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CHEMICAL MIXTURES OF XENOESTROGENS AND THEIR METABOLITES ALTER ESTRADIOL-INDUCED NON-GENOMIC SIGNALING

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CHEMICAL MIXTURES OF XENOESTROGENS AND THEIR METABOLITES ALTER ESTRADIOL-INDUCED NON-GENOMIC SIGNALING.

by

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Dedicación

Quiero dedicar esta tesis a las personas más importantes que me ayudaron a lograr un sueño más en vida, la de doctorarme en toxicología. Esta tesis es para mis padres, Dr. René Gilberto Viñas y Lic. Leonor Trillo, y a mí querida esposa Carolina. Estoy eternamente agradecido con ustedes.

Dedication

I would like to dedicate this dissertation to the most important individuals that have helped me reach this goal; to my parents Dr. René Gilberto Viñas and Leonor Trillo, BSN, BA, and to my dear wife, Carolina.

I am eternally grateful to you.

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I would first like to acknowledge my mentor, Cheryl Watson, for all of her support and guidance through my PhD career. I have learned so much from her and I cannot thank her enough for being a true mentor in every respect. A “thank you” to my committee members: Fernanda Laezza, Xiaodong Cheng, Terumi Midoro-Horiuti, and Viroj Boonyaratanakornkit for their time and insightful comments. I would also like to thank the graduate school, in particular Dorian Coppenhaver for his patience, guidance, and support throughout the difficult moments as a graduate student. I am also very grateful to my “external advisor”, Ernest Smith, who first introduced me to the field of toxicology, and has since guided and counseled me throughout my adventure of becoming a toxicologist. Also a special thanks to Sol Bobst, who has become my “industry advisor” and friend, and who has provided me with invaluable information and insights into the world of regulatory toxicology. Special thanks to my friends and colleagues who have made this journey all the more enjoyable.

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ABSTRACT:

BACKGROUND: Xenoestrogens (XEs) pose a threat to human health by disrupting natural responses of physiologic estrogens. Bisphenol-A (BPA), a plastics monomer, is potently estrogenic via non-genomic signaling; however, bisphenol-S (BPS), a BPA substitute, has unknown endocrine actions. Nonylphenol is a surfactant and a ubiquitous contaminant. Our goals were to use a well-characterized estrogen-responsive cell model, the $GH_3/B_6/F_{10}$ pituitary line, to characterize the potentially potent nongenomic signaling and functional responses to these compounds alone, as mixtures, as metabolites and congeners, and in combination with estradiol (E_2), while also automating these assays to facilitate future screening of a potentially large number of XEs.

METHODOLOGY: Environmentally relevant concentrations of XEs (10^{-15}-10^{-7}M) were assessed individually and as mixtures by challenging 1nM E_2-induced responses. We quantified phospho-activation of extracellular signal-regulated kinases (ERKs) and c-Jun-N-terminal kinases (JNKs) by multi-well plate immunoassays. Cell proliferation was assessed by crystal violet assay, while apoptosis (caspase-8, -9) was assessed via the release of 7-amino-4-trifluoromethylcoumarin. Prolactin release was measured by radio-immunoassay after 1 min XE exposures. The BIOMEK FXP workstation was used to develop an automated screening system for changes in MAPKs activities due to XE exposures.

RESULTS: XEs often activated MAPKs in a non-monotonic dose- and oscillating time-dependent (2.5-60 min) manner and attenuated 1nM E_2 responses. While individual bisphenols did not activate JNK as NP did, the combination of all XEs with E_2 generated an enhanced non-monotonic JNK dose-response. E_2 and all XE compounds induced cell proliferation, while mixtures of these compounds with E_2 suppressed proliferation. Caspase 8 activity was suppressed by E_2, and elevated by BPS, while caspase 9 activity was inhibited by E_2 and some XE combinations at later times. Mono- and di-chlorinated BPA activated, while tri-chlorinated BPA dephosphorylated ERK. Di- and tri-chlorinated BPAs caused JNK dephosphorylation. Phase II metabolites (sulfated and glucuronidated) were mostly unable to activate either kinase and in some cases severely inactivated them.

CONCLUSIONS: Novel chemical analogues and conjugated forms of BPA individually or as mixtures with other known XEs had dramatic disrupting effects on physiologic estrogens, disrupting mechanisms of cell regulation and their downstream functional responses.

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Introduction[1]

1.1.0 Introduction

Trace levels of industrial and naturally occurring chemicals have been shown to perturb endocrine systems. These endocrine disrupting chemicals (EDCs) are currently the subject of intense research and regulatory action. A large number of these EDCs act via the estrogen receptor (ER), imperfectly mimicking and interfering with the physiologic actions of endogenous estrogens. Xenoestrogens (XEs) can bind to ERs in the cell nucleus, where the complex recognizes DNA response elements and alters gene expression; in the non-genomic pathway XEs can bind to membrane-bound ERs and rapidly initiate signaling cascades that culminate in kinase and phosphatase activations, ultimately influencing cellular function by post-translational modifications of a variety of proteins (Watson et al. 2011a).

Functional consequences observed at the organismal level include decreased fecundity in aquatic organisms, altered sexual behavior and memory in rats, and malformations and decreased mobility of human sperm (Goncalves et al. 2010; Li DK et al. 2011; Oehlmann et al. 2009; Sohoni et al. 2001; Wolstenholme et al.

[1] Chapter 1 taken from: **René Viñas,** Yow-Jiun Jeng and Cheryl S. Watson. 2012. Non-genomic Effects of Xenoestrogen Mixtures. International Journal of Environmental Research and Public Health, 9(8): 2694-2714 (open source).

2011; Zhou et al. 2011). XEs have also been implicated in the development of such chronic diseases as obesity, diabetes mellitus, asthma, and cancer (Alonso-Magdalena et al. 2006; Bouskine et al. 2009; Midoro-Horiuti et al. 2010; Newbold et al. 2009; Newbold 2011). Many XEs, such as nonylphenol, are persistent organic byproducts of our industrialized society that can remain in the environment for extended periods of time, thereby increasing the likelihood of human and wildlife exposure (Topp and Starratt 1999; Wang et al. 2007). Human exposure to XEs can result from eating or drinking contaminated food and water that has been kept in plastic containers constructed from XEs such as bisphenol-A (BPA) (Kubwabo et al. 2009). Waste water and subsequently drinking water have also become plentiful sources of XEs (e.g., pharmaceuticals, surfactants) that are not removed by treatment plants (Dave and Herger 2012; Dolar et al. 2012); in addition, use of chlorine as a typical means of water purification has given rise to poly-chlorinated conjugated compounds that can also interfere with endocrine regulation (Kuruto-Niwa et al. 2005).

In real-world situations, however, humans and wildlife are not exposed to one chemical at a time, but rather to complex mixtures. The potential health hazard from EDC mixtures is one of the most difficult challenges for a regulatory agency to evaluate. Nevertheless, several legislative mandates (Superfund Amendments and Reauthorization Act (1986); Food Quality and Protection Act (1980); Safe Drinking Water Act (1996)) require the U.S. Environmental Protection Agency to

examine mixtures of chemicals in regulatory decision making (Teuschler et al. 2002a). Studies examining known XEs in combination with endogenous estrogens (binary mixtures) have shown additive, synergistic, or antagonistic changes in cell signaling (mitogen-activated kinases (MAPK)) and functional (proliferation and peptide hormone (prolactin (PRL) release) endpoints (Alyea et al. 2008; Alyea and Watson 2009b; Jeng et al. 2009; Jeng et al. 2010b; Jeng and Watson 2009; Jeng and Watson 2011; Kochukov et al. 2009), demonstrating the difficulty of predicting the estrogenic responses in XE mixtures. There is also strong evidence that several types of XE mixtures can produce non-monotonic dose-responses at low concentrations, making the task of risk assessment all the more difficult (Vandenberg et al. 2012). Others have recently reviewed the literature on the genomic responses contributing to endocrine disruption, and we refer readers to those reviews (Hayes et al. 2006; Kortenkamp 2007; Vandenberg et al. 2012). In this review we will focus on the non-genomic actions of diverse estrogenic chemicals, as single entities and as mixtures, as well as current approaches used to evaluate their toxicity levels.

