High-soy diet decreases infarct size after permanent middle cerebral artery occlusion in female rats

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Schreihofer, Derek A., Khoi D. Do, and Ann M. Schreihofer. High-soy diet decreases infarct size after permanent middle cerebral artery occlusion in female rats. Am J Physiol Regul Integr Comp Physiol 289: R103–R108, 2005; doi:10.1152/ajpregu.00642.2004.—Estrogen is a powerful neuroprotective agent in rodent models of ischemic stroke. However, in humans, estrogen treatment can increase risk of stroke. Health risks associated with hormone replacement have led many women to consider alternative therapies including high-soy diets or supplements containing soy isoflavones, which act as estrogen receptor ligands to selectively mimic some of estrogen’s actions. We hypothesized that a high-soy diet would share the neuroprotective actions of estrogen in focal cerebral ischemia. Female Sprague-Dawley rats were ovariectomized and divided into three groups: isoflavone-free diet plus placebo (IF-P), isoflavone-free diet + estradiol (IF-E), or high-soy diet + placebo (S-P). Two weeks after being placed on diets, rats underwent left permanent middle cerebral artery occlusion (MCAO). Reductions in ipsilateral cerebral blood flow were equivalent across groups (~50%). Twenty-four hours later neurological deficit was determined, and brains were collected for assay of cerebral infarct by TTC staining. In the IF-P rats MCAO produced a 50 ± 4% cerebral infarct. Estrone and high-soy diet both significantly reduced the size of the infarcts to 26 ± 5% in IF-E rats and to 37 ± 5% in S-P rats. Analysis at five rostro-caudal levels revealed that estrogen treatment was slightly more effective at reducing infarct size than high soy diet. Overall neurological deficit scores at 24 h correlated with infarct size; however, there were no statistically significant differences among the treatment groups. These data show that 2 wk of a high-soy diet is an effective prophylactic strategy for reducing stroke size in a rat model of focal cerebral ischemia.

ESTROGEN PROTECTS THE BRAIN from insults as varied as trauma (6, 13), kainate toxicity (3), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (7), and several models of focal and global cerebral ischemia in rodents (37). However, the use of estrogen as a prophylactic agent in humans is controversial. Recent data from both the Women’s Health Initiative and Women’s Estrogen for Stroke Trial demonstrated an increased risk of occurrence of stroke in postmenopausal women treated with estrogen alone (2, 54) or estrogen plus progesterone hormone replacement therapy (55). Furthermore, other risks associated with hormone replacement, including breast cancer, have led many women to eschew traditional hormonal therapy for what are perceived as safer alternatives, including diets high in soy (16, 38).

Soy contains phytoestrogens, which are nonsteroidal polyphenolic compounds with the ability to bind and activate nuclear estrogen receptors (ER). Like estrogen, binding of phytoestrogens leads to transcriptional activation of ER-regulated genes. The two soy phytoestrogens genistein and daidzein, which belong to the isoflavone class, have been proposed as selective ER modulators (48). Although these compounds have estrogenic actions in many cell types, very little is known about their ability to mimic estrogen’s neuroprotective effects. In vitro, physiologically attainable concentrations of genistein can protect primary cortical neurons from thapsigargin-induced apoptosis (32). Similarly, both genistein and daidzein have moderate neuroprotective actions in primary hippocampal neurons exposed to glutamate or β-amyloid toxicity (59). In vivo, intraperitoneal injection of high doses of genistein delays the onset of symptoms in a mouse model of amyotrophic lateral sclerosis in male, but not female mice, suggesting an estrogenic action (53). The same regimen reduces cerebral infarct size in a murine model of free radical-induced stroke; however, no sexual dimorphism is observed (53). In addition, whether adding these compounds to the diet will result in neuroprotection is not known.

Because estrogen is neuroprotective during stroke, we hypothesized that dietary soy containing high levels of phytoestrogens would also reduce the extent of brain injury in a rat model of focal cerebral ischemia. Our results demonstrate that 2 wk on a high-soy diet leads to a significant reduction in cerebral infarct size in ovariectomized female rats subjected to permanent middle cerebral artery occlusion (MCAO).

