CHAPTER 3

PHYTOESTROGENS GENISTEIN AND DAIDZEIN ENHANCE THE ACETYLCHOLINESTERASE ACTIVITY OF THE RAT PHEOCHROMOCYTOMA CELL LINE PC12 BY BINDING TO THE ESTROGEN RECEPTOR

3.1. Introduction

Phytoestrogens are a class of plant-derived compounds that have both estrogenic and antiestrogenic properties. Among the widely known phytoestrogens are the isoflavones genistein and daidzein, which are abundant in soy and soy products. Based on cellular biology experiments and animal studies, phytoestrogens may potentially confer health benefits related to cardiovascular diseases, cancer, osteoporosis, and menopausal symptoms (Tham et al., 1994).

However, exposure to very high concentrations of phytoestrogens, esp. isoflavones, may also cause deleterious affects. Infertility in sheep, "clover disease," has been traced to isoflavone concentrations as high as 5% of the dried weight of clover (Verdeal K and Ryan DS, 1979). For some time, it has also been recognized that goiter in animals and humans is associated with feeding infants soy-based formula (Shepard et al., 1960).

Recently, phytoestrogens have attracted serious concern since their chemical structures are similar to that of the female hormone estrogen. They are likewise considered as endocrine disruptors due to their ability to bind with the estrogen receptor (ER) (Martin et al., 1978).

Estrogen receptor expression in NGF-treated PC12 cells has been shown by Sohrabji et al. (1994). Using in situ hybridization histochemistry, RT-PCR and a modified nuclear exchange assay, they have found both ER mRNA and estrogen binding in PC12 cells. They also observed that while estrogen binding was relatively low in naïve PC12 cells, long-term exposure to NGF enhanced estrogen binding in these cells by sixfold.

In this study, the author exposed the rat pheochromocytoma cell line PC12 to varying concentrations of genistein and daidzein to determine their effect on the cells' acetylcholinesterase activity. This is to determine if phytoestrogens also have the same impact on PC12 cells as the alkylphenols used in Chapter 2. The author also exposed the cells to these isoflavones in the presence of tamoxifen, a known ER antagonist. Expressing neurochemical and ultrastructural properties associated with their normal counterparts, PC12 cells provide a useful model for assessing the effects of these compounds on the mammalian nervous system *in vitro*. Moreover, to confirm the estrogenic potential of genistein and daidzein, he also exposed the human breast cancer cell line MCF7 to these compounds and determined their effect on cell proliferation in response to estrogenic activity, both in the presence or absence of tamoxifen.

As mentioned in Chapter 2, in the neuromuscular junction, the neurotransmitter acetylcholine binds to the acetylcholine receptor inducing a conformational change that opens this transmitter-gated ion channel. This short-lived opening is followed by a closed state, whereby the acetylcholine dissociates from the receptor and is hydrolyzed by a specific enzyme called acetylcholinesterase (AChE). AChE activity in neuronal cells is of particular interest since it plays an important role in cholinergic synaptic transmission.

3.2. Materials and methods

Cell lines and maintenance

The rat pheochromocytoma PC12 cell line was obtained from the National Institute of Bioscience and Human-Technology (Tsukuba, Ibaraki, Japan) and routinely maintained in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) supplemented with 5% fetal bovine serum (Sigma), 10% horse serum (Sigma), and 1% streptomycin (5000 I.U. ml^{-1})-penicillin (5000 μg ml^{-1}) solution (ICN Biomedicals, Inc.) in tissue culture flasks. On the other hand, the MCF7 cell line was obtained from Dr. H. Shinmoto of the National Food Research Institute (Tsukuba, Japan) and routinely maintained in phenol-red free RPMI –1640 (Sigma) supplemented with 10% fetal bovine serum (Sigma) and 1% streptomycin (5000 I.U. ml^{-1}) – penicillin (5000 μg ml^{-1}) solution (ICN Biomedicals Inc.) in 75-cm^2 tissue culture flasks. Both cells were incubated at 37 °C in a 95% air-5% CO_2 incubator. Cell passage was carried out at 80% confluence at 1:3 ratio using trypsin.

