrial respiratory activity

Mitochondrial respiratory activity was measured polygraphically using a Clarke-type oxygen electrode (Hansatech Instruments Ltd., Norfolk, UK) at 37 C. A total of 100  $\mu$ g isolated mitochondria was added in a magnetically stirred chamber filled with 500  $\mu$ l respiration buffer [25 mM sucrose, 75 mM mannitol, 5 mM  $KH_2PO_4$ , 100 mM KCl, 0.05 mM EDTA, 20 mM HEPES, and 5 mM  $MgCl_2$  (pH 7.4)-KOH]. After a basal respiration recording, mitochondrial state 4 respiration was measured after the addition of 10.5  $\mu$ l substrates, malate (2.5 mM)/glutamate (2.5 mM). State 3 respiration was measured after the addition of 2.5  $\mu$ l ADP (350  $\mu$ M). Respiratory control ratio (RCR) was calculated as the ratio between the rate of oxygen uptake at state 3 and the rate of oxygen uptake at state 4.

### Mitochondrial cytochrome c oxidase (COX) activity

Mitochondrial COX activity was measured using a microplate immunocapture method (MS427) developed by MitoSciences (Eugene, OR), which spectrophotometrically monitors the change in absorbance at 550 nm after the oxidation of reduced cytochrome *c*. Briefly, COX in 25  $\mu$ g isolated mitochondria was first immunocaptured onto the assay plate, followed by the addition of the substrate, reduced cytochrome *c*. Colorimetric absorbance was measured at 550 nm at 30 C, and recorded every 5 min for 115 min, on a Benchmark Plus spectrophotometer equipped with a Microplate Manager version 5.2 Build 103 software (Bio-Rad Laboratories, Inc., Hercules, CA). Because the reaction is product inhibited, COX activity was expressed as the initial rate of oxidation of reduced cytochrome *c*, and determined by calculating the initial slope between two time points within the linear region (5–15 min).

### Western blot

Protein extraction and concentration determination were performed as previously described (26). A total 20–40  $\mu$ g protein samples was loaded per lane and separated by electrophoresis on 10–12% SDS-PAGE. Proteins were then electrotransferred to polyvinylidene fluoride membranes, which were probed with primary antibodies against Bcl-2 (1:250; Zymed Laboratories, Inc., San Francisco, CA), Bcl-xL (1:500; Zymed Laboratories), or insulin-degrading enzyme (IDE) (1:1000; Calbiochem, San Diego, CA), at 4 C overnight and then with horseradish peroxidase-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA).  $\beta$ -Tubulin (Abcam, Inc., Cambridge, MA) was used as the loading control. Bands were visualized with a 3,3',5,5'-tetramethylbenzidine peroxidase kit (Vector Laboratories) or by chemiluminescence using an enhanced chemiluminescence detection kit (Amersham Biosciences Inc., Piscataway, NJ). Relative intensities of the immunoreactive bands were quantified by an OD analysis using Un-Scan-It version 5.1 (Silk Scientific, Orem, UT).

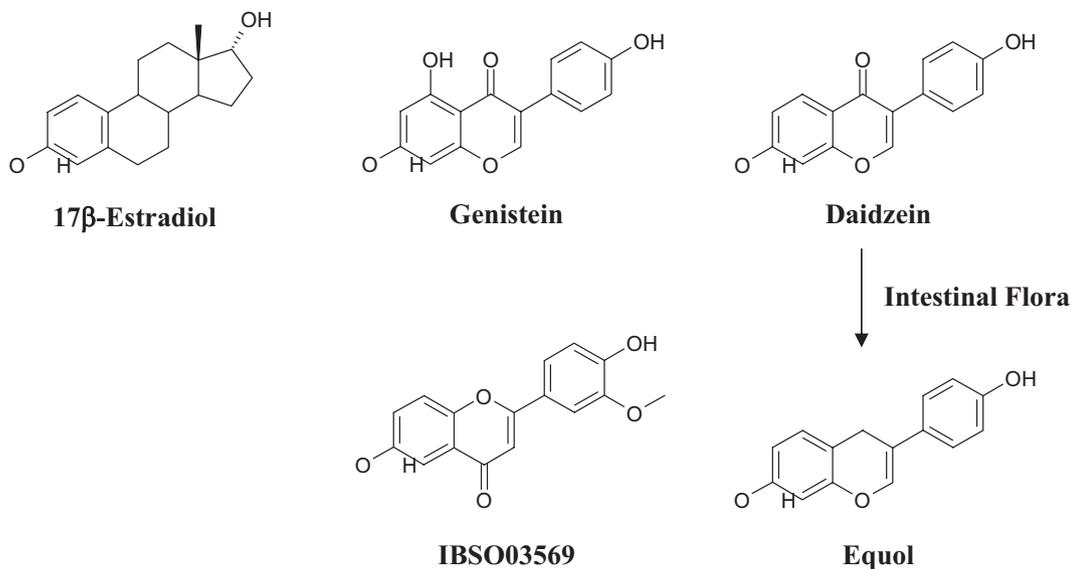
### Statistical analyses

Data are presented as group means  $\pm$  SEM. Statistically significant differences were determined by a one-way ANOVA followed by a Student-Newman-Keuls *post hoc* analysis.