1.2.0 Mechanisms of Estrogenic Actions—Genomic & Non-Genomic Pathways

1.2.1 Different Types of Estrogen Receptors

Estrogens, other steroids, and related compounds were traditionally thought to mediate their actions exclusively via nuclear actions, so the name “nuclear

receptor" has become exchangeable with "steroid receptor." ERs are one category of nuclear receptors in the larger family through which EDCs can act. Others are the receptors for compounds such as androgens, thyroid hormones, aryl hydrocarbons, and pregnane-x compounds (Decherf et al. 2010; Diamanti-Kandarakis et al. 2009; Kretschmer and Baldwin 2005; Qin et al. 2011; Vandenberg et al. 2009). Nuclear actions result in various macromolecular syntheses initiated by transcription factors, and require extended time periods to elicit a response. Genes regulated by estrogens via this pathway influence reproduction, development, bone integrity, cardiovascular function, behavior (Deroo and Korach 2006), and a growing list of tissue-specific functions. However, compelling more recent evidence has resulted in wide acceptance of an alternative non-genomic, often rapid, signaling pathway for estrogenic actions (Li et al. 2003; Pappas et al. 1994; Pietras et al. 2005; Pietras and Szego 1999). Membrane-bound ER subtypes (α, β) have been identified and linked to the initiation of non-genomic responses (Kang et al. 2010; Li et al. 2003; Pappas et al. 1994). Even more recently, another entirely different receptor type has been associated with rapid estrogenic responses—a seven-transmembrane G protein-coupled receptor called GPR30 (also referred to as GPER) (Thomas et al. 2005; Thomas and Dong 2006).

The reason for the existence of different ER localizations and subtypes is not yet clear. We have yet to document enough examples of the serpentine-type GPR30

receptor actions to determine if it fits discernible subcategories of functional responses. Different ERs (membrane and nuclear) could exist to accommodate the wide variety of estrogenic molecules with distinct functions (Watson and Gametchu 1999), as mERs in lipid membrane environments as opposed to nuclear aqueous environments might take on different conformations and thus accept different subsets of ligands. Unfortunately, this ability of ERs to accommodate many different lipophilic compounds also makes them vulnerable to EDC binding. It is also possible that multiple ERs must all participate for a complete and complex cellular response (Watson et al. 1999).

Membrane-initiated signaling may be a “first alert system” for the cell to eventually prepare for more permanent make-overs (Watson and Gametchu 1999). Activation of MAPKs and other kinases do in some cases lead to activation of transcriptional events (Jeng and Watson 2009), resulting in new proteins and altered cellular differentiation. However, some non-genomic responses end in complete functional endpoints without the eventual induction of genomic endpoints. For example, we have previously shown that in pituitary cells, mER-initiated pathways are capable of activating caspases 8 and 9, as well as inducing ion influxes triggering the release of PRL from secretory vesicles (Jeng and Watson 2009; Kochukov et al. 2009).

Our group and others have explored the similarities between the mERα and the intracellular estrogen receptor (iERα) (Campbell and Watson 2001; Powell et al. 2001); a close similarity was established between alternatively localized forms when nine iERα-specific antibodies (Abs) recognized seven different mERα epitopes in unpermeabilized cells where Abs cannot cross the plasma membrane (Campbell and Watson 2001; Watson et al. 1999). Additionally, the ability of the ERα-recognizing Ab H151 to elicit responses or block subsequent responses to estrogenic ligands also added strength to this identification (Norfleet et al. 2000; Watson et al. 1999).

Other results have also suggested identity of ERα in both subcellular locations by virtue of the proteins with which they associate. Recently, we used Duolink immunofluorescence imaging to visualize the partial co-localization of mERα and the Gαi subclass of G proteins at the cell membrane. Interactions of ERα and caveolin-I were also demonstrated by epitope proximity ligation studies which supported the idea that these proteins jointly participate in estrogen-induced signaling in the membrane (Razandi et al. 2002; Razandi et al. 2003; Schlegel et al. 1999; Watson et al. 2011).

The mERα was also deemed identical to its nuclear counterpart in MCF7 breast cancer cells by membrane isolation (affinity chromatography) and mass spectrometry analysis (Pedram et al. 2006). These results are in line with the

blocking of responses by antisense (Norfleet et al. 1999b) and siRNA knockdowns (Alyea et al. 2008; Pedram et al. 2006) of ERα, and the use of a variety of immunohistochemistry techniques for mER identification (Norfleet et al. 1999a; Norfleet et al. 1999b). More recently, the mechanism for membrane attachment has been shown to be via post-translational palmitoylation (Pedram et al. 2007). Thus overall, it is very likely that mERα is indeed closely related to iERα, modified for targeting to the plasma membrane.

1.2.2 Types of Non-Genomic Signaling Induced by Estrogens and Xeno estrogens and Their Functional Consequences

BPA and other XEs have been found to be “weak” inducers of estrogenic activity via the genomic pathway in comparison to E_2 (1,000-fold difference) (Kuiper et al. 1997). However, BPA is equipotent with E_2 in its ability to initiate rapid non-genomic responses from membrane receptors (Wozniak et al. 2005) (see figure 1). Non-genomic signaling can occur within seconds-minutes of the initial steroid-receptor contact, yet sustained activation of cell signaling can influence more permanent changes such as cell proliferation, differentiation, movement, or apoptosis. Membrane steroid receptor-mediated signals include the activation of kinases that regulate the phosphorylated states of important functional proteins, each linked to different pathways of actions.

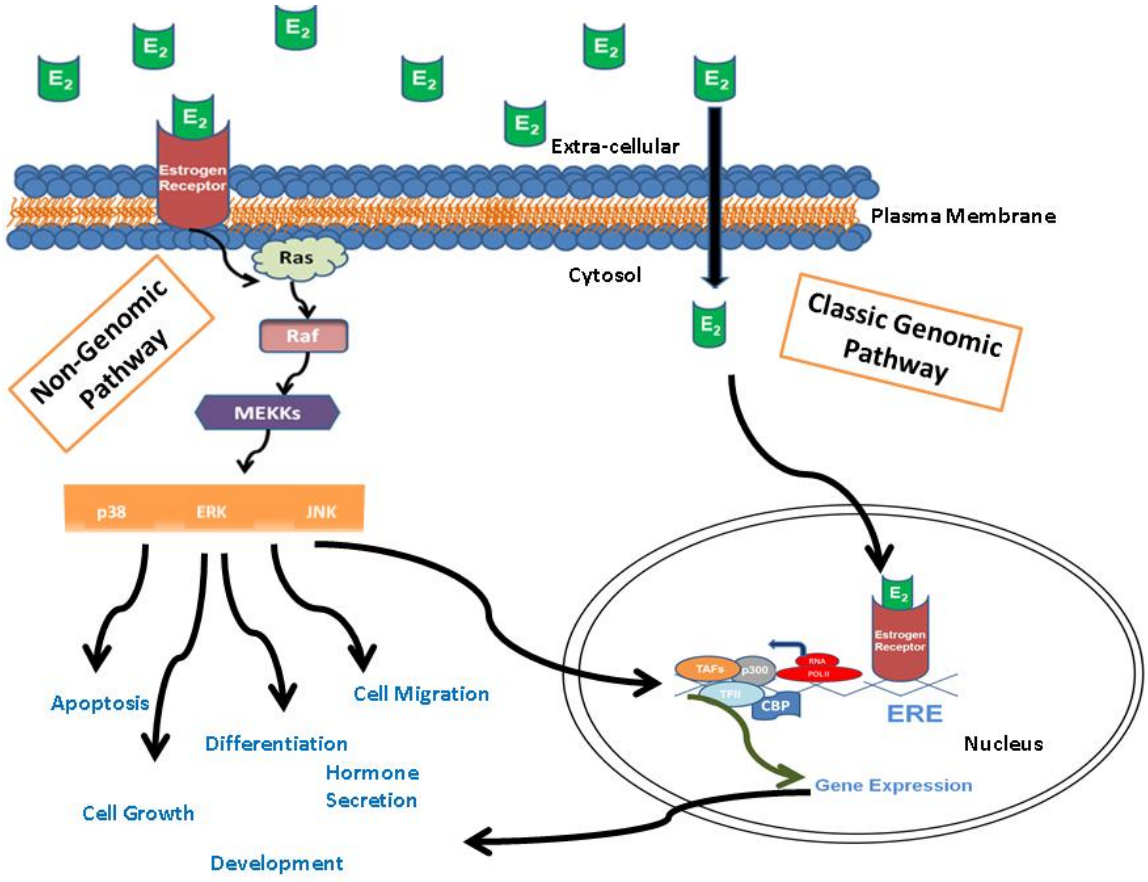

Figure 1. Comparison of estrogen induced genomic and non-genomic pathways.

In our studies, we have extensively examined the estrogen- and XE-induced MAPK signaling activations, specifically those of the extracellular regulated kinases (ERKs 1 and 2), the c-Jun N-terminal kinases (JNKs 1 and 2), and the p38 kinase. Activation of ERKs is commonly associated with cell growth and survival, whereas activation of JNKs has long been linked to the induction of apoptosis (Hunter 1995; Junttila et al. 2008; Nordstrom et al. 2009; Xia et al. 1995). In the case of E_2, coumesterol, and BPA, we have been able to correlate

strong and sustained ERK activity together with weak JNK activation responses to the induction of cell proliferation in the $GH_3/B_6/F_{10}$ pituitary cell line (Jeng and Watson 2009; Kochukov et al. 2009)—the ERK response apparently predominating. Others have speculated that extended stimulation of the JNK pathway may lead to a shutdown of ERK and its associated effects (Junttila et al. 2008). Therefore, there is interplay between these integrator kinases to render a final outcome.