MATERIALS AND METHODS

The Medical College of Georgia Institutional Animal Care and Use Committee approved all animal protocols. Female Sprague-Dawley rats (225–250 g, Harlan) were acclimated for 1 wk in the animal facility on a 12:12-h light/dark cycle. All rats were placed on one of two soy-free, casein-based diets: 5K96 (www.Labdiet.com) or a custom isoflavone-free diet (www.Zeiglerfeed.com) (30). Both are complete diets without any isoflavone-containing ingredients. One week after initiation of diets, rats were bilaterally ovariectomized under halothane anesthesia and randomized into three groups: isoflavone-free diet + placebo (IF-P), isoflavone-free diet + estradiol (IF-E), or high-soy diet + placebo (S-P). In the IF-P and S-P groups half of the rats were maintained on each diet. In the IF-E group five rats received the 5K96 diet; the remaining seven received the custom isoflavone-free diet. Rats on the IF diet received subcutaneous 21-day time-release pellets containing 0.25 mg of 17β-estradiol or placebo (www.Innovrsrch.com), and rats on the high-soy diet received placebo pellets. The estradiol pellet size was chosen to mimic proestrus serum estradiol levels of ~80–140 pg/ml as cited by the manufacturer’s reference tables. After implantation of pellets, rats in the IF groups were maintained on the IF diets, and rats in the S-P group were switched to Teklad 8604 rodent diet (www.Teklad.com). This diet has been shown to contain 600 mg soy isoflavones/g and has been
RESULTS

Two weeks after ovariectomy, rats treated with a placebo or high-soy diet gained weight (Table 1). In contrast, rats treated with estrogen had significantly lower body weights at the time of infarct compared with either placebo group (Table 1). Serum estradiol concentrations in the estrogen-treated group (159 ± 21 pg/ml) were 15-fold higher than in the placebo-treated groups and close to that anticipated by the implant manufacturer (Table 1). The wet weight of uteri from rats treated with estrogen were nearly 3.5 times more than those of placebo-treated rats on either soy or soy-free diets (Table 1). These results confirm the effectiveness of estrogen treatment and the lack of uterotrophic effects of soy (40, 41). Occlusion of the left middle cerebral artery produced equivalent decreases in left LDF in all three groups (Fig. 1). On average we observed a 54 ± 5% reduction in left LDF. The reduction in blood flow persisted for the 15-min observation period and remained comparable among the treatment groups (Fig. 1). Nevertheless, when all animals were combined, there was a weak, but statistically significant correlation between neurological deficit score and infarct size ($R^2 = 0.23, P < 0.05$).

Permanent MCAO in ovariectomized IF-P rats resulted in a 50 ± 4% infarct on the side ipsilateral to the occlusion (Figs. 1 and 2). Table 1. Effects of treatment with estrogen or high-soy diet on body and uterine weights, serum estradiol concentrations, and neurological deficit scores

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Initial Body Weight, g</th>
<th>MCAO Body Weight, g</th>
<th>Uterine Weight, g</th>
<th>Serum Estradiol, pg/ml</th>
<th>Neurological Deficit Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>IF-P</td>
<td>12</td>
<td>238±5</td>
<td>291±7*</td>
<td>0.50±0.01</td>
<td>9.34±0.92</td>
<td>2.67±0.30</td>
</tr>
<tr>
<td>S-P</td>
<td>16</td>
<td>242±2</td>
<td>277±4*</td>
<td>0.48±0.02</td>
<td>13.43±1.28</td>
<td>2.44±0.28</td>
</tr>
<tr>
<td>IF-E</td>
<td>12</td>
<td>233±6</td>
<td>222±4†</td>
<td>1.72±0.18†</td>
<td>159.02±21.34†</td>
<td>2.09±0.34</td>
</tr>
</tbody>
</table>

Values are mean ± SE. Initial body weight recorded at time of ovariectomy and middle cerebral artery occlusion (MCAO) body weight recorded 2 wk after ovariectomy and isoflavone-free (IF) or high-soy (S) diet on day of MCAO. *Significantly different from initial body weight within group, $P < 0.05$. †Significantly different from IF-placebo (P) and S-P, $P < 0.05$.

Differences among the three groups of rats were assessed by analysis of variance with post hoc comparisons between groups performed with Fisher’s protected least-significant-difference test with a Bonferroni correction for multiple comparisons. For behavioral analysis, which was assessed on an ordinal scale, data were analyzed using a Kruskal-Wallis test. Data are expressed as means ± SE. A difference of $P < 0.05$ was considered significant.