Acetylcholinesterase assay

The acetylcholinesterase assay was performed as follows. PC12 cells were inoculated onto 96-well microplates at 1 x 10^4 cells per well in 100 μl of medium. The cells were incubated with 40 ng ml^{-1} nerve growth factor 2.5S (NGF) (Funakoshi, Tokyo, Japan) and allowed to attach for at least 6 h before genistein (Wako, Japan) and daidzein (Wako, Japan), with or without 1.5 μM tamoxifen, were added to obtain the required final concentrations. Similar experiments were also conducted using the natural hormone 17 β-estradiol. After incubating for 24h, the

medium was carefully removed and the cells carefully washed twice with 200 µl of PBS(-). After washing, 20 µl of 5.6 mM acetylthiocholine iodide and 180 µl of buffer solution, pH 7.5 (0.12 M NaCl, 0.2% TritonX-100, 1 mM EDTA, 50 mM HEPES) were added into each well. After incubating for 2 hours in room temperature, 20 µl of the cell lysates were transferred to another multiwell plate and incubated for 1 h with 160 µl buffer solution, pH 5.0 (1 mM EDTA, 0.2% TritonX-100, 50 mM acetate buffer) and 20 µl of 0.4 mM 7-diethylamino-3(4'-maleimidyl-phenyl)-4-methylcoumarin in acetonitril. The absorbance in each well was then measured using a fluorescence plate reader (Fluoroskan Ascent FL) at 460 nm.

Cell proliferation assay

For the cell proliferation assay, MCF-7 cells were trypsinized and plated onto 96-well plates at initial concentrations of 3,000 cells per well. After allowing the cells to attach for 24 h, the seeding medium (10% FBS in phenol-red free RPMI) was removed and replaced with the experimental medium (10% charcoal-treated FBS in phenol-red free RPMI). Genistein or daidzein, with or without tamoxifen, was then added to the cells to obtain the required final concentrations. The plates were incubated for 6 days, after which 10 µl of 50 µg/ml MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (Dojindo, Japan) was added to each well and then incubated for 4 h. Sodium dodecyl sulfate (10%) was then added at 100 µl per well and the plates incubated for 24 h. The absorbance was then measured at 570 nm using a microplate reader.

Protein kinase C assay

The protein kinase C assay was performed as follows. About 1 x 10^6 PC12 cells were plated onto sterile Petri plates and allowed to attach for 24 h. After which, the seeding medium was removed and replaced with medium containing 0.08 μM genistein or daidzein. The plates were then incubated for 24 h, after which, the cells were then washed twice with equal volumes of PBS (-), and then incubated with lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 50 mM sodium fluoride, 5 mM β-glycerophosphate, 2 mM EDTA, 2 mM EGTA, 1 mM sodium vanadate, 1% (v/v) Nonidet P-40, 1mM PMSF, 5 μg/ml leupeptin hydrochloride, 1 μM E64, and 50 mM 2-mercaptoethanol) for 10 min at 4 °C. The cells were then collected using a sterile plastic cell scraper, transferred to sterile centrifuge tubes, and spun at 15300 rpm for 10 min at 4 °C. The supernatant was then used for the PKC assay using the Pep Tag Assay Kit (Promega).

3.3. Results and Discussion

Results in **Figure 5** *(page 73)* show that at concentrations as low as 0.08 μM, the plant estrogens genistein and daidzein enhanced the AChE activity of PC12 cells; however, at higher concentrations (≥0.625 μM), the AChE activity was similar to that of the control. This enhancement of AChE activity at lower phytoestrogen concentrations was effectively blocked by the ER antagonist tamoxifen, suggesting that this enhancement may be due to the binding of the phytoestrogen to the ER in PC12 cells.

Results in **Figure 6a** *(page 74)* clearly indicate that genistein and daidzein are estrogenic, as shown by the increased proliferation of MCF7 cells in the presence of increasing concentrations of these compounds. The results further show that this estrogenic property is due to the binding of the phytoestrogen to the ER, since the effect was effectively blocked by tamoxifen (**Figure 6b**, *page 74*). On the other hand, there was no significant difference in the cell number of PC12 cells incubated in the presence or absence of these isoflavones, indicating that these compounds are not mitogenic to neuronal cells.

To determine whether the enhanced AChE activity of PC12 cells was due to the binding of estrogenic compounds to the ER, we incubated the cells with varying concentrations of 17 β-estradiol (E2) and E2 plus 0.08 μM genistein, 0.08 μM daidzein or 1.5 μM tamoxifen. Results in **Figure 7** *(page75)* show that there is no significant enhancement of AChE activity under these conditions. This suggests that binding of E2 to the ER does not enhance AChE activity, and that possibly the effect of genistein and daidzein on AChE activity cannot be expressed in the presence of

E2, considering that it requires 1,000- to 10,000-fold molar excess of these phytoestrogens to compete with the ability of E2 to bind to the ER (Miksicek RJ, 1993). Another possible explanation is that exposure of PC12 cells to saturating concentrations of E2 decreases specific estrogen binding in NGF-treated PC12 cells, suggesting that estrogen down-regulates its own receptor (Sohrabji et al., 1994).