Ion fluxes (Ca^{2+}, K^+. Na^+, H^+) are a common signaling responses to steroids and related compounds, often leading to changes in cell mobility, downstream signaling processes, and peptide hormone secretion (Watson and Gametchu 1999). Using an mERα-enriched $GH_3/B_6/F_{10}$ prolactinoma cell line we examined functional consequences of elevated Ca^{2+} levels (PRL release) upon stimulation with low picomolar and sub-picomolar E_2 concentrations (Bulayeva et al. 2005; Jeng et al. 1994; Jeng and Watson 2009; Kochukov et al. 2009; Pappas et al. 1994; Wozniak et al. 2005). BPA, o′,p′-dichlorodiphenyl-ethylene (DDE), nonylphenol, coumesterol and other known XEs caused release of PRL from secretory vesicles within a minute, with non-monotonic dose response characteristics. Changes in Ca^{2+} influx were monitored in order to establish a correlation between Ca^{2+} levels and PRL secretion (Bulayeva et al. 2005; Kochukov et al. 2009; Wozniak et al. 2005). Interestingly, Ca^{2+} fluxes were not non-monotonic, and thus did not explain the bi-modal nature of the changes seen

with PRL release. Therefore, for this and other reasons, it is likely that regulation of peptide secretion also involves additional signaling pathways (Kochukov et al. 2009; Watson and Gametchu 1999; Wozniak et al. 2005).

We have found that non-genomic effects of estrogens also modulate transporter functions (Alyea et al. 2008; Alyea and Watson 2009a; Alyea and Watson 2009b). Using the rat pheochromocytoma (PC12) cell model, we examined dopamine efflux via the dopamine transporter upon exposure to several physiologic estrogens at 10^{-14}–10^{-8} M concentrations. Our studies found that like amphetamines, multiple estrogens (Binda et al. 2008) are capable of reversing transport direction of the dopamine transporter via kinase regulation (Alyea and Watson 2009b; Foster et al. 2006). Upon XE exposure, all compounds tested (nonylphenol, BPA, dieldrin, endosulfan and DDE) elicited dopamine efflux with non-monotonic response characteristics, resembling a U-shaped curve, or with even more fluctuations in the responses, thus making it difficult to extrapolate low dose effects from high ones to assign chemical safety margins for regulatory purposes.

1.2.3 Non-Monotonic Dose Responses of Xenoestrogens

According to the International Dose-Response Society (http://www.dose-response.org), hormesis is defined as “a dose-response phenomenon characterized by low-dose stimulation and high-dose inhibition” (2012). The

occurrence of such non-monotonic responses to XEs at low concentrations (below the so-called toxic threshold) has in recent years gained increasing awareness by the scientific and regulatory community (U.S. Food and Drug Administration 2012). Still, there is considerable debate as to the fundamental mechanisms responsible and their practical use in evaluating chemical safety. Typical dose-response studies in regulatory testing involve in-vivo or in-vitro models exposed to high concentrations of chemicals uncommonly found in human populations or the environments to which they are exposed (Fenner-Crisp 2000; Lucier 1997). Past evaluations assumed that all chemical responses follow a linear monotonic path that eventually reaches an asymptote; safe doses for humans or wildlife were then determined to be just below the lowest measurable response-causing concentrations or the no-observed-effect-level (NOEL) (Vandenberg et al. 2012) . However, most XE exposures occur at low doses and exhibit non-monotonic responses that make it difficult to predict low-dose effects from high-dose effects (Vandenberg et al. 2012). Furthermore, because XEs are rarely present at concentrations that produce immediate death or illness, traditional toxicology testing is irrelevant, and in any case insufficient for understanding XE mechanisms (Myers et al. 2009; Teuschler et al. 2002b; Vandenberg et al. 2012; Watson et al. 2011b). Therefore, more recent XE studies have begun to investigate low dose exposures focusing on very sensitive endpoints such as cell signaling or gene expression that could have dire

repercussions on tissue and whole-animal functioning and health over time (Soto et al. 2009).

Various theories have been offered as explanations for non-monotonic dose responses; these have been previously reviewed (Calabrese 2009; Calabrese 2010; Calabrese et al. 2012; Vandenberg et al. 2006; Vandenberg et al. 2012; Watson 2003; Watson et al. 2011b) and so will only be summarized here. We and others observed that XEs are capable of initiating multiple receptor-proximal signaling cascades, responding with different rates and dose dependencies; these eventually contribute to composite response patterns of downstream phospho-activated MAPKs (i.e., pERK, pJNK, p38) (Bermudez et al. 2008; Wang et al. 2005; Yu et al. 2004; Zivadinovic and Watson 2005). It is well known that inhibition or negative feedback regulation of MAPKs is crucial for preventing unfavorable effects from extended pathway stimulation (Hunter 1995); hence, as seen in many of our studies, when concentrations of both physiologic estrogens and XEs increase (10^{-15}–10^{-7} M), MAPK responses eventually decrease (Bulayeva et al. 2004; Jeng et al. 2009; Jeng et al. 2010b; Jeng and Watson 2009; Jeng and Watson 2011; Kochukov et al. 2009). Furthermore, assessment of resulting functional endpoints has also shown that low doses and short exposure periods induce responses (e.g., proliferation and PRL secretion in pituitary cells), while higher doses and longer exposure periods cause inhibition (Calabrese et al. 2012; Jeng and Watson 2009; Stormshak et al. 1976;

Vandenberg et al. 2012; Wiklund et al. 1981; Wozniak et al. 2005). Other plausible explanations for non-monotonic dose-responses as a means of preventing overstimulation from XEs at higher concentrations include receptor down-regulation or desensitization, changes in receptor selectivity when going from low (selective ER binding) concentrations to high (non-selective) concentrations, the presence of co-factors or co-regulators that influence hormone-receptor binding at certain selective concentrations, and the presence of multiple receptor subtypes that bind to the same XE, but each with a different (stimulatory or inhibitory) response pattern (Calabrese 2008b; Vandenberg et al. 2012; Watson et al. 2010b; Watson et al. 2011b).

For mixtures toxicology, the significance of non-monotonicity has not been adequately characterized (Calabrese 2008a). This is partly due to the impossibility of testing so many chemical interactions in mixtures where components can target various mechanisms and vary by tissue. In addition, chemical interactions such as synergy and potentiation occur in the low dose stimulatory zone, below the traditionally identified toxicological threshold (Calabrese 2008a). Furthermore, such responses are probably limited by various biological constraints, to modest increases of 30–60% above controls (Calabrese 2008b; Yang and Dennison 2007). In addition, evidence exists for EDCs inducing biological effects even at very low analytically undetectable concentrations. “No-threshold” responses (Sheehan et al. 1999; Sheehan 2006; Vandenberg et al.

2012) can be due to the presence of endogenous or exogenous mimetic hormones already present. These obscure low dose responses of compounds being experimentally tested (Sheehan et al. 1999; Sheehan 2006; Vandenberg et al. 2012), unless effectively removed (such as in well-controlled cell culture experiments).

1.3.0 Types of Estrogens and Estrogen Mimetics

1.3.1 Physiologic Estrogens

Produced primarily in the testes and ovaries, estrogens such as estradiol (E_2), estrone (E_1), and estriol (E_3) play diverse roles in human and wildlife physiology beyond those required just for reproductive success, affecting metabolism, bone integrity, cardiovascular functions, behavior and mood, and other functions (Cornwell et al. 2004; Watson et al. 2010a). Physiologic estrogens play selective roles in women's life stages. For example, the predominant hormone driving sexual development, function of reproductive organs (e.g., breast and uterus) and the menstrual cycle is E_2. E_1 is found at elevated levels (~150–200 pM) during post-menopausal stages, while E_3 is high during pregnancy (~10–100 nM); males also have lower development stage-specific blood levels of endogenous estrogens (Greenspan and Gardner 2004). Excessive estrogenic activities have been associated with the development of cancer in estrogen-responsive tissues (e.g., breast and uterus). Decreased levels of E_3 have been

linked to complications of eclampsia and an increased probability of Down's syndrome in offspring (Benn 2002; Shenhav et al. 2003).