![Fig. 1. Laser Doppler blood flow (LDF) during the induction of middle cerebral artery occlusion (MCAO). Onset of MCAO is at time = 0. Values are means ± SE of percent change in LDF in the region served by the left middle cerebral artery as determined by LDF signal from the skull surface. IF-P, isoflavone-free diet with placebo; IF-E, isoflavone-free diet with estradiol; S-P, high-soy diet with placebo.](http://ajpregu.physiology.org/)
and display both overlapping and distinct distributions (50). In isoforms, ER\textsubscript{A} and ER\textsubscript{B} act as transcriptional activators through two nuclear receptor lines exposed to varied apoptotic stimuli (18, 57). Estrogen acts protective in primary cortical (56) and hippocampal culture (20) and ischemia and in primary neurons and neuronal cell models of ischemia and in primary neurons and neuronal cell protective agent in animal models of cerebral ischemia. Ovariectomized female rats on a high-soy diet for 26 ± 5% (P < 0.01, Figs. 2 and 3A). These results agree with previous observations of estrogen’s neuroprotective actions in ovariec-tomized rats on standard diets (11, 42, 44, 49). A high-soy diet also significantly reduced overall infarct size to 37 ± 5% (P < 0.05, Fig. 3A), which was not different from the overall infarct size of estrogen-treated rats (Fig. 3A). Separate analyses of the cortex and striatum revealed neuroprotective effects of estrogen and soy in both regions (Fig. 3A). However, in the cortex, estrogen was significantly more protective than the high-soy diet (Fig. 3A). Analysis at five rostro-caudal levels revealed a trend for the infarct size in S-P rats to be smaller than IF-P rats, with significant differences observed in sections 1 and 4 (P < 0.05, Fig. 3B). In contrast, IF-E rats had a significantly smaller infarct size at all five levels examined compared with IF-P rats (P < 0.05, Fig. 3B). In addition, at each of the five levels examined there was a trend for the infarct size to be smaller in IF-E rats compared with S-P rats with a significant difference observed at level 5 (P < 0.05, Fig. 3B).

DISCUSSION

The present study is the first to demonstrate a beneficial role for a high-soy diet in an animal model of focal cerebral ischemia. Ovariectomized female rats on a high-soy diet for only 2 wk showed a significant decrease in infarct size compared with animals on soy-free diets. Because individual estrogenic isoflavones present in soy have previously been shown to be neuroprotective in vitro (32, 59) and when administered at high intraperitoneal doses (53), the present study suggests that the isoflavones present in high-soy diets can act as neuroprotective estrogens in vivo. However, our results also suggest that isoflavones may not be as effective as estrogen in reducing infarct size after MCAO.

Estrogen at physiological levels is now well recognized as a neuroprotective agent in animal models of cerebral ischemia including transient and permanent focal ischemia by suture-mediated MCAO (37), global ischemia (23, 26, 51), and photothrombotic MCAO (15). Similarly, estrogen is neuroprotective in primary cortical (56) and hippocampal culture (20) models of ischemia and in primary neurons and neuronal cell lines exposed to varied apoptotic stimuli (18, 57). Estrogen acts as a transcriptional activator through two nuclear receptor isoforms, ER\textsubscript{A} and ER\textsubscript{B}. Both receptors are present in the brain and display both overlapping and distinct distributions (50). In vivo the neuroprotective actions of estrogen in cerebral ischemia have been attributed to activation of the ER\textsubscript{A} subtype (12) using ER\textsubscript{A} and ER\textsubscript{B} knockout mice, although both isoforms can be neuroprotective in vitro (14, 17, 46). However, others have failed to find a significant role for ER\textsubscript{A} in vivo (45), and an ER\textsubscript{B}-selective agonist has recently been shown to be neuroprotective in global ischemia (9). The mechanism(s) of estrogen’s neuroprotective actions are not fully understood, but they likely involve both the activation of antiapoptotic genes and other nongenomic mechanisms including interactions with cytoplasmic signaling pathways, modulation of intracellular calcium levels, and free radical scavenging (14, 24, 39, 57).

The estrogen levels achieved in the present study were close to endogenous proestrus levels. Previous results have demonstrated that estrogen is neuroprotective over a broad range of concentrations (25), and studies in spontaneously hypertensive, stroke-prone rats have shown that high endogenous proestrus levels of circulating estradiol are more neuroprotective than basal meestrus levels (8).

Soy isoflavones act as transcriptional activators of ERs in vitro. These effects are generally greater in the presence of ER\textsubscript{B}, but significant activation through ER\textsubscript{A} is also observed (1, 19, 28, 43). In the brain, soy diets and isolated phytoestrogens can also mimic estrogen’s transcriptional actions. Soy isoflavones increase brain-derived neurotrophic factor (41) and choline acetyl transferase (40) mRNA levels in the frontal cortex and increase vasopressin levels in the hypothalamus.
Males but not females (53). In vitro daidzein and genistein have neuroprotective effects. Genistein delays the onset of symptoms and death (47). Phytoestrogens can also mimic estrogen’s neuroprotective effects. Genistein protects primary cortical neurons from thapsigargin-induced apoptosis via actions on ERα (32). Which ER isoform is responsible for soy’s actions in the present study is not known, and either or both ERs may play a role.