Apart from early findings that genistein and daidzein can bind to the ER in estrogen-responsive cells (Martin et al., 1978, Miksicek RJ, 1993), previous studies have also shown that the precise molecular target(s) of these compounds is not known (Barnes S and Peterson TG, 1995). Hence, it is also possible that some of the effects elicited by these compounds are independent of their estrogenic properties.

Previous studies have shown varied results as far as the effect of genistein on AChE activity is concerned. Studies using the neuroblastoma cell lines TS12 and SJNKP showed that genistein induced neurite outgrowth, increased AChE activity and cell growth inhibition in both cell lines, possibly due to tyrosine protein kinase inhibition (Rocch et al., 1995). However, when the mouse erythroleukemia cell line F55 was used, genistein did not enhance AChE activity, suggesting the absence of genistein receptors in this cell line (Okafuji et al., 1995).

The inhibition of protein kinases, such as tyrosine kinase or nucleotide-dependent and Ca^{2+}-phospholipid-dependent protein kinases (protein kinase C), is a possible cause of AChE induction in PC12 cells treated with genistein and daidzein. Genistein is a well-known tyrosine kinase inhibitor that induces AChE activity in mouse megakaryoblastic cells (Honma et al., 1991), and in PC12 cells after priming with certain neurotrophic active compounds (Pradines et al., 1995). In this study, the author examined the protein kinase C activity in PC12 cells after treatment with

genistein and daidzein, but neither phytoestrogens inhibited PKC activity, suggesting that PKC inhibition is not involved in the observed phenomena.

The enhanced AChE activity of PC12 cells when incubated with 0.08 μM of genistein and daidzein may possibly be due to its potentiating effect on NGF, but that further increasing its concentration cancels this out. Green and Rukenstien (1981) hypothesized that NGF may work via parallel or branching pathways such that its effect on AChE activity can be dissociated from its effects on neurite outgrowth and proliferation. Regulation of AChE activity involves NGF receptors with binding properties that are different from the binding properties of the receptors that mediate actions such as neurite outgrowth, ornithine decarboxylase induction, and maintenance of survival (Green and Rukenstien, 1981).

The author hypothesizes that the enhanced AChE activity of PC12 cells in the presence of 0.08 μM genistein or daidzein may be due to the following possible reasons: binding of the phytoestrogens to the ER in PC12 cells increases cellular responsiveness to NGF by altering the number or ratio of NGF receptors, or enhancement of the NGF-receptor-mediated pathway through its effect on the enzymes involved in AChE production.

That phytoestrogens can enhance AChE activity in neuronal cells may have implications in medical cases involving AChE deficiency (Nobuo et al., 2002, Donger et al., 1998) as well as in animal (Minic et al., 2002) and insect physiology (Greenspan et al., 1980).

3.4. Summary

Some compounds derived from plants have been known to possess estrogenic properties and can thus alter the physiology of higher organisms. Genistein and daidzein are examples of these phytoestrogens, which have recently been the subject of extensive research. In this study, genistein and daidzein were found to enhance the acetylcholinesterase (AChE) activity of the rat neuronal cell line PC12 at concentrations as low as 0.08 μM by binding to the estrogen receptor (ER). Results have shown that this enhancement was effectively blocked by the known estrogen receptor antagonist tamoxifen, indicating the involvement of the ER in AChE induction. That genistein and daidzein are estrogenic was confirmed in a cell proliferation assay using the human breast cancer cell line MCF7. This proliferation was also blocked by tamoxifen, again indicating the involvement of the ER. On the other hand, incubating the PC12 cells in increasing concentrations of 17 ß-estradiol (E2) did not lead to enhanced AChE activity, even in the presence of genistein or daidzein. This suggests that mere binding of an estrogenic compound to the ER does not necessarily lead to enhanced AChE activity. Moreover, the effect of the phytoestrogens on AChE activity cannot be expressed in the presence of E2 since they either could not compete with the natural ligand in binding to the ER or that E2 down-regulates its own receptor. This study clearly suggests that genistein and daidzein enhance AChE activity in PC12 cells by binding to the ER.