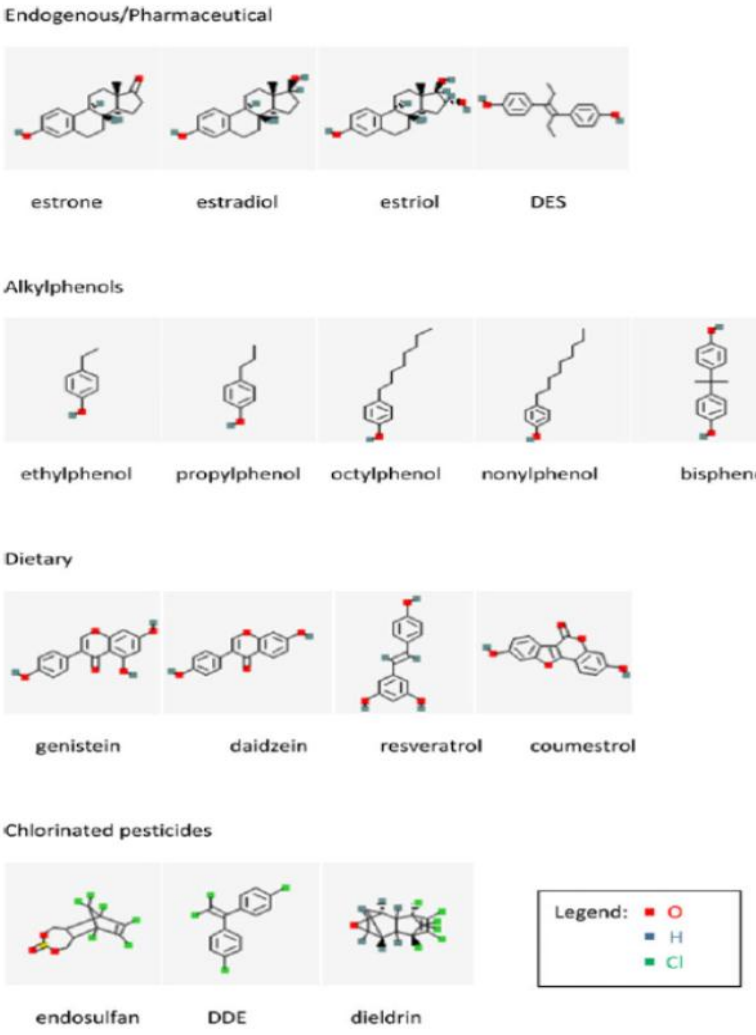

Figure 2. Chemical structures from different classes of estrogenic compounds.
Image taken from Watson, C. S., Jeng, Y. J., and Kochukov, M. Y. (2010b). Nongenomic signaling pathways of estrogen toxicity. Toxicol. Sci. 115(1), 1-11.

Therefore, understanding the mechanisms that influence or disrupt all estrogenic actions is crucial for preventing negative outcomes. Compared to E_2, these other endogenous estrogens have yielded weak genomic responses (Greenlee et al. 2007; Riza et al. 2001). However, we and others have shown that many of these same compounds can potently activate non-genomic signaling

pathways (Alyea and Watson 2009b; Jeng et al. 2010b; Jeng and Watson 2011; Mermelstein et al. 1996; Schwarz and Pohl 1994; Watson et al. 2008), which means that they can affect health and life stage-selective functions.

1.3.1 Pharmaceutical and Personal Care Product Estrogens

Pharmaceuticals and personal care products enter urban sewage networks and wastewater treatment plants, and eventually streams and waterways, via human use. Pharmaceuticals are excreted after use and therefore enter sewage from various locations, but especially from hospitals (Jean et al. 2012). Once in aquatic environments, low molecular weight and hydrophilic compounds will dissolve in water or will degrade in the sewage sludge (Fent et al. 2006). Still, a large amount of pharmaceuticals and their metabolites will remain, and expose humans via drinking/bathing water or by consuming fish and other aquatic animals that have ingested or absorbed pharmaceutical residues. Examples of estrogenic compounds commonly found in aquatic systems are ethinyl estradiol and mestranol (commonly used as hormonal contraceptives), trenbolone (used for growth promotion in cattle), tamoxifen (and other anti-estrogens used for breast cancer treatment that can sometimes be estrogenic in certain tissues), and equine or other estrogens used for postmenopausal hormone replacement, many of which are ubiquitously present in our water supplies in the ng/L, ppb, nM range (Fent et al. 2006; Gall et al. 2011; Lu et al. 2011; Soto et al. 2004; Touraud

et al. 2011; Zhou et al. 2012). Their estrogenic effects via the non-genomic pathway, as single chemicals and as mixtures, are currently unexplored.

1.3.3 Phytoestrogens

Some plant-derived components of the diet can act as either estrogenic agonists or antagonists of mERs, depending on concentration and tissue specificity (Jeng and Watson 2009). Common sources of phytoestrogens include soy-based products such as tofu (isoflavones and their metabolites); sprouts, red clover, or alfalfa (coumestans); and flaxseed, sesame seed, or nut products (lignans). In Asian cultures traditional culinary dishes are rich in phytoestrogens that are a major component of dietary intake. Better bone health, lower cardiovascular and cancer risks, and extended lifespans are often attributed to phytoestrogens in Asian diets. The intake of soy can be as high as 50 g a day, with measured genistein plasma concentrations from 0.1–10 μM (Mustafa et al. 2007; Whitten and Patisaul 2001). In contrast, Western diets typically have ten-fold lower concentrations (Adlercreutz et al. 1993). Another phytoestrogen, the stilbenoid resveratrol, is plentiful in red wine and other grape products, and has enjoyed much attention for its potential anti-diabetic, anti-cardiovascular disease, and cancer prevention effects, especially in cultures with a rich wine heritage (Baur and Sinclair 2006; Lippi et al. 2010).

An ever-expanding number of studies explore the physiologic and biochemical outcomes of phytoestrogen use. Such studies have been prompted by widespread phytoestrogen use as replacements for estrogen loss at menopause (hormone replacement therapy); phytoestrogens may prevent the increased risk of cancer that can occur from taking other estrogenic hormone supplements (Eden 2012; Pitkin 2012; Sunita and Pattanayak 2011). In a recent study we determined that unlike the pharmaceutical estrogen diethylstilbestrol, high levels of phytoestrogens do not promote precancerous growth of the pituitary and other estrogen-responsive tissues in Fischer 344 rats (Jeng et al. 2010a).Though high concentrations of phytoestrogens may not cause adverse effects in an adult individual, for an infant, the effects could lead to adverse developmental repercussions (Adlercreutz et al. 1999; Jefferson et al. 2012). Serum levels of genistein have been detected in a range of 1–10 μM in infants exclusively fed soy-based formulas (Cao et al. 2009; Setchell et al. 1998). Phytoestrogens have been reported to have low transcriptional activity via yeast-based receptor assays (Gaido et al. 1997), but they are capable of inducing MAPK signaling via the membrane ER (mERα) at doses far below or equivalent to the reported plasma concentrations achieved with even Western diets (Jeng and Watson 2009).

Unless taken individually as dietary supplements, phytoestrogens are typically found in the diet as chemical mixtures. Therefore the health benefits of

resveratrol, for example, could be the result of an additive effect with one of the other hundreds of “minor phenols” found in red wine (Mazerolles et al. 2010; Zhu et al. 2012), and also the grape species type that affects the wine’s chemical composition. Our lab has shown that in a prolactinoma cell line model, resveratrol attenuates cell proliferation when found in combination with E_2 (Jeng and Watson 2009). We therefore must examine mixtures of phytoestrogens and their combinations with endogenous estrogens more carefully for their beneficial (and deleterious) effects, as well as the cross-talk between genomic and non-genomic pathways. Much research remains to be done to even begin to understand how phytoestrogen mixtures may combine the signaling effects of their component compounds.

1.3.4 Synthetic/Anthropogenic Estrogens

Anthropogenic or man-made chemicals with estrogenic capabilities have become abundant pollutants in our environment (air, soil and water); many are by-products of plastics, preservatives, industrial surfactants, and pesticides. Agricultural compounds (e.g., pesticides such as dieldrin and endosulfan) have been detected in breast milk, urine, maternal blood, and serum in appreciable amounts, with links to low birth weight, fetal death, and childhood cancers (Mnif et al. 2011; Shaw and deCatanzaro 2009; Soto et al. 1994). Alkylphenols are surfactant breakdown products that are have been shown to be highly estrogenic via non-genomic and some genomic pathways (Bonefeld-Jorgensen et al. 2007;

Isidori et al. 2007; Jeng and Watson 2011; Kochukov et al. 2009). After the recent (2010) Deepwater Horizon oil spill in the Gulf of Mexico, large amounts (2,900,000 L) of oil dispersants containing alkylphenols were used, increasing safety concerns over their estrogenic effects (Kujawinski et al. 2011). In particular, nonylphenol can remain in the environment long enough to bio-accumulate in humans and wildlife (including human food supplies), leading to developmental abnormalities (Ahel et al. 1993).