## Results

### ER $\alpha$ / $\beta$ binding profile

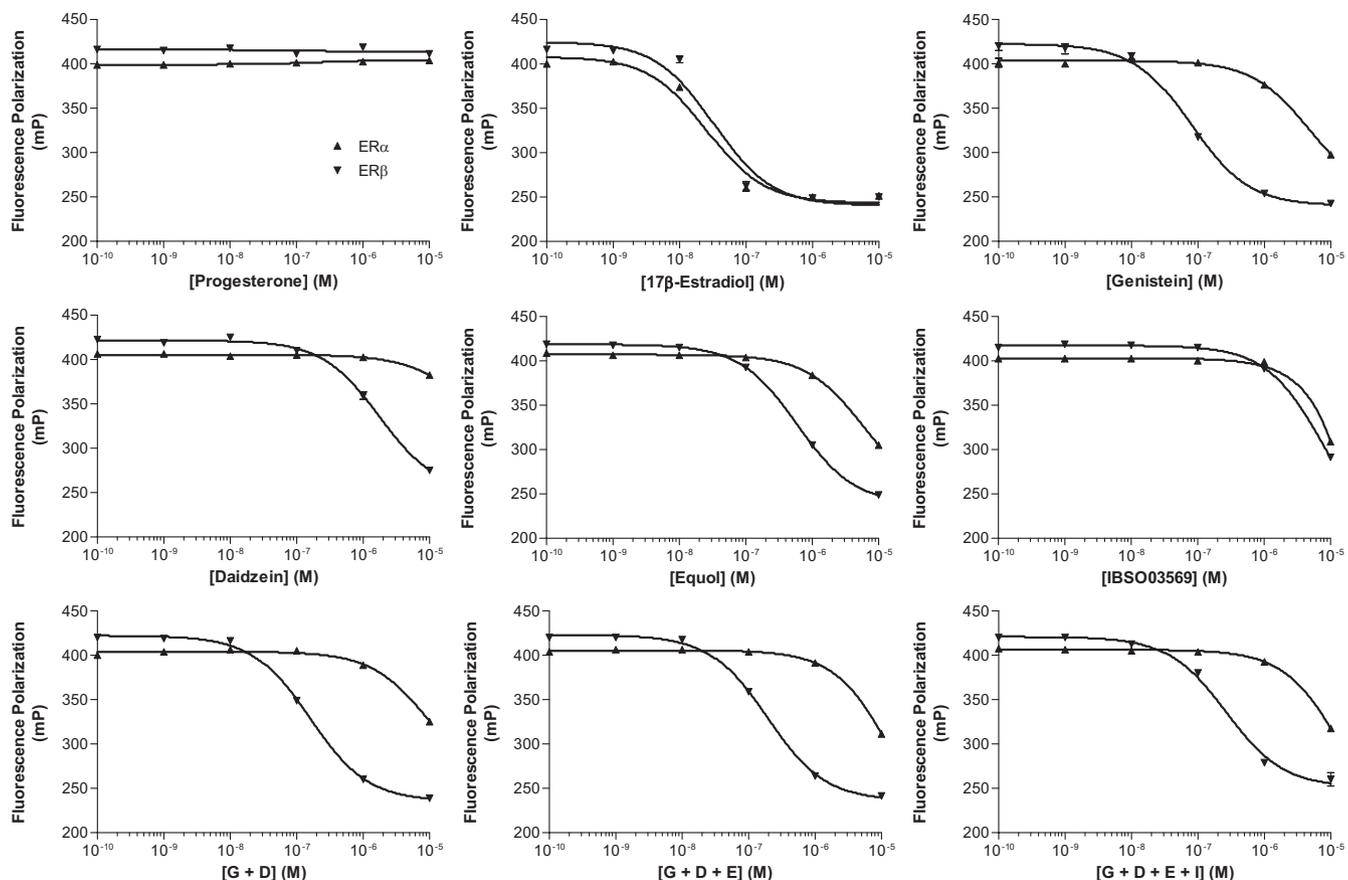
A fluorescence polarization-based competitive binding assay, which has been validated in our previous analyses (34, 37), was first conducted to determine the ER $\alpha$ / $\beta$  binding profile of test phytoestrogens when used alone or in combination (Fig. 1). Figure 2 illustrates the binding curves, and data derived from the binding curves are summarized in Table 1. The binding  $IC_{50}$  of 17 $\beta$ -E2, 25.3 nM for ER $\alpha$  ( $R^2 = 0.98$ ), and 32.5 nM for ER $\beta$  ( $R^2 = 0.96$ ) were consistent with the values reported previously (34, 37). As expected the negative control compound, progesterone, did not bind to either ER subtypes. Among individual phytoestrogens, G exhibited the maximal binding to both ER $\alpha$  ( $IC_{50} = 4.74$   $\mu$ M;  $R^2 = 0.98$ ) and ER $\beta$  ( $IC_{50} = 78.9$  nM;  $R^2 = 0.99$ ), with an approximate 60-fold preference for ER $\beta$  over ER $\alpha$ . The binding  $IC_{50}$  of G to ER $\beta$  was approximately 41% of the value from 17 $\beta$ -E2. In comparison with G, the binding affinities of D to both ER $\alpha$  ( $IC_{50} = 26.7$   $\mu$ M;  $R^2 = 0.79$ ) and ER $\beta$  ( $IC_{50} = 1.74$   $\mu$ M;  $R^2 = 0.99$ ) were weaker but maintained an approximate 14-fold binding preference for ER $\beta$ . E exhibited a similar binding affinity to G for ER $\alpha$  ( $IC_{50} = 5.88$   $\mu$ M;  $R^2 = 0.99$ ), but with a less, approximately 10-fold, binding preference



**FIG. 1.** Chemical structures of 17β-E2 and phytoestrogens tested in the present study. G and D are active aglycones of glucoside conjugates, genistein and daidzin, two main isoflavone constituents widely distributed in legumes such as soybeans. E is not of plant origin yet can be produced from D by enteric microfloral metabolism in a subset of human populations. I is a flavone derivative of D with an additional hydroxyl group attached to the 2-phenol ring.

to ERβ (IC<sub>50</sub> = 0.58 μM; R<sup>2</sup> = 1.00). Although I exhibited the greatest binding selectivity toward ERβ (>100-fold), the binding affinity was the lowest, with an IC<sub>50</sub> at 1695 μM for ERα (R<sup>2</sup> = 0.99) and 7.82 μM for ERβ (R<sup>2</sup> = 1.00). The combination of G

and D exhibited a decreased binding affinity compared with G alone, with IC<sub>50</sub> values (IC<sub>50</sub> = 9.90 μM for ERα; R<sup>2</sup> = 0.99; IC<sub>50</sub> = 0.16 μM for ERβ; R<sup>2</sup> = 1.00) approximately 50% of G alone. However, the binding selectivity of G plus D was slightly



**FIG. 2.** Competition binding curves for ERα and ERβ. Data were generated with a fluorescence polarization-based competitive binding assay using full-length human ERα and ERβ, and plotted against the logarithm of serially diluted concentrations of the test compounds or combinations. Progesterone served as a negative control. 17β-E2 served as a positive control.

**TABLE 1.** Binding profile for ER $\alpha$  and ER $\beta$ 

| Compounds/combinations | ER $\alpha$  |                      |                 | ER $\beta$                                |         |                | Selectivity ( $\beta/\alpha$ ) <sup>d</sup> |
|------------------------|--|----------------------|-----------------|---|---------|----------------|---|
|                        | IC <sub>50</sub> ( $\mu$ M) <sup>a</sup><br>Nonbinding | RBA (%) <sup>b</sup> | R <sup>2c</sup> | IC <sub>50</sub> ( $\mu$ M)<br>nonbinding | RBA (%) | R <sup>2</sup> |   |
| Progesterone           |  |                      |                 |   |         |                |   |
| 17 $\beta$ -E2         | 0.0253   | 100.0                | 0.9791          | 0.0325                                    | 100.0   | 0.9611         | 0.78  |
| G                      | 4.735  | 0.5343               | 0.9811          | 0.0789                                    | 41.12   | 0.9908         | 60.0  |
| D                      | 26.65  | 0.0949               | 0.7876          | 1.738                                     | 1.867   | 0.9883         | 14.27                                       |
| E                      | 5.876  | 0.4306               | 0.9948          | 0.5825                                    | 5.571   | 0.9986         | 10.09                                       |
| I                      | 1695   | 0.0015               | 0.9917          | 7.819                                     | 0.415   | 0.9959         | >100  |
| G+D                    | 9.896  | 0.2557               | 0.9865          | 0.1574                                    | 20.62   | 0.9970         | 62.87                                       |
| G+D+E                  | 15.71  | 0.1610               | 0.9925          | 0.1902                                    | 17.06   | 0.9969         | 82.60                                       |
| G+D+E+I                | 15.85  | 0.1596               | 0.9932          | 0.2615                                    | 12.41   | 0.9891         | 60.61                                       |

<sup>a</sup> The concentration of the test compounds or combinations resulting in a half-maximal shift in polarization value was determined from the binding curve (Fig. 2) by a nonlinear least-squares analysis.

<sup>b</sup> The relative binding affinity (RBA) of the test compounds or combinations that is expressed as the percentage of the binding affinity of 17 $\beta$ -E2 (relative binding affinity = 100%).

<sup>c</sup> The goodness of fit of nonlinear regression between the binding curve and the data. Ranging between 0.0 and 1.0, higher values indicate that the curve fits the data better. A fit with a R<sup>2</sup> at 1.0 indicates that all points lie exactly on the curve with no scatter.

<sup>d</sup> Selectivity ( $\beta/\alpha$ ) refers to the fold binding selectivity of the test compounds or combinations for ER $\beta$  over ER $\alpha$ , which was calculated as the ratio between the binding IC<sub>50</sub> for ER $\alpha$  and the IC<sub>50</sub> for ER $\beta$ .

