

## **CHAPTER 5**

### **GENERAL DISCUSSION AND CONCLUSION**

Because of the complexity of the endocrine system, there are no generally accepted, validated methods to screen for or monitor exposure to chemicals that could cause adverse hormonal activity (Committee on Hormonally Active Agents in the Environment, 1999). No single assay can predict accurately the effects of endocrine disruptors. Moreover, their significant effects at lower concentrations is an inherent property. A number of studies have even shown conflicting or opposite results between *in vitro* and *in vivo* experiments.

As mentioned in Chapter 1, *in vitro* assays are utilized because of their rapidity, relatively low cost and reproducibility, thereby allowing a larger number of samples or compounds to be screened simultaneously. And as far as investigating the mechanism of action of endocrine disruptors and their interaction with endocrine-response pathways are concerned, *in vitro* assays are the methods of choice.

*In vivo* assays, on the other hand, are relatively expensive, labor intensive, and have low sensitivity. And since these assays deal with animals which have highly complex responses that can be modulated by other mechanisms, the results obtained might not be selective enough for the compounds of interest. As such, they are not suitable for large-scale screening.

The discrepancy between *in vitro* and *in vivo* results can be illustrated using kepone (chlordecone), a persistent organochlorine pesticide. In the short time frame of *in vitro* assays, kepone is 100,000 to 1 million times less potent than estradiol (Soto et al., 1995). However, in a rat uterine weight assay, kepone is only 1,000 to 5,000 times less potent than estradiol (Hammond et al., 1979). As shown in the latter, the combination of increased bioavailability and prolonged receptor association can lead to increased potency of many endocrine-altering compounds (Crain et al., 2000).

The involvement of serum binding proteins further complicates the dynamics of endocrine disruption. Natural hormones have a high binding affinity towards these proteins that are found in the blood and cytoplasm, and this binding regulates the hormones' metabolism, clearance and bioavailability.

On the other hand, the lack of affinity between serum and/or cytosolic binding proteins and various steroid-mimicking contaminants can increase the availability of these chemicals to intracellular steroid receptors and, therefore, increase the cellular potency of such contaminants relative to native hormones (Crain et al., 2000).

Arnold et al. (1996) has shown that the activity of the phytoestrogen genistein was reduced by serum proteins, although the reduction was smaller than that of estradiol. In contrast, the transcriptional activity of synthetic estrogens, diethylstilbestrol, *op'*-DDT, *pp'*-DDD, octylphenol, and kepone, was only minimally reduced by the addition of serum proteins. Hence, the presence of binding proteins may explain the discrepancy between *in vitro* and *in vivo* studies.

In order to focus more on the possible mechanisms behind endocrine disruption, this study has primarily examined the effect on neuronal cells *in vitro* of two types of endocrine disruptors, namely, xenoestrogens as represented by alkylphenolic compounds such as nonylphenol, and phytoestrogens as represented by the soy isoflavones genistein and daidzein. Work also focused on food factors from the edible fungus *A. blazei*, which is known to have anti-cancer properties and its interaction with nonylphenol in human breast cancer cells. No claims are made that the results obtained in these *in vitro* studies necessarily and immediately apply to *in vivo* findings. Further studies are required to establish these connections.

Chapter 2, which focused on alkylphenolic compounds as examples of synthetic xenoestrogens, revealed the following conclusions:

- Results using the lactate dehydrogenase cytotoxicity assay to determine cell injury rate reveal that alkylphenols (*p*-pentylphenol, *p*-hexylphenol, *p*-heptylphenol, *p*-octylphenol, and *p*-nonylphenol) did not induce cell necrosis in the rat neuronal cell line PC12 beyond 30%, even at concentrations as high as 300  $\mu$ M in a 15-minute incubation period.
- Exposing PC12 cells to alkylphenols for 4 h and testing for DNA fragmentation showed that nonylphenol and octylphenol also did not induce apoptosis, even at concentrations as high as 500 and 100  $\mu$ M, respectively.
- However, incubating PC12 cells with alkylphenols (*p*-pentylphenol, *p*-hexylphenol, *p*-heptylphenol, *p*-octylphenol, and *p*-nonylphenol) for 24 h significantly inhibited acetylcholinesterase activity at concentrations as low as 0.8  $\mu$ M.

Chapter 3, which aimed to determine whether the naturally-occurring endocrine disruptors genistein and daidzein also inhibit AChE activity, revealed the following conclusions:

- The soy phytoestrogens genistein and daidzein were found to enhance the acetylcholinesterase (AChE) activity of PC12 cells at concentrations as low as 0.08  $\mu$ M by binding to the estrogen receptor

(ER). This enhancement was blocked by the known ER antagonist tamoxifen, indicating the involvement of the ER.

- That genistein and daidzein are estrogenic were confirmed in a cell proliferation assay using the human breast cancer cell line MCF7. This proliferation was also blocked by tamoxifen.
- Incubating PC12 cells in increasing concentrations of  $17\beta$  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.

Chapters 2 and 3 showed that alkylphenols inhibit AChE activity in neuronal PC12 cells, while genistein and daidzein had an opposite effect. Chapter 4, which aimed to determine whether other food factors with anti-cancer properties can also mitigate the mitogenic effect of nonylphenol on estrogen-responsive cell lines, revealed the following conclusions:

- Incubating human breast cancer MCF7 cells with 1 nM  $17\beta$ -estradiol (E2), 1  $\mu$ M *p*-nonylphenol (NP) and a 5 percent aqueous extract (AE) from the edible mushroom *A. blazei* Murill combined, or NP plus AE, resulted in increased cell proliferation compared to the untreated control by 93% and 67%, respectively.
- The aqueous extract from *A. blazei* Murill significantly enhanced the expression of the c-Jun/activator protein-1 (AP1) in the human breast cancer cell line MCF7; thereby suggesting that the enhanced proliferation of MCF7 cells in the presence of nonylphenol and the

aqueous extract may be due to the involvement of the AP1 gene regulatory complex.

A synthesis of these findings provides us with the following general conclusions:

- Endocrine disruptors that are of synthetic origin (such as alkylphenolic compounds) induce toxicity in neuronal cells by inhibiting acetylcholinesterase activity; however, plant-based estrogens (such as genistein and daidzein), on the other hand, not only do not have toxic effects on cells, they also enhance AChE activity by binding to the estrogen receptor found in PC12 cells.
- While it is known that alkylphenolic compounds such as nonylphenol have mitogenic effects on breast cancer cells, cellular proliferation is oftentimes not too significant compared to untreated cells. However, it was discovered that if these cells are incubated with nonylphenol plus an aqueous extract from the fungus *A. blazei*, cell proliferation is dramatically enhanced. This phenomenon was found to be concomitant with the increased expression of the proto-oncogene cJun in breast cancer cells treated solely with the fungal extract. It is therefore hypothesized that this increased proliferation is due to the interaction of nonylphenol with the activator protein-1 (AP1) gene regulatory complex, which has been activated by food factors from *A. blazei*.