The highly controversial XE, BPA, has also been detected in significant levels (in urine samples) in 93% of U.S. residents' ≥ 6 years of age. BPA's prevalence is due to its incorporation into many manufactured goods that we as consumers use on a daily basis; these include: plastic food and water containers, thermal coatings on cashier paper receipts, linings in canned foods, and dental repair materials, among many others (Deblonde et al. 2011; Fleisch et al. 2010; Geens et al. 2011; Huang et al. 2012; Myers and Hutz 2011). Exposure to BPA during critical developmental stages has been implicated in the onset of a variety of health problems, including breast and prostate cancer, asthma, diabetes and reproductive dysfunction (Alonso-Magdalena et al. 2006; Alonso-Magdalena et al. 2010; Midoro-Horiuti et al. 2010; Soto et al. 2008). Growing concern over BPA has prompted 11 U.S. states, Canada (2008), and Europe (2011) to ban its use in plastic feeding bottles for infants (Canada Gazette 2012; European Union 2011; Akst 2012; Layton and Lee 2008). Though much information has been

amassed on BPA's estrogenic actions, only recently have we and others explored its effects on functional endpoints at the cellular level (peptide hormone release and cell proliferation) via non-genomic mechanisms (Alyea and Watson 2009b; Bouskine et al. 2008; Bulayeva and Watson 2004; Jeng et al. 2009; Jeng and Watson 2009; Jeng and Watson 2011; Otto et al. 2008) as a potential mechanistic explanation for EDC-induced disease.

Stricter regulatory mandates on the production of and use of BPA have led to the synthesis of alternative bisphenol analogues (Gallar-Ayala et al. 2011; Liao et al. 2012a; Liao et al. 2012b). Among these alternatives is 4,4'-dihydroxydiphenyl sulphone, also known as bisphenol S (BPS). The structural similarity between BPS and BPA raises concerns over its safety and endocrine-disrupting potential. BPS was recently detected in a variety of paper products, including 87% of paper currencies sampled from 21 different countries (geometric mean of 0.029 µg/g) (Liao et al 2012a). Urinary BPS was detected in 97% of urinary samples (n=31) among from residents of Albany, NY in appreciable amounts [geometric mean of 0.299ng/ml;1.2nM] (Liao et al. 2012b). Despite the wide implementation of BPS in various products, its overall ability to act as a XE via the non-genomic (or otherwise) has been poorly explored, but is the central topic of study in Chapter 3.

Elimination or metabolism of XEs into inactive (or less active), water-soluble compounds occurs through oxidative metabolism (Phase I) or conjugative enzymatic reactions (Phase II). Phase I oxidative metabolism of estrogens and XEs is in large part catalyzed by the NADPH-dependent cytochrome P450 family of enzymes (Zhu and Conney, 1998). Depending on the compound and P450 enzymes involved, this pathway may result in wide variety of reactions such as epoxidation or hydroxylation (Zhu and Conney, 1998). Certain XEs are known inducers of specific P450s, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 3-Methylcholanthrene (3-MC), and poly-brominated compounds, among others (Swedenborg et al. 2008). Other compounds such as BPA are dominated by Phase II conjugation reactions such as glucuronidation and sulfonation (Doerge and Fisher, 2010). These reactions are catalyzed by uridine diphosphate (UDP) glucuronosyltransferase (UGT) or sulfotransferase (SULT) enzymes thus converting BPA into a charged, water-soluble compound, unable to bind to the iER (Reed et al, 2005; Tukey and Strassburg, 2000; Mathews et al. 2001). The signaling activities of Phase II BPA conjugates via the non-genomic pathway are currently unknown and will be examined in Chapter 5.

Pesticides/herbicides are perhaps the most studied of this class of chemicals in mixtures, as multiple remedies are often applied against different insect and weed categories simultaneously for efficiency (Hayes et al. 2006; Yang et al. 2012), and increased regulatory constraints have demanded more scrutiny

(Reffstrup et al. 2010). However, very few studies have evaluated the low-dose non-genomic estrogenic effects of this class of compounds when administered either alone or in mixtures (Watson et al. 2007). With increased awareness of the need to study chemical mixtures' actions via more recently revealed estrogenic signaling mechanisms, an increasing number of studies have emerged, which we will discuss in the following section.

1.4.0 Non-Genomic Actions of Estrogen/Xenoestrogen Mixtures

Various approaches for evaluating chemical mixtures have been proposed by the scientific community (Reffstrup et al. 2010); however, there is no internationally agreed-upon procedure. The proposed approaches fall within two general categories, the whole-mixture (evaluation as though mixtures are single entities) and the component-based (evaluating individual chemicals in a mixture to estimate response) approaches (ATSDR 2011; Boobis et al. 2011; Teuschler et al. 2002b; US EPA 2000). Whole-mixture approaches can be impractical due to the multiple interactions that can potentially occur in real-world mixtures, some of which do not necessarily occur via a common mode of action by structurally similar compounds. Furthermore, this approach does not identify which types of chemical interactions are responsible for additive, synergistic, or antagonistic effects. Most studies have thus resorted to using the component-based approach, which requires information on each individual component within the mixture (Boobis et al. 2011). The component-based approach operates on the

calculated sum from either of the following methods: (1) concentration or dose addition method, which assumes that mixture components act on a similar target and therefore elicit a common response; and (2) the response addition method, which assumes that components act on different targets (the overall response is calculated from individual components) (Reffstrup et al. 2010; US EPA 2000). The latter method is not commonly used for XE mixtures.

The overall goal of every method is to establish principles of how chemicals behave based on structure and mode of action. Once enough examples are processed and the model optimized, then predictions for unknown mixtures should be possible. In a review, Kortenkamp extensively discussed mixture effects of several classes of EDCs (i.e., estrogenic, anti-androgenic, and thyroid-disrupting agents) (Kortenkamp 2007), focusing primarily on their genomic and functional responses. Below, we will briefly focus on the few existing non-genomic studies of synthesized estrogen mixtures.

Jeng and Watson studied the phospho-activation of MAPK (pERK) upon exposure to binary mixtures of endogenous estrogens (E_1, E_2, and E_3) at single physiologic (nM) concentrations with increasing (10^{-15}–10^{-7} M) concentrations of alkylphenol compounds in the $GH_3/B_6/F_{10}$ pituitary cell line (Jeng and Watson 2011). Individual compounds caused non-monotonic dose-responses, but with varying weak, moderate or strong response levels compared to E_2. The

composite responses were not additive, and often showed attenuation at the higher concentrations. The degree of attenuation was based on the response magnitude and potency of the paired xenoestrogen. The stronger the XE's activating response, the more it was able to attenuate the physiologic estrogenic response.

When assessing the effects of XEs on dopamine efflux through its transporter in PC12 cells, Alyea and Watson also found that 10^{-14}–10^{-8} M DDE caused a weak efflux as a lone compound, but in a binary mixture with 10^{-9} M E_2 it additively enhanced dopamine efflux. BPA in contrast evoked a strong efflux response on its own, but when mixed with 10^{-9} M E_2 it inhibited efflux (Alyea and Watson 2009a).

The overall pattern observed in these two studies was that when a compound with a weak response is paired with a physiologic estrogen, the response is enhanced. But, when a compound elicits a potent estrogenic effect, then it inhibits the paired physiologic estrogen's response. This progression is summarized graphically in Figure 3. In a very recent tertiary mixture study, we have observed further inhibition of responses to the physiologic estrogen E_2 by two added XEs However, the same mixture resulted in a synergistic positive response for pJNK (Viñas and Watson, unpublished); hence, when assessing non-genomic pathways one has to take into consideration the variety of signaling

responses, and probably the interactive nature of signaling “webs”. Response inhibitions by combinations of estrogens may be governed by cellular protective mechanisms against combined hormone overstimulation. Overstimulation can be wasteful and even dangerous when the enhanced function (such as peptide release or cell proliferation, for example) can lead to diseases like cancer.