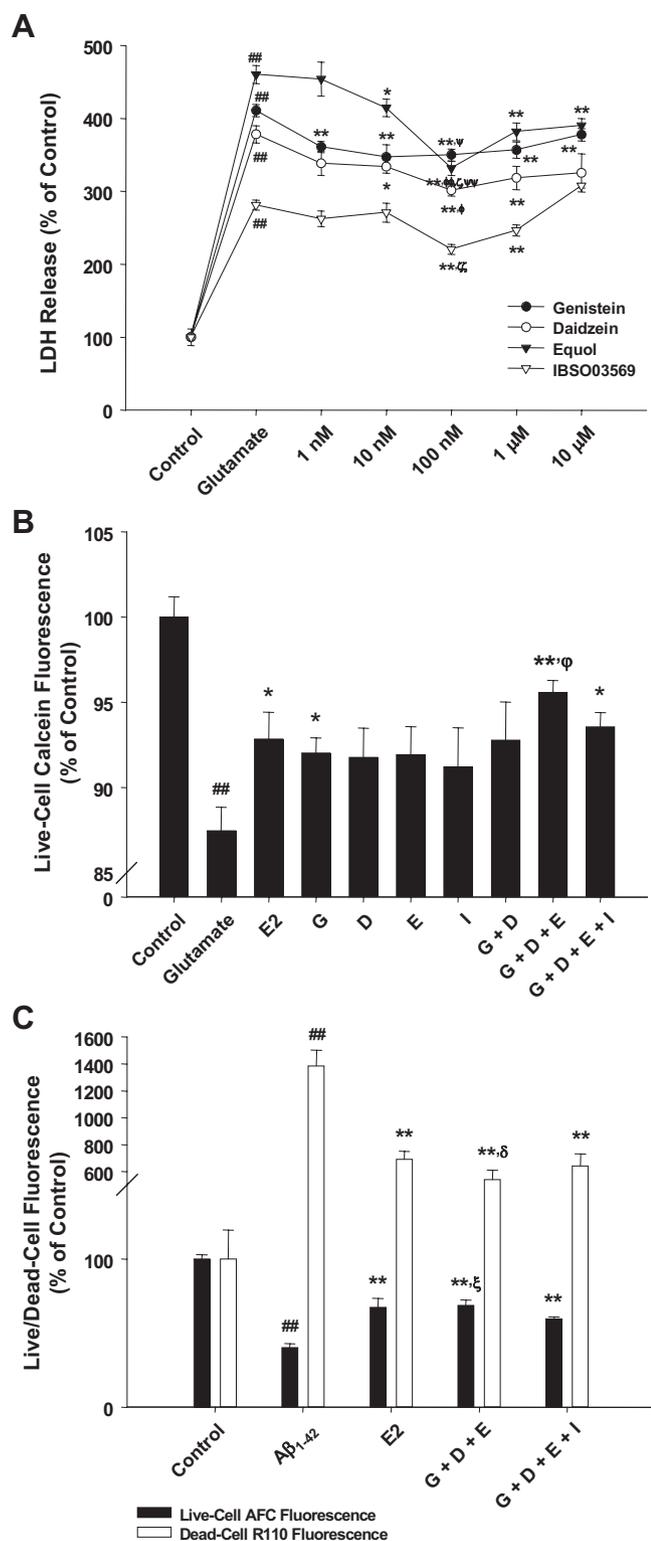
higher, with an approximate 63-fold binding preference for ER $\beta$ . The combination of G, D, and E exhibited a greater decrease in the binding affinity toward ER $\alpha$  (IC<sub>50</sub> = 15.7  $\mu$ M; R<sup>2</sup> = 0.99) than the decrease toward ER $\beta$  (IC<sub>50</sub> = 0.19  $\mu$ M; R<sup>2</sup> = 1.00), which was slightly lower than the value from G plus D. However, G plus D plus E exhibited a much improved, approximately 83-fold, binding preference for ER $\beta$ , which represents an approximate 30% increase compared with G alone or G plus D. The combination of G, D, E, and I exhibited a similar binding affinity to G plus D plus E toward ER $\alpha$  (IC<sub>50</sub> = 15.9  $\mu$ M; R<sup>2</sup> = 0.99) but with a lower, approximately 61-fold, binding preference for ER $\beta$  (IC<sub>50</sub> = 0.26  $\mu$ M; R<sup>2</sup> = 0.99), which is similar to the selectivity from the combination of G plus D.

### Neuroprotective activity *in vitro*

Neuroprotective effects of test phytoestrogens, when administered alone or in combination, were evaluated in rat primary hippocampal neuronal cultures challenged with neurotoxic glutamate or A $\beta$ <sub>1–42</sub>. A dose-response analysis was first conducted to determine the concentration for each phytoestrogen resulting in the maximal neuronal survival after an acute exposure to a supraphysiological concentration of glutamate. LDH release in the culture medium served as an indicator of neuronal membrane integrity, a minimum requirement for neuroprotection (38). Data summarized in Fig. 3A demonstrated that cultures exposed to glutamate alone had significantly increased LDH release in the medium as compared with vehicle-treated control cultures (<sup>##</sup>,  $P < 0.01$ ), although the relative amount of release varied across cultures. All four phytoestrogens induced a concentration-dependent and moderate reduction of LDH release as compared with glutamate alone-treated cultures (<sup>\*</sup>,  $P < 0.05$  and <sup>\*\*</sup>,  $P < 0.01$ ). A maximal reduction occurred at 100 nM, exhibiting significant differences from neuronal responses induced by some of the neighboring concentrations (<sup>ϕ</sup>,  $P < 0.05$  and <sup>ϕϕ</sup>,  $P < 0.01$ , <sup>ξ</sup>,  $P < 0.05$  and <sup>ξξ</sup>,  $P < 0.01$ , <sup>ψ</sup>,  $P < 0.05$ , and <sup>ψψ</sup>,  $P < 0.01$ ,

compared with cultures treated with 10 nM, 1  $\mu$ M, and 10  $\mu$ M phytoestrogens, respectively).

Further analyses were conducted to determine whether the test phytoestrogens administered at the concentration (100 nM) that produced the maximal protection against glutamate-induced damage in neuronal membrane integrity would be effective in protecting neurons against glutamate-induced deficits in metabolic activity, and whether combined use of these phytoestrogens would increase neuroprotective response when compared with single administrations. Neuronal viability was assessed by the live-cell calcein staining, which served as an indicator of neuronal metabolic activity (38). Data shown in Fig. 3B demonstrated that a 5-min exposure to 100 nM glutamate was not only deleterious to neuronal membrane integrity as previously determined, but also toxic to neuronal metabolic viability, as indicated by a significant reduction in calcein staining (87.4  $\pm$  1.4% relative to control cultures) compared with vehicle-treated control cultures (<sup>##</sup>,  $P < 0.01$ ). Cultures treated with 17 $\beta$ -E2 (10 nM) produced an average of 43.0% increase in calcein fluorescence (92.8  $\pm$  1.6% relative to control cultures) compared with glutamate alone-treated cultures (<sup>\*</sup>,  $P < 0.05$ ). When used alone, only G induced a significant increase (36.4%) in calcein fluorescence (92.0  $\pm$  0.9% relative to control cultures) compared with glutamate alone-treated cultures (<sup>\*</sup>,  $P < 0.05$ ). Although the other three phytoestrogens, D, E and I, when administered at 100 nM, were protective at the level of neuronal membrane integrity, they were insufficient to induce a statistically significant effect at the level of neuronal metabolic activity. In comparison, combined use of select phytoestrogens (with each individual phytoestrogen at 100 nM) exerted an increased neuroprotective efficacy. The combination of G plus D induced an average of 42.5% increase in calcein fluorescence (92.8  $\pm$  2.2% relative to control cultures), which, however, was not statistically significant. The combination of G plus D plus E induced a maximal effect (95.6  $\pm$  0.7% relative to control cultures) against gluta-



**FIG. 3.** Neuroprotective activity against glutamate (A and B) and A $\beta_{1-42}$ -induced toxic insults (C) in rat primary hippocampal neurons. Results are presented as the percentage of values produced by vehicle-treated control cultures. A "break" in y-axis (B and C) was used to demonstrate the differences among treatment groups. ##,  $P < 0.01$  compared with vehicle-treated control cultures; \*,  $P < 0.05$  and \*\*,  $P < 0.01$  compared with glutamate or A $\beta_{1-42}$  alone-treated cultures. In A,  $\phi$ ,  $P < 0.05$  and  $\phi\psi$ ,  $P < 0.01$  between 10 and 100 nM;  $\xi$ ,  $P < 0.05$  and  $\xi\psi$ ,  $P < 0.01$  between 100 nM and 1  $\mu$ M;  $\psi$ ,  $P < 0.05$  and  $\psi\phi$ ,  $P < 0.01$  between 100 nM and 10  $\mu$ M. In B and C,  $\delta$ ,  $P < 0.05$  between 17 $\beta$ -E2 and G + D + E-treated cultures;  $\phi$ ,  $P < 0.05$  between G + D + E and G-treated cultures;  $\xi$ ,  $P < 0.05$  between G + D + E and G + D + E + I-treated cultures.