In the present study, the reduction of infarct size by ingestion of a high-soy diet was not as great as that produced by estrogen at all levels or in all areas of the brain. Several factors may account for the intermediate effect of soy on infarct size. First, ERα appears to be critical for protection from permanent cerebral ischemia, although this result has recently been questioned (9, 45). Because soy isoflavones have a lower affinity for ERα than estradiol, our results may simply reflect compounds that are less active in the presence of ERα. Alternatively, increased ERβ activity might mitigate the neuroprotective actions of ERα. Further supporting a role for ERβ is the slight weight loss and dramatic uterine growth in the IF-E group but not the S-P group. Estrogen actions on body weight and uterine size are dependent on ERα (21). Second, the concentration of soy phytoestrogens in the brain may be lower than that needed to fully activate the necessary neuroprotective pathways. Third, estrogen may have neuroprotective actions through other nontranscriptional mechanisms, which may not be shared by phytoestrogens (14, 24, 39). Fourth, more than one phytoestrogen is present in soy and in the circulation after digestion including genistein, daidzein, and equol. The relative activity of each individual compound or combination of compounds must be considered. Finally, additional, nonestrogenic, components in soy protein may play a role in neuroprotection, although there are presently no data to support this notion.

Neuroprotective actions in vitro have focused on individual phytoestrogens, not dietary combinations. In rodents, ingested soy results in high concentrations of circulating equol, a daidzein metabolite that has not been studied for its neuroprotective actions (35). However, genistein, daidzein, and equol are all found in the brains of rat on high-soy diets (35). The high-soy diet used in this study has been used and evaluated extensively and found to have a total isoflavone content of 600 µg/g diet (35). This leads to a serum concentration of >4,000 ng/ml, similar to that of Asian populations on high-soy diets (48). In rodents, most daidzein is converted to equol by intestinal flora, and >90% of circulating phytoestrogens are in this form (35). However, the brain does appear to concentrate all three isoflavones from soy. Lund et al. (36) found that the concentration of total isoflavones in male rats on the high-soy diet we used ranged from 35 to 1,300 ng/g of tissue in the hippocampus and frontal cortex, respectively. This concentration compares favorably to concentrations of estradiol reported in the cycling female rat brain that has been reported to be as high as 0.2 ng/g in the proestrous hypothalamus (5). Given that phytoestrogens are two to three orders of magnitude less potent than estradiol, these concentrations would be expected to have similar effects to endogenous estradiol on ER activity. To our knowledge, there is no strong evidence for actions of genistein, daidzein, or equol at other steroid receptors at sub-mM concentrations (4). There is also limited evidence for isoflavone activity on aryl hydrocarbon receptors at 10 µM, but this activation is <10% that of a cognate ligand (58). In addition, equol has been shown to bind dihydrotestosterone (DHT), effectively acting as a DHT antagonist (34). Although most studies of soy isoflavone focus on estrogenic actions, additional actions of phytoestrogens cannot be ruled out as potential mechanisms for soy-dependent neuroprotection.

In the present study infarct size as measured with TTC staining was used as the primary experimental measure of the outcome of MCAO. Whether the differences in infarct size at 24 h reflect long-term functional changes in behavior in the recovering animal remains to be determined. Although there were no statistical differences in neurological deficit score among treatment groups, the 24-h time point may reduce the ability to detect differences due to lingering surgical trauma or other acute effects of MCAO. In many other studies, a clear correlation between infarct size and behavioral outcome is lacking, and treatments that affect one variable do not often affect the other (10). Only one other study has investigated the role of estrogen in behavioral outcomes following MCAO. In mice subjected to a 90-min transient MCAO and reperfusion Li et al. (31) found that some behavioral tests were unable to distinguish MCAO from sham-operated animals 24 h after stroke although evidence for a beneficial effect of estrogen was apparent 7 and 12 days after recovery. Other behavioral tests showed gender differences but no significant effects of estrogen. Another study showed that, in gerbils, estrogen improves performance in the Morris water-maze memory task 3 days after transient global ischemia (27). Additional detailed and sensitive behavioral testing will be required to determine whether the soy-induced reduction in infarct size observed in the present study is associated with improved behavioral outcomes.

Overall, the results of the present study show that 2 wk of a high-soy diet is neuroprotective in the rat model of permanent focal cerebral ischemia. Whether this effect of dietary soy can be mimicked by ingested soy supplements or individual isoflavones remains to be determined. Although the precise mechanism(s) underlying this neuroprotective effect remain to be elucidated, other in vitro and in vivo studies suggest soy may act through the regulation of neuroprotective genes by actions upon ER.

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GRANTS

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REFERENCES

DIETARY SOY NEUROPROTECTION IN STROKE