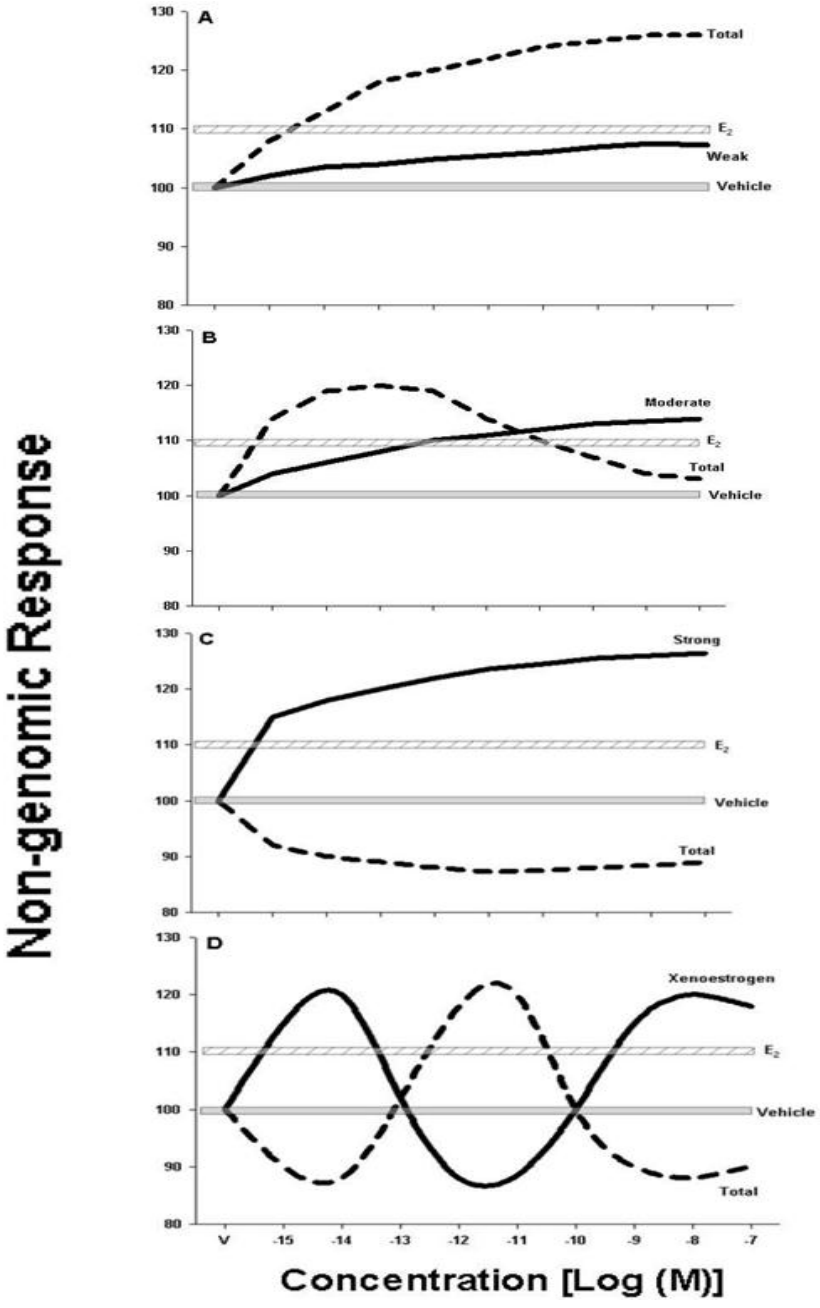

Figure 3. Working model of xenoestrogen (XE) alteration of physiologic estrogen non-genomic response effects. XEs of increasing dose were used to challenge the responses of the physiologic estrogen estradiol (10^{-9} M E_2). These combinations examples are mainly summarized from (Jeng and Watson 2011; Kochukov et al. 2009). In each case the vehicle control (V) and 10^{-9} M E_2 responses are shown by horizontal bars. The response to an XE alone is shown by a solid line, and the combination of 10^{-9} M E_2 plus the XE is shown by a dashed line. The types of combination responses are: (A) A weak XE enhances the physiologic estrogen E_2 response; (B) A moderate XE response enhances the E_2 response at low concentrations, and inhibits it at higher concentrations; (C) The strongest XE inhibits the E_2 response at all concentrations, with increasing inhibition as the XE concentration increases; and (D) If the XE exhibits a fluctuating non-monotonic estrogenic response, the effect on the E_2 response also fluctuates, in line with cases A–C above. These idealized data summarize what we have seen in combinations using a variety of XEs. Depending on the estrogenic potency of the XE, when paired with a physiologic estrogen, an inverse relationship in responses occurs (enhancement or attenuation).

Interestingly, not all signaling pathways culminating in different functional responses may behave in the same fashion. When another type of response (PRL release) was monitored under the same (binary mixture) circumstances, BPA's strong pERK response when present by itself did not correlate with a strong PRL secretion. However, PRL release did decrease when BPA was paired with either E_2 or E_1 (but not the weaker E_3) in pituitary cells (Jeng et al. 2010b). This means that one has to study sufficient examples of compounds over a wide range of times points and concentrations, assessed for a spectrum of different signals and functional endpoints. It will take an adequately representative set of such data to finally hone our predictive principles. In addition, for some complex responses such as cell proliferation, there will undoubtedly be both genomic and

non-genomic contributory components to consider, as well as cross-talk between signaling pathways.

1.5.0 Objectives of Dissertation Project

Due to public health concerns and the ever increasing regulatory mandates imposed on current chemicals, development of “greener” chemical replacements to meet those new standards is of key importance. The work found within this dissertation is built upon the premise of developing better screening methodologies for creating safer chemical ingredients before they are used in the manufacturing of consumer products. As a consequence, this project first focuses on assessing the estrogenic potential of a chemical replacement for BPA via the rapid non-genomic pathway, 4,4’- dihydroxydiphenyl sulphone, commonly known as bisphenol S (BPS). Therefore, we first characterized the ability of BPS to initiate non-genomic responses, alone and together with E_2, and correlated those signaling pathways to downstream cellular functions – proliferation, caspase activation, and PRL release (Chapter 3). We then compared the estrogenic effects of BPS to those of BPA and NP individually, and assessed their effects as mixtures on E_2-induced non-genomic signaling and associated functional responses (Chapter 4). In addition, we ventured to expand the usefulness of our in-vitro screening assays by developing an automated high-throughput system. As our test compounds for this newly developed system, we used poly-chlorinated conjugates of BPA (mono-, di-, and tri- chlorinated BPA)

and its phase II metabolites (glucuronide and sulfate), as little is known about their ability to induce non-genomic activity (Chapter 5).

Chapter 2

Methodology

Materials and methodology are consistently used for studies in chapters 3 and 4 with slight modifications in chapter 5 which will include the use of the BIOMEK FX^P Automation Workstation to carry out in- vitro studies. A full description of the materials and methods used for non-genomic assessment of BPA-chlorinated and phase II conjugated compounds will be included separately in chapter 5.

2.1.0 Cells and Culture Conditions

The $GH_3/B_6/F_{10}$ rat prolactinoma cell line was selected on the basis of its naturally high expression of mERα (Bulayeva et al. 2004; Pappas et al. 1994; Pappas et al. 1995a). Cells were routinely sub-cultured with phenol red-free Dulbecco's Modification of Eagle's Medium (DMEM, high glucose) (Mediatech, Herdon,VA) containing 12.5% horse serum (Gibco BRL, Grand Island, NY) and defined supplemented calf and fetal serum (Thermo Fisher, Waltham, MA) at 2.5% and 1.5%, respectively. Cells of passages 10-20 were used for these experiments.

2.2.0 Quantitative ERK and JNK Phosphorylation Assays

A fixed cell-based immunoassay was employed to quantify phospho-activation of ERK (pERK) and JNK (pJNK), as previously developed and described in detail

(Bulayeva and Watson 2004). Cells (10^{e4}/well) were plated in 96-well plates (Corning Incorporated, Corning, NY) and allowed to attach for 24hrs. The cells were then cultured in DMEM containing 1% charcoal-stripped (4X) serum for 48hrs to deprive cells of serum hormones. Medium was then removed and cells were exposed to individual XEs alone or as mixtures with a physiologic level of E_2 (10^{-9}M) (all chemicals were acquired from Sigma-Aldrich, St. Louis, MO) to assess time- (0-60min) and concentration-dependent (10^{-15}-10^{-7}M) changes (at 5min). Both the short time points and range in concentrations chosen were based upon recently published studies from our group (Jeng et al. 2009; Jeng et al. 2010b; Jeng and Watson 2011; Kochukov et al. 2009). The short time points ensure that we are indeed observing a rapid non-genomic activation of ERK and JNK without genomic interferences, while the concentrations are reflective of levels found in the environment. Test compounds were dissolved in ethanol then diluted in DMEM containing 1% charcoal-stripped serum. Vehicle control (V) was 0.001% ethanol in DMEM. To stop mER-initiated signaling cells were fixed with a 2% paraformaldehyde/0.2% picric acid solution (Fisher Scientific, Pittsburgh, PA) and incubated at 4°C for 48 hrs. Cells were then incubated with phosphate–buffered saline (PBS) containing 0.2% fish gelatin and 0.1% Triton X-100 (Sigma-Aldrich) for 1hr at room temperature (RT), followed with overnight incubation at 4°C with primary antibodies (Abs) against pERK or pJNK (Cell Signaling Technology, Beverly, MA) (1:500 in PBS/0.2% fish gelatin/0.1% TritonX-100).Cells were then washed with PBS (3X) before biotin-conjugated Ab

(Vector Labs, Burlingame, CA) was applied and incubated (1hr) at RT (1:500 in PBS/0.2% fish gelatin). The cells were again washed in PBS (3X) and incubated with Vectastain ABC-AP solution (Vector Labs) (50µL/well) for 1hr at RT, followed by Vectastain alkaline phosphatase substrate (pNpp solution) (50µL/well). Plates were incubated in the dark for 30min at 37°C. The signal for the product of para-nitrophenol phosphate (pNpp) (Thermo Scientific, Rockford, IL) breakdown to pNp was read at A405 in a model 1420 Wallac microplate reader (Perkin Elmer, Boston, MA). The pNp signal was normalized to cell number, determined by the crystal violet (CV) assay at A590, described in; (Campbell et al. 2002).