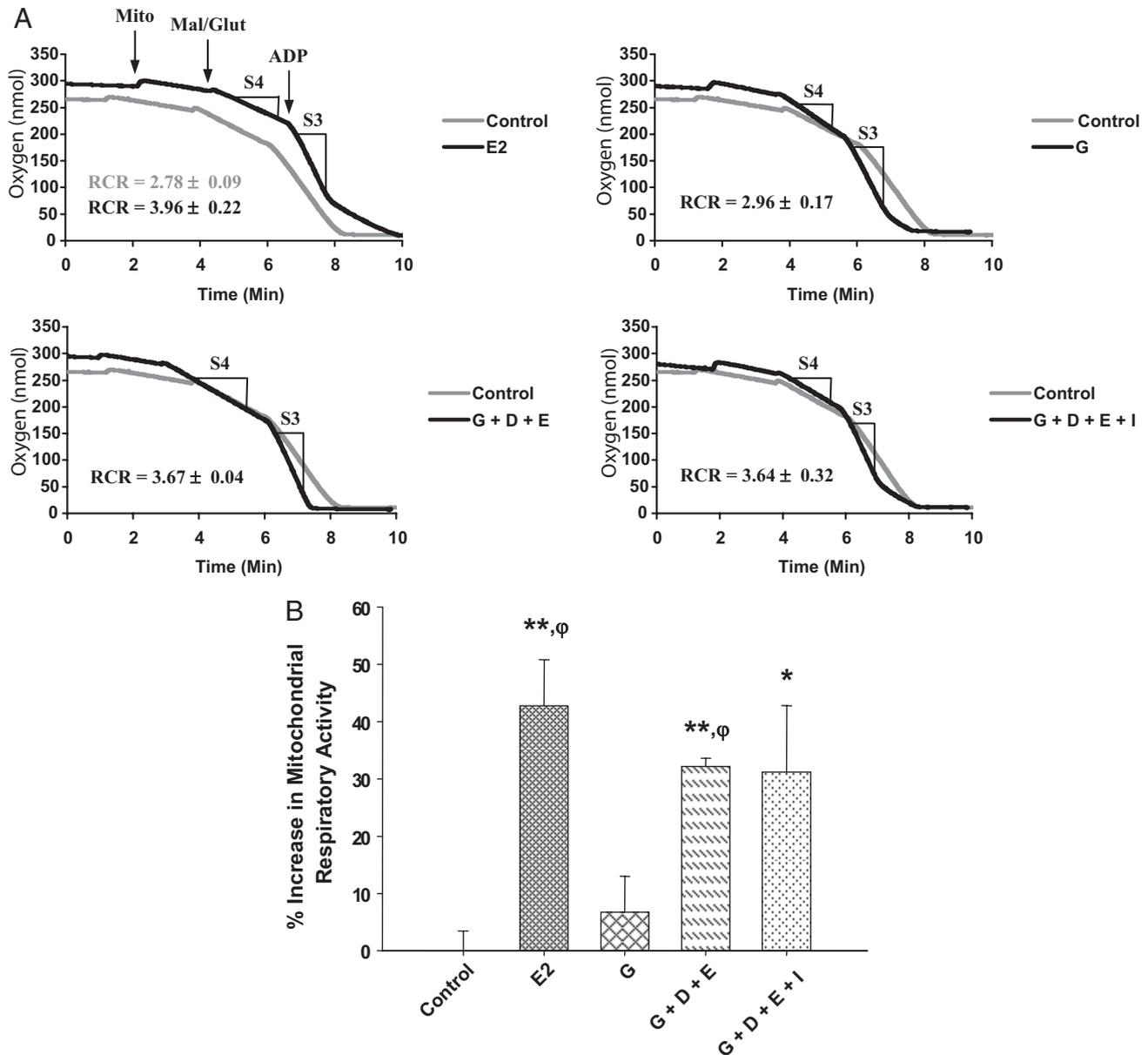
mate insult ( $64.8 \pm 5.6\%$  increase in calcein fluorescence compared with glutamate alone-treated cultures; \*\*,  $P < 0.01$ ), which was significantly greater than the effect induced by 17 $\beta$ -E2 ( $\phi$ ,  $P < 0.05$ ). The combination of G plus D plus E plus I induced an average of 48.5% increase in ( $93.5 \pm 0.9\%$  relative to control cultures) compared with glutamate alone-treated cultures (\*,  $P < 0.05$ ).

Based on the significant neuroprotective activity exhibited by combinations of G plus D plus E and G plus D plus E plus I, subsequent experiments were conducted to determine the impact of these combined formulations on neuronal survival when challenged with an extended exposure to an aggregated form of A $\beta_{1-42}$ . Neuronal viability was assessed by a dual measurement of live-cell AFC and dead-cell R110 staining, as indicators of neuronal metabolic activity and membrane integrity, respectively. Data shown in Fig. 3C demonstrated that a 2-d exposure to 3.0  $\mu$ M A $\beta_{1-42}$  was significantly neurotoxic, as evidenced by both reduced live-cell AFC ( $40.2 \pm 2.6\%$  relative to control cultures) and increased dead-cell R110 staining ( $1384.9 \pm 118.8\%$  relative to control cultures) compared with vehicle-treated control cultures (##,  $P < 0.01$ ). Treatment with 17 $\beta$ -E2 induced an average of 45.4% increase in AFC ( $67.3 \pm 6.2\%$  relative to control cultures) and an average of 54.1% reduction in R110 staining ( $690.4 \pm 60.3\%$  relative to control cultures) compared with A $\beta_{1-42}$  alone-treated cultures (\*\*,  $P < 0.01$ ). Consistent with the data against glutamate, the combination of G plus D plus E exerted the greatest effect on both neuronal metabolic viability measured by AFC staining ( $68.6 \pm 3.7\%$  relative to control cultures; an average of 47.6% increase compared with A $\beta_{1-42}$  alone-treated cultures; \*\*,  $P < 0.01$ ;  $\xi$ ,  $P < 0.05$  compared with G plus D plus E plus I-treated cultures) and membrane integrity measured by R110 staining ( $538.8 \pm 71.1\%$  relative to control cultures; an average of 65.8% reduction compared with A $\beta_{1-42}$  alone-treated cultures; \*\*,  $P < 0.01$ ;  $\delta$ ,  $P < 0.05$  compared with 17 $\beta$ -E2-treated cultures). The combination of G plus D plus E plus I induced an average of 32.2% increase in AFC staining ( $59.5 \pm 1.4\%$  relative to control cultures) and an average of 58.0% reduction in R110 staining ( $639.6 \pm 92.9\%$  relative to control cultures), both of which were statistically significant relative to A $\beta_{1-42}$  alone-treated cultures (\*\*,  $P < 0.01$ ).

### Regulation of brain mitochondrial bioenergetics *in vivo*

To determine whether the *in vitro* findings were predictive of *in vivo* efficacy, after experiments conducted in ovariectomized adult female rats compared *in vivo* estrogenic responses in neural and uterine tissues, induced by 17 $\beta$ -E2 and three phytoestrogen formulations: G alone and combinations of G plus D plus E and G plus D plus E plus I, which were found to be significantly neuroprotective *in vitro*. The dosage of 17 $\beta$ -E2 (70  $\mu$ g/kg  $\cdot$  d) was designed to be commensurate with a commonly used dose (0.625 mg/kg  $\cdot$  d) reported in epidemiological and clinical studies. The dosage of phytoestrogen formulations (6 mg/kg  $\cdot$  d) was commensurate with 50 mg/d exposure in humans based on an estimated amount of total phytoestrogen intake in Asian populations.

We have previously found that 17 $\beta$ -E2 promotes brain mitochondrial proteome involved in oxidative phosphorylation (36), which could serve as a proactive buffer against mitochon-

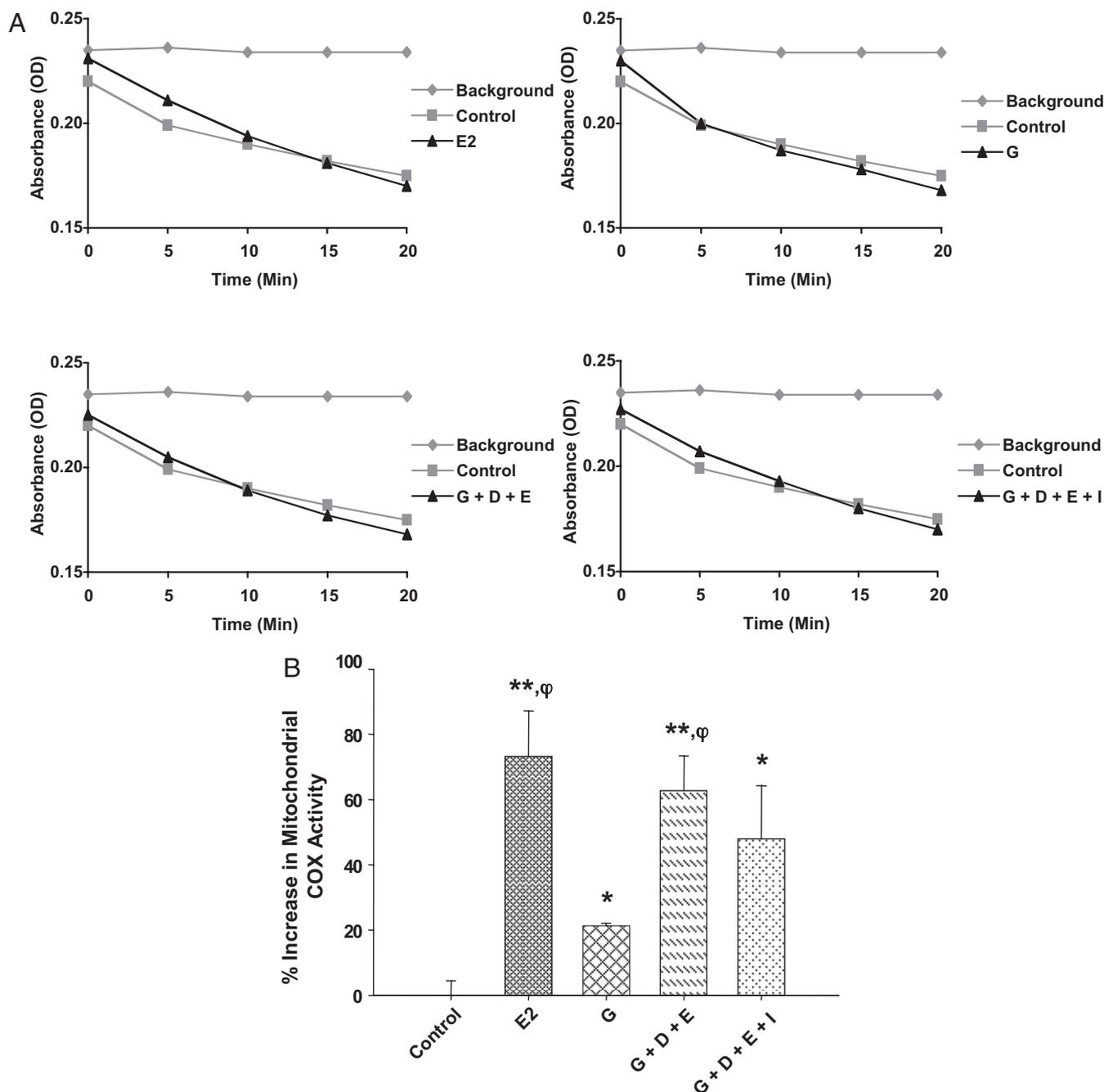


**FIG. 4.** Regulation of forebrain mitochondrial respiratory activity in ovariectomized adult female rats. A, Time-lapse oxygen uptake. B, Percent (%) increase in mitochondrial respiratory activity compared with vehicle-treated control rats ( $n \geq 4$ ). \*,  $P < 0.05$  and \*\*,  $P < 0.01$ ;  $\phi$ ,  $P < 0.05$  compared with G-treated rats. Mal/Glut, Malate/glutamate; Mito, mitochondria.

drial functional decline that has been implicated in a variety of neurodegenerative diseases, including AD and Parkinson's (39–42). In this experiment, rat forebrain mitochondria were analyzed to determine whether phytoestrogen formulations would have a similar impact on mitoenergetics as determined by respiratory efficiency as a primary and COX enzymatic activity as a secondary indicator. As shown in Fig. 4, consistent with our previous report (36), rats treated with  $17\beta$ -E2 ( $70 \mu\text{g}/\text{kg} \cdot \text{d}$  for 2 d) exhibited a significantly enhanced mitochondrial oxygen consumption with an average increase of 42.7% ( $\pm 7.9\%$ ) in RCR (RCR =  $3.96 \pm 0.22$ ) compared with vehicle alone-treated control rats (RCR =  $2.78 \pm 0.09$ ; \*\*,  $P < 0.01$ ). Oxygen consumption in rats exposed to G alone ( $6 \text{ mg}/\text{kg} \cdot \text{d}$  for 2 d) did not exhibit a significant change (RCR =  $2.96 \pm 0.17$ ) as compared with control rats. In contrast, RCR in rats exposed to the com-

bination of G plus D plus E ( $6 \text{ mg}/\text{kg} \cdot \text{d}$  for 2 d; RCR =  $3.67 \pm 0.04$ ) was significantly enhanced with an average increase of 32.2% ( $\pm 1.5\%$ ) as compared with control rats (\*\*,  $P < 0.01$ ). Similarly, exposure to the combination of G plus D plus E plus I ( $6 \text{ mg}/\text{kg} \cdot \text{d}$  for 2 d; RCR =  $3.64 \pm 0.32$ ) induced an average increase of 31.2% ( $\pm 11.5\%$ ) compared with control rats (\*,  $P < 0.05$ ). Among four treatment groups, there was a statistically significant difference between  $17\beta$ -E2 and G ( $\phi$ ,  $P < 0.05$ ) as well as between G plus D plus E and G treatment groups ( $\phi$ ,  $P < 0.05$ ). The variance within the G plus D plus E plus I-treated group prohibited a significant difference from G alone-treated group.