2.3.0 Receptor Inhibitor Studies

Prototypic selective receptor antagonists were used to determine the involvement of the three different types of mERs (ERα, ERβ, and GPR30) in ERK activation upon exposure to BPS [10^{-14}M]. Receptor involvement in responses to BPA and NP have been determined previously (Bulayeva et al. 2005; Bulayeva and Watson 2004; Jeng and Watson 2011). Cells (10^4/well) were plated in 96-well plates, allowed to attach for 24hrs and then treated with DMEM containing 1% charcoal-stripped (4X) serum for 48hrs to deprive cells of serum hormones. Media were then removed and cells pre-incubated for 1hr at 37°C with media (50µl) containing antagonists for ERα ([MPP]-1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride), ERβ ([PHTTP]- 4-

[2-Phenyl-5,7-bis(trifluoromethyl) pyrazolo[1,5-a]pyrimidin-3-yl]phenol), and GPER/GPR30 ([G15]- (3aS*,4R*,9bR*)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinolone); all compounds were acquired from Tocris Bioscience (Bristol, UK) and target both membrane and intracellular versions of estrogen receptors. DMEM media (50µl) containing [10-14M] BPS was then applied to cells for a period of 5 min followed by fixation with a 2% paraformaldehyde/0.2% picric acid solution and the quantitative ERK phosphorylation assays were performed as described above.

2.4.0 Effects on Cell Numbers

We have previously described this method for estimating cell numbers in detail (Jeng and Watson 2009). Briefly, sub-confluent cells were seeded into 96-well plates that had been coated with poly-D-lysine (5000 cells/well) and allowed to attach overnight. Plating medium was then replaced with DMEM containing 1% 4X charcoal-stripped serum for 48 hrs, then treated with media containing increasing concentrations of individual XEs (10^{-15}-10^{-7}M) or mixtures of BPS + 10^{-9}M E_2, BPS + BPA + 10^{-9}M E_2, and BPS + BPA + NP + 10^{-9}M E_2 . After 3 days, cells were fixed (2% paraformaldehyde/0.1% glutaraldehyde in PBS; 50µl/well) and cell numbers were assessed by CV assay to compare the proliferative effects of XE-mixtures at different concentrations.

2.5.0 Determination of Caspase Activities

Assessment of caspase-8 and -9 activities were performed as previously described (Jeng and Watson 2009). Sub-confluent $GH_3/B_6/F_{10}$ cells were seeded into 96-well plates ($5x10^3$/well) and allowed to attach overnight. Treatments began the next day; cells were exposed for 24hr period with 1 nM E_2, 10^{-14}M and 10^{-8}M individual XEs, and mixtures in DMEM-1% 4X charcoal-stripped serum; treatment medium was suctioned off and cells lysed with 50µL lysis buffer (10mM Hepes; 2mM EDTA; 0.1% CHAPS; pH 7.4) to which 1mM DTT (1:2000, freshly prepared, Sigma-Aldrich) was added. Plates were then stored at -70˚C until assay. Staurosporine [500nM] (Sigma-Aldrich) dissolved in DMSO was used as a positive control for activation of caspase-8 and -9. The released fluorescent product 7-amino-4-trifluoromethylcoumarin (AFC) was read using a Flexstation 3 spectrofluorometer (Molecular Devices, Sunnyvale, CA) at 400nm excitation, and 505nm emission wavelengths.

2.6.0 Prolactin Release

These assay conditions were based on our previous studies (Kochukov et al. 2009; Wozniak et al. 2005). Cells ($0.5–0.7\times 10^6$) were plated into poly-d-lysine–coated 6-well plates overnight and hormone-deprived in DMEM-1% 4X charcoal-stripped serum for 48 hrs. Cells were then pre-incubated for 30 min in DMEM/0.1% BSA and exposed for 1min to different concentrations of individual XEs alone (10^{-15} -10^{-7}M), or as mixtures with 10^{-9}M E_2 , then centrifuged at 4°C,

350×g for 5 min. The supernatant was collected and stored at –20°C until radioimmunoassay (RIA) for PRL. Cells were then fixed with 1ml of 2% paraformaldehyde/0.1% glutaraldehyde in PBS, and cell numbers determined via the CV assay. Concentrations of PRL secreted into the media were determined using components of the rat PRL RIA kit from the National Institute of Diabetes and Digestive and Kidney Disease and the National Hormone and Pituitary Program (http://www.humc.edu/hormones/; Baltimore, MD). We combined 100µL of cold standard (rat PRL-RP-3) or unknown sample with 500µL rPRL-s-9 antiserum [final dilution of 1:437,500 in RIA buffer containing 80% phosphate-buffered saline (PBS), 20% DMEM, and 2% normal rabbit serum] and 200µL of ^{125}I-labeled rat PRL (Perkin Elmer, Wellesley, MA; using 15,000 counts/tube diluted in RIA buffer). The samples were then incubated and shaken overnight at 4°C. Anti-rabbit IgG was then added (200µL of 1:9 final dilution in RIA buffer) and the samples incubated and shaken for 2 hr at RT. Polyethylene glycol (PEG) solution (1ml; 1.2 M PEG, 50 mM Tris, pH 8.6) was added, incubated and then shaken at room temperature for 15min. The samples were centrifuged at 4,000×*g* for 10 min at 4°C, the supernatants decanted, and the pellets counted in a Wizard 1470 Gamma Counter (Perkin Elmer). PRL concentrations were calculated and normalized to CV values representing cell number.

2.7.0 Statistical Analysis

Statistical analyses were performed using Sigmaplot version 12.3 (Systat Software Inc). One-way analysis of variance (ANOVA) was applied to the dose- and time-dependent studies to assess the statistical significance of mean values produced by varying exposures. A Holm-Sidak comparison against vehicle control or against E_2 treatment was used to evaluate significance. The overall α level selected for the statistical analysis was 0.05.

Chapter 3[2]

Bisphenol-S Disrupts Estradiol-induced Non-genomic Signaling in Rat Pituitary Cell Line: Effects on Cell Functions

3.1.0 Introduction

Bisphenol A (BPA), a leachable monomer of polymerized polycarbonate plastics, has been used commercially since 1957 (Bisphenol A Global Industry Group 2002), and is also found in food can liners and coatings on thermal cashier receipt paper (Zalko D et al. 2011). Humans are exposed to BPA primarily from food and H_2O contaminated by manufactured products, particularly during the heating of plastic containers (Kubwabo et al. 2009). According to the National Health and Nutrition Examination Survey (NHANES), BPA levels range from 0.4 - 149 µg/L (1.8 - 660nM) in urine samples from 92.6% of U. S. residents ≥ 6 years of age (Calafat et al. 2008). An extensive review of studies that tested blood samples from a variety of human specimens, including healthy adults and pregnant women, concluded that levels of unconjugated BPA were in a range of 0.5-10ng/ml (2-45pM) (Vandenberg LN et al. 2012b).

Exposure to BPA in humans has been implicated in the development of chronic diseases, including diabetes, asthma and cancer (Alonso-Magdalena et al. 2010; Li et al. 2011; Midoro-Horiuti et al. 2010; Watson et al. 2010), while also causing

[2] Chapter 3 taken from: Vinas R, Watson CS. 2013. Bisphenol S Disrupts Estradiol-Induced Nongenomic Signaling in a Rat Pituitary Cell Line: Effects on Cell Functions. Environ Health Perspect; doi:10.1289/ehp.1205826 [Online 17 January 2013].

decreased fecundity in wildlife via disrupted spermatogenesis and ovulation (Li et al. 2011; Oehlmann et al. 2009; Sohoni et al. 2001; Zhou et al. 2011). The European Food Safety Authority has set a tolerable daily intake (TDI) for BPA of 0.05 mg/kg body weight/day, a value accepted by many regulatory agencies, including the U. S. Environmental Protection Agency (EPA 1993). Due to increased concern over the safety of BPA, Health Canada (Health Canada 2009), and more recently the European Union (European Commission 2011) and the US FDA (FDA 2012) have banned its use in plastic feeding bottles for infants.