A similar trend was observed for the enzymatic activity of COX as shown in Fig. 5. Consistent with our previous report (36),  $17\beta$ -E2 treatment induced a significant increase ( $73.3 \pm 13.9\%$ ) in mitochondrial COX activity as compared with vehicle



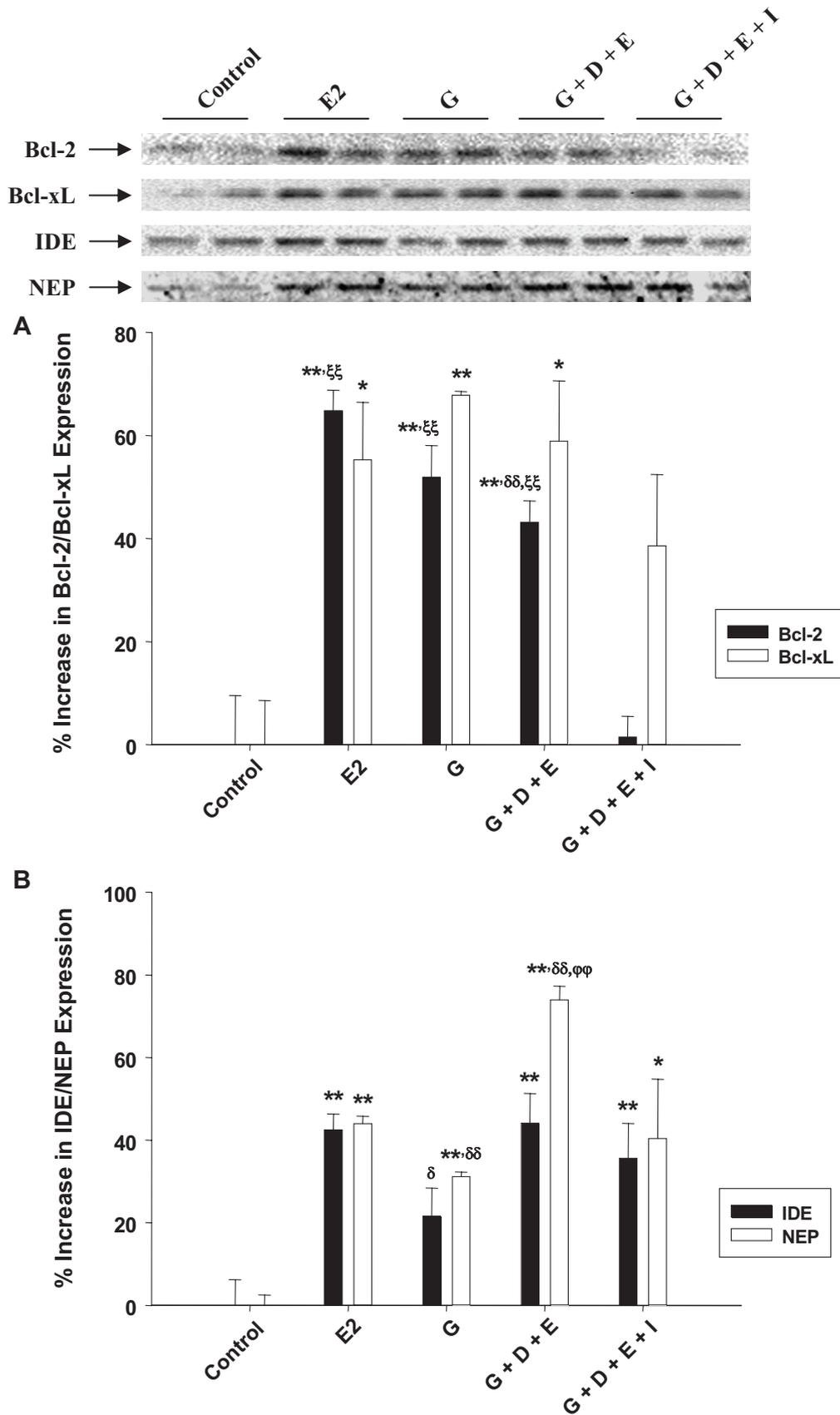
**FIG. 5.** Regulation of forebrain mitochondrial COX activity in ovariectomized adult female rats. A, Time-lapse change in absorbance (first 20 min). B, Percent (%) increase in mitochondrial COX activity compared with vehicle-treated control rats ( $n \geq 4$ ). \*,  $P < 0.05$  and \*\*,  $P < 0.01$ ; φ,  $P < 0.05$  compared with G-treated rats.

alone-treated control rats (\*\*,  $P < 0.01$ ). In contrast to the lack of an effect on mitochondrial respiration, rats treated with G alone exhibited a moderate but statistically significant enhancement in COX activity ( $21.3 \pm 0.8\%$  increase relative to control rats; \*,  $P < 0.05$ ). Similar to the data on respiration, rats treated with the combination of G plus D plus E or G plus D plus E plus I induced an average increase of  $62.7\% (\pm 10.8\% \text{ **}, P < 0.01)$  and  $48.0\% (\pm 16.2\%; *, P < 0.01)$ , respectively, in COX activity as compared with control rats. Among the four treatment groups, a statistically significant difference occurred between  $17\beta$ -E2 and G (φ,  $P < 0.05$ ), as well as between G plus D plus E and G treatment groups (φ,  $P < 0.05$ ). Consistent with the re-

spiratory activity, there was no significant difference between G plus D plus E plus I and G treatment groups.

#### Regulation of brain mitochondrial antiapoptotic proteins *in vivo*

In association with impact on mitochondrial bioenergetic efficiency, up-regulation of brain mitochondrial antiapoptotic proteins Bcl-2 and Bcl-xL has also been indicated as part of neuroprotective mechanisms induced by  $17\beta$ -E2 (43, 44). In this experiment, Western blot analyses of hippocampal protein samples prepared from the opposite hemispheres of treated rats were conducted to determine whether the test compounds or combi-



**FIG. 6.** Regulation of mitochondrial antiapoptotic proteins, Bcl-2/Bcl-xL (A), and  $\beta$ -degrading enzymes, IDE/NEP (B), expression in hippocampal tissues derived from ovariectomized adult female rats. Results are presented as the percent (%) increase compared with vehicle-treated control rats ( $n \geq 4$ ). \*,  $P < 0.05$  and \*\*,  $P < 0.01$ ;  $\delta$ ,  $P < 0.05$  and  $\delta\delta$ ,  $P < 0.01$  compared with 17 $\beta$ -E2-treated rats;  $\phi\phi$ ,  $P < 0.01$  compared with G-treated rats;  $\xi\xi$ ,  $P < 0.01$  compared with G + D + E + I-treated rats.

nations would regulate the expression of Bcl-2 and Bcl-xL, and if so, whether such a regulation correlated with their regulation of brain mitochondrial bioenergetic efficiency. Consistent with our previous reports (45, 46) and the present data for RCR and COX activity, treatment with 17 $\beta$ -E2 (70  $\mu$ g/kg  $\cdot$  d for 2 d) induced a significant increase in both Bcl-2 (64.8  $\pm$  4.0% increase compared with vehicle alone-treated control rats; \*\*,  $P$  < 0.01) and Bcl-xL expression (55.2  $\pm$  11.2% increase compared with control rats; \*,  $P$  < 0.05) in rat hippocampal tissues (Fig. 6A). In contrast to respiratory and COX activities, which were markedly lower in rats treated with G alone than the other three treatment groups, G alone (6 mg/kg  $\cdot$  d for 2 d) induced a magnitude of increase comparable to 17 $\beta$ -E2-treated rats in both Bcl-2, with an average increase of 51.9% ( $\pm$  6.2%; \*\*,  $P$  < 0.01), and Bcl-xL expression, with an average increase of 67.8% ( $\pm$  0.7%; \*\*,  $P$  < 0.01), compared with control rats (Fig. 6A). Increase in Bcl-2 expression (43.2  $\pm$  4.1% increase compared with control rats; \*,  $P$  < 0.05) induced by the combination of G plus D plus E (6 mg/kg  $\cdot$  d for 2 d) was significantly lower than that induced by 17 $\beta$ -E2 ( $\delta\delta$ ,  $P$  < 0.01). There was a greater variance in Bcl-xL expression in G plus D plus E-treated rats, with an average increase of 58.9% ( $\pm$  11.7%) compared with control rats (\*,  $P$  < 0.05). Rats treated with G plus D plus E plus I (6 mg/kg  $\cdot$  d for 2 d) did not exhibit a significant change in either protein, although there was an average increase of 38.6% ( $\pm$  11.7%) in Bcl-xL expression, which, however, was not statistically significant due to the large variance among animals. Bcl-2 expression (1.5  $\pm$  4.0% increase compared with control rats) induced by G plus D plus E plus I was significantly lower than all other treatment groups ( $\xi\xi$ ,  $P$  < 0.01).

### Regulation of brain A $\beta$ -degrading enzymes *in vivo*

A major neuropathological hallmark of AD is the significant deposition of A $\beta$  peptide, which can lead to formation of A $\beta$  plaques. Clinical investigations have revealed a strong link between a deficit in expression and activity of A $\beta$ -degrading enzymes and AD pathogenesis (47, 48). Several A $\beta$ -degrading enzymes have been identified, including IDE and neprilysin (NEP), which appear to play a more significant role than others in regulating A $\beta$  catabolic clearance in the brain. In this experiment, Western blot analyses of the same hippocampal protein samples used in Bcl-2/Bcl-xL analyses were conducted to determine whether the test compounds or combinations would potentially impact brain A $\beta$  metabolism via regulation of the expression of

A $\beta$ -degrading enzymes such as IDE and NEP. Results shown in Fig. 6 demonstrated that all treatment groups exhibited an enhanced expression of both IDE and NEP (Fig. 6B). Specifically, rats treated with 17 $\beta$ -E2 (70  $\mu$ g/kg  $\cdot$  d for 2 d) exhibited an average increase of 42.4% ( $\pm$  3.9%) in IDE and a 44.0% ( $\pm$  1.8%) increase in NEP expression, both of which were statistically significant compared with vehicle alone-treated control rats (\*\*,  $P$  < 0.01). Among three groups treated with different phytoestrogen formulations, the combination of G plus D plus E (6 mg/kg  $\cdot$  d for 2 d) induced a magnitude of up-regulation of IDE similar to 17 $\beta$ -E2 (44.1  $\pm$  7.2% increase compared with control rats; \*\*,  $P$  < 0.01). In comparison, G alone (6 mg/kg  $\cdot$  d for 2 d) induced an average increase of 21.6% ( $\pm$  3.9%) compared with vehicle-treated control rats, which was not statistically significant ( $\delta$ ,  $P$  < 0.05 compared with 17 $\beta$ -E2-treated rats). The combination of G plus D plus E plus I (6 mg/kg  $\cdot$  d for 2 d) induced a slightly smaller magnitude of change in IDE expression (35.6  $\pm$  8.4% increase compared with control rats; \*,  $P$  < 0.05) than that induced by G plus D plus E. For induction of NEP expression, the combination of G plus D plus E induced a maximal effect among all treatment groups, with an average increase of 73.8% ( $\pm$  3.4%) (\*\*,  $P$  < 0.01 compared with control rats), which was statistically greater than that induced by either 17 $\beta$ -E2 ( $\delta\delta$ ,  $P$  < 0.01) or G alone ( $\varphi\varphi$ ,  $P$  < 0.01). Although G alone induced a significant increase in NEP expression (31.2  $\pm$  1.2% increase compared with control rats; \*\*,  $P$  < 0.01), the magnitude of induction was lower than that induced by G plus D plus E or 17 $\beta$ -E2 ( $\delta\delta$ ,  $P$  < 0.01). Rats treated with G plus D plus E plus I had a greater variance and exhibited an average increase of 40.4% ( $\pm$  14.4%) in NEP expression relative to control rats (\*,  $P$  < 0.05).

### Impact on uterine growth *in vivo*

Induction of proliferative responses and risk of cancers in reproductive tissues has been a major concern to women who receive the current form of estrogen-containing HT (17). In this experiment, both the wet and dry weights of uteri excised from treated rats at the time of being killed were recorded as an indicator of estrogenic activity on uterine growth. Data shown in Table 2 revealed that as expected, treatment with 17 $\beta$ -E2 (70  $\mu$ g/kg  $\cdot$  d for 2 d) induced a marked increase in both wet (120.2  $\pm$  25.1% increase compared with vehicle alone-treated control rats; \*\*,  $P$  < 0.01) and dry uterine weight (76.7  $\pm$  15.6% increase compared with control rats; \*\*,  $P$  < 0.01). Treatment

**TABLE 2.** Effect on uterine weight in ovariectomized adult female rats

| Treatment                         | Uterine weight                  |                                 |                               |                                |
|-----------------------------------|---------------------------------|---------------------------------|-------------------------------|--------------------------------|
|                                   | Wet weight (mg)                 | Increase (%) <sup>a</sup>       | Dry weight (mg)               | Increase (%)                   |
| Control (vehicle)                 | 127.62 $\pm$ 10.75              | 0.00 $\pm$ 8.42                 | 26.42 $\pm$ 2.45              | 0.00 $\pm$ 9.27                |
| 17 $\beta$ -E2 (70 $\mu$ g/kg BW) | 281.06 $\pm$ 32.00 <sup>b</sup> | 120.23 $\pm$ 25.07 <sup>b</sup> | 46.70 $\pm$ 4.13 <sup>b</sup> | 76.74 $\pm$ 15.63 <sup>b</sup> |
| G (6 mg/kg BW)                    | 144.11 $\pm$ 10.18              | 12.92 $\pm$ 7.97                | 28.14 $\pm$ 2.04              | 6.49 $\pm$ 7.71                |
| G+D+E (6 mg/kg BW)                | 119.84 $\pm$ 1.19               | -6.10 $\pm$ 0.93                | 23.71 $\pm$ 0.04              | -10.26 $\pm$ 0.13              |
| G+D+E+I (6 mg/kg BW)              | 146.99 $\pm$ 18.45              | 15.17 $\pm$ 14.46               | 28.73 $\pm$ 3.67              | 8.73 $\pm$ 13.90               |

<sup>a</sup> Increase (%) in uterine weight compared with vehicle-treated control rats and expressed as the percentage of control (set as zero).

<sup>b</sup>  $P$  < 0.01 compared with any other treatment groups.

with G alone (6 mg/kg · d for 2 d,  $12.9 \pm 8.0\%$  and  $6.5 \pm 7.7\%$  increase in wet and dry weight, respectively, compared with control rats), the combination of G plus D plus E (6 mg/kg · d for 2 d,  $-6.1 \pm 0.9\%$  and  $-10.3 \pm 0.1\%$  increase in wet and dry weight, respectively, compared with control rats), or G plus D plus E plus I (6 mg/kg · d for 2 d,  $15.2 \pm 14.5\%$  and  $8.7 \pm 13.9\%$  increase in wet and dry weight, respectively, compared with control rats) did not produce a significant impact on uterine weight. Although there was not a statistical significance, a trend toward a slightly declined uterine weight was observed in rats treated with the combination of G plus D plus E, but not in those treated with either G alone or the combination of G plus D plus E plus I, compared with vehicle-treated control rats.

## Discussion

In the present study, we conducted a range of comparative analyses of: 1) the ER $\alpha/\beta$  binding profile; 2) *in vitro* to *in vivo* neural responses, including neuronal survival against glutamate and A $\beta_{1-42}$ -induced toxic insults in rat primary hippocampal neurons, regulation of rat forebrain mitochondrial bioenergetic activity, and hippocampal expression of antiapoptotic and A $\beta$ -degrading proteins; and 3) the impact on uterine growth induced by four clinically relevant phytoestrogens when used alone or in combination.

One major finding of these analyses is that the ER $\alpha/\beta$  binding profile associated with individual phytoestrogens can be modified when they are used in combination. Figure 2 and Table 1 reveal that, of four test phytoestrogens, G exhibited the maximal binding affinity for both ERs, particularly for ER $\beta$  (~41% of 17 $\beta$ -E2), followed by E (~14% of G), D (~34% of E), and then I (~22% of D), despite their binding selectivity for ER $\beta$  with the following disparate order: I (>100-fold) >G (~60-fold) >D (~14-fold) >E (~10-fold). These profiles were altered when these phytoestrogens were used in combination. The combination of G with D (G plus D), or D and E (G plus D plus E), or D, E, and I (G plus D plus E plus I) yielded a 50% or greater decrease in the ER $\beta$ -binding affinity, at approximately 50, 41, and 30% of G for G plus D, G plus D plus E, and G plus D plus E plus I, respectively. Nevertheless, the ER $\beta$ -binding selectivity of these combinations remained the same or increased compared with G alone. Combinations of G plus D and G plus D plus E plus I exhibited a similar selectivity for ER $\beta$  to G, at approximately 63- and 61-fold, respectively. In comparison, G plus D plus E exhibited an approximate 30% increase (~83-fold) compared with G. These results indicate that the addition of a weak ER $\beta$  ligand in a formulation could induce a competitive binding and lead to a decrease in the overall binding affinity. However, such a reduction in the binding affinity could be offset by a substantial increase in the binding selectivity for ER $\beta$ .

Another major finding is that a select combination of phytoestrogens can produce increased estrogenic activities in the neural system compared with administration of single phytoestrogens. Such an increase was first demonstrated in *in vitro* analyses of neuroprotective responses in rat primary hippocampal neurons. We observed that despite the fact that individual

phytoestrogens were effective in promoting neuronal membrane integrity against glutamate-induced insult, only G was sufficient in exerting significant protection on neuronal metabolic viability against the insult. Of three combinations on the same measure of neuronal metabolic activity, no effect was evident from G plus D. In comparison, G plus D plus E not only induced a significant increase in neuronal metabolic viability against the insult from glutamate when compared with neuronal response to G alone, it was also highly protective of both neuronal membrane integrity and metabolic viability against the insult induced by A $\beta_{1-42}$ . Neurons treated with G plus D plus E plus I also exhibited enhanced survival against both glutamate and A $\beta_{1-42}$ , however, the overall magnitude was lower than responses in neurons treated with G plus D plus E. In particular, after A $\beta_{1-42}$  exposure, metabolically live neurons were significantly less evident in cultures treated with G plus D plus E plus I than those treated with G plus D plus E.

An increase in neural responses induced by a select combination of test phytoestrogens was also evident *in vivo*. Adult female rats treated with the combination of G plus D plus E, at a clinically relevant dosage, exhibited a significant increase in forebrain mitochondrial bioenergetic function, including respiratory efficiency and COX enzymatic activity. In comparison, rats treated with G alone at the same dosage did not show a significant change in respiration, although there was an increase in COX enzymatic activity but at a much lower magnitude relative to G plus D plus E-treated rats. On both measurements, the effects induced by G plus D plus E were significantly greater than those from G alone. Consistent with *in vitro* neuroprotection, the magnitude of change induced by G plus D plus E plus I was lower than that induced by G plus D plus E, although there was no significant difference between the two combinations. The enhanced mitochondrial efficiency was to some extent paralleled by an increase in the expression of the mitochondrial antiapoptotic proteins Bcl-2/Bcl-xL. Although G was ineffective at enhancing mitochondrial function, it exerted a significant impact on the expression of Bcl-2/Bcl-xL, with an efficacy on par with that of G plus D plus E. However, the effect produced by G plus D plus E was negated by the presence of I. These results may suggest a dissociation between regulation of antiapoptotic proteins and activation of signaling cascades that mediate mitochondrial function. It appears that induction of Bcl-2/Bcl-xL more closely correlates with the ER $\beta$ -binding affinity, which, however, was not the main determinant of brain mitochondrial function. Moreover, combinations of G plus D plus E and G plus D plus E plus I exerted a marked effect on the expression of the A $\beta$ -degrading enzymes IDE and NEP. It was particularly notable that G plus D plus E induced an increase in NEP expression significantly greater than that induced by 17 $\beta$ -E2. For G alone, there was no change in the expression of IDE, although there was a significant increase in the expression of NEP, but at a much lower magnitude than that induced by either 17 $\beta$ -E2 or G plus D plus E. Consistent with most of the observations presented in this study, G plus D plus E was more efficacious than G plus D plus E plus I.

These observations indicate that the overall neural activities associated with a phytoestrogen combination are not a simple sum of individual effects but, rather, the net result of comple-

mental or competitive interactions among phytoestrogens. In addition to an ER $\beta$ -mediated signaling network, there may be additional ER $\beta$ -independent mechanisms linked to a neuroprotective outcome (33, 49–51). A greater effect produced by a select combination could be the result of concurrent activation and interaction of these complex signaling cascades. Although the combination of G plus D plus E exhibited an efficacy comparable to or even greater than 17 $\beta$ -E2 in neural tissues, the combination did not induce a significant effect on the uterus, which may largely be attributable to its high binding selectivity for ER $\beta$  over ER $\alpha$ . In comparison, 17 $\beta$ -E2, which shows no binding preference to either ERs, induced a 2-fold increase in uterine weight under the same treatment paradigm. Of particular interest, the combination of G plus D plus E exerted an approximate 10% reduction in uterine weight relative to vehicle-treated control rats. Despite the absence of a significant difference between the two groups, it can be speculated that such a slight down-regulation of uterine weight may become apparent from a long-term use, leading to a preventive impact against reproductive cancers.

Clinically, a phytoestrogen combination composed of rationally defined content (*e.g.* the combination of G plus D plus E) has the potential to address the compositional complexity and potential antagonistic interactions that occur in soy extracts. Soy-derived extract preparations have been the most common form used in phytoestrogen intervention studies. However, variations in the constitutive composition could be significant among soy extracts obtained from different sources of soy plants and manufactured using different protocols (17, 52). Possible antagonistic interactions could come from many sources (17). In addition to those derived from intrinsic antiestrogens and between ER $\alpha$  and ER $\beta$ -selective components, some newly formed substances generated from the extraction process could also pose undesirable effects counteracting the favorable health-giving properties of other substances. The antagonistic impact could conceivably diminish the overall effect and present an undetectable or undesirable clinical outcome. As demonstrated in the present study, the addition of I to the combination of G plus D plus E not only weakened the ER $\beta$ -binding affinity and selectivity, it negatively impacted the overall activity of G plus D plus E plus I compared with G plus D plus E. These analyses underlie a critical notion that the compositional complexity of a random mixture makes its safety and efficacy unpredictable. In point of fact, a rationally designed formulation with a clear composition could maximize the therapeutic potential of individual components and lead to a clinically meaningful effect. Another potential clinical advantage relates to E. Unlike G and D, E is not of plant origin, yet can be exclusively produced through the metabolism of D catalyzed by intestinal microbial flora after the intake of soy products (53). In comparison with D, E has been demonstrated as a much stronger ER binder and more potent transcriptional inducer (54). Interestingly, wide variations in the ability to produce E from D metabolism exist across human populations, with 20–35% of Western adults (55, 56) and 55–60% of Asian populations being E producers (57, 58). Therefore, it can be speculated that the E-producing phenotype could serve as a critical modulator of human response to phytoestrogen treatment. An

enhanced response could occur in E producers compared with nonproducers. To date, a number of clinical studies have confirmed this speculation (59–61). This may also hold true for the strong link between the many health benefits associated with phytoestrogen intake and the high prevalence of E-producing phenotype in Asians. The failure to factor the interindividual variations of E-producing phenotype could be another major cause of the disparity in clinical outcomes across studies. Inclusion of E in the combination of G plus D plus E presents the opportunity to minimize these variations, and make E accessible in both E producers and nonproducers.

In conclusion, the present study provides evidence in support of the hypothesis that the ER $\alpha/\beta$  binding profile and neural activities derived from individual phytoestrogens could be modified when they are used in combination. Specifically, the combination of G plus D plus E, which enhanced ER $\beta$ -binding selectivity and *in vitro* to *in vivo* neural responses without eliciting a significant impact on uterine growth, has the potential of a relatively safe alternative approach to conventional HT for reducing the increased risk of cognitive decline and neurodegenerative disease associated with menopause in women.

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