More stringent global regulations on BPA production and use have led to the development of alternative; more heat-stable bisphenol compounds (Gallar-Ayala H et al. 2011; Liao et al. 2012a; Liao et al. 2012b). Among these alternative compounds is 4,4'-dihydroxydiphenyl sulphone (bisphenol S (BPS)) (Figure 4). Because of the novel nature of BPS, at the time of writing this manuscript in-vivo toxicity studies have not been reported, nor has the ability of BPS to disrupt the actions of physiologic estrogens been explored. Several studies have tested the effects of BPS via genomic mechanisms at extremely high concentrations, unlikely to be leached from BPS-containing products. At concentrations as high as 0.1 to 1 mM BPS showed only slight estrogenic activity in a 4hr recombinant two-hybrid yeast test system (Hashimoto et al. 2001; Hashimoto and Nakamura 2000). Another such study (Chen et al. 2002) showed that 40μM BPS had 15-fold lower genomic estrogenic activity than BPA. However, BPS was equipotent to

BPA in an ERE-driven green fluorescent protein (GFP) expression system in MCF7 breast cancer cells (Kuruto-Niwa et al. 2005a). Discrepancies between these studies were attributed to species (yeast vs. mammalian) differences (Kuruto-Niwa et al. 2005a). However, as tissues frequently differ in responses, this could also be the case. No studies prior to ours have examined BPS for non-genomic mechanisms of action, or at the low concentration ranges likely to be present in foods, environmental samples, or humans.

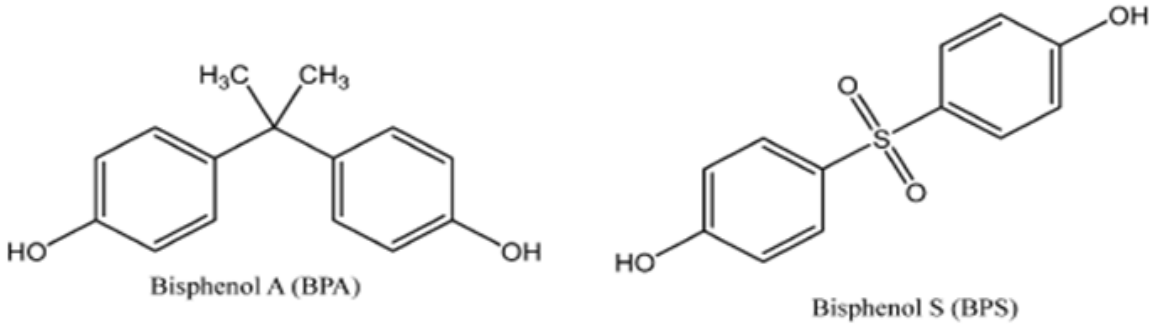

Figure 4. Comparison between chemical structures of BPA and BPS.

We know that BPA can potently interfere with the actions of endogenous estrogens in pituitary cells via several types of non-genomic signaling [e.g. mitogen-activated protein kinases (MAPKs), Ca^{2+} influx] (Kochukov et al. 2009; Wozniak et al. 2005) acting via membrane estrogen receptors (mERα, mERβ, GPER/GPR30), and thus alter functional responses [cell proliferation, prolactin (PRL) release, and transporter function] at picomolar- and sub-picomolar concentrations (Alyea and Watson 2009; Jeng et al. 2010; Jeng and Watson

2011; Wozniak et al. 2005). Physiologic estrogen actions are disrupted by BPA and other XEs for both timing and magnitude of responses, enhancing or inhibiting, depending upon their concentrations (Jeng et al. 2010; Jeng and Watson 2011). Introduction of a new active bisphenol compound (BPS) into the environment poses an unknown threat for signaling and functional disruptions.

Therefore, our present study examined the effects of BPS on non-genomic signaling at concentrations that will allow full assessment of potency given the non-monotonic concentration responses that we expect based on our previous studies of BPA (Jeng et al. 2010; Jeng and Watson 2011). To simulate likely exposures, we tested BPS both alone and in combination with the physiologic estrogen estradiol (E_2). With the use of prototypic receptor inhibitors we sought to identify the predominant mER through which BPS initiates non-genomic signaling. Effects of BPS on associated downstream (from MAPKs) functional endpoints were also examined, including cell number changes (proliferation or decline), and caspase activations or inhibitions occurring via external stimuli (caspase 8) vs. internal stimuli (caspase 9). Together these mechanisms can contribute to effects on cell number. Finally we examined the effect of BPS on peptide hormone release (PRL).These measurements employ high-throughput plate immunoassays to facilitate quantitative comparisons between responses to different compounds and mixtures.

3.2.0 Results

Exposure to BPS caused ERK activation in $GH_3/B_6/F_{10}$ cells at 5 min (Figure 5A) at concentrations similar to that caused by E_2 (Jeng et al. 2010; Jeng and Watson 2011). The lowest tested BPS concentrations evoked a higher pERK response than did 10^{-9}M E_2; the response steadily decreased with increasing BPS, indicating a non-monotonic dose-response (Vandenberg et al. 2012). The combination of increasing concentrations of BPS with constant 10^{-9}M E_2 caused a lower pERK activity than did BPS alone, and was significantly lower than the nM E_2 response at the highest (10-100 nM) concentrations. In contrast, BPS did not produce significant pJNK activation (Figure 5B), but instead caused deactivation significantly below vehicle levels at the highest (10^{-7}M) concentration.

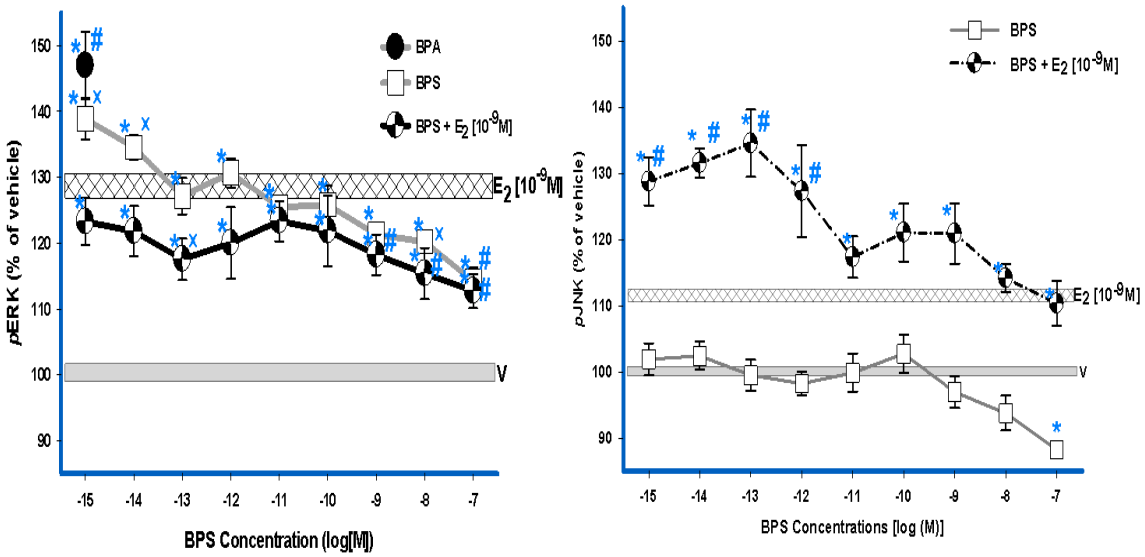

Figure 5. Concentration –responses of phosphorylated-ERK (pERK) and -JNK (pJNK) responses to a range of BPS concentrations, single concentrations of E_2 or BPA, and 1nM E_2-BPS concentration range combinations. pERK (A) and pJNK (B) pNp signals were measured by plate immunoassay after a 5 min exposure and normalized to cell number estimates. Absolute absorbance values (normalized to cell number) of the vehicle control are as follows: ERK (mean =0.834); JNK (mean =0.395).The width of vehicle and E_2 [10^{-9}M] bars represent means ± S.E. (n=24 over 3 experiments) * = $p<0.05$ when compared to vehicle (V). # =$p< 0.05$ when compared to 10^{-9}M E_2. X = $p<0.05$ when compared to E using Student's t-Test. In panel B error bars for 10^{-7}M BPS (± 1.2%) are about the size of the symbol and therefore are difficult to see.

However, when BPS and E_2 were administered together, JNK was very strongly activated over the level seen with E_2 alone, and again featured a non-monotonic dose-response curve with the lowest concentrations evoking the largest responses. We also examined the time dependence of these responses at optimal response concentrations (10^{-14}M BPS, 10^{-9}M E_2; Figures 6A and 6B). E_2 produced a typical oscillating two-peak ERK response, with the first peak within 5 min, followed by a second peak at 30 min (Bulayeva et al. 2004; Bulayeva and Watson 2004; Jeng et al. 2009; Jeng and Watson 2011). BPS phospho-activated ERK within 2.5 minutes but did not show significant oscillation. BPS-and E_2-induced responses were not significantly different from each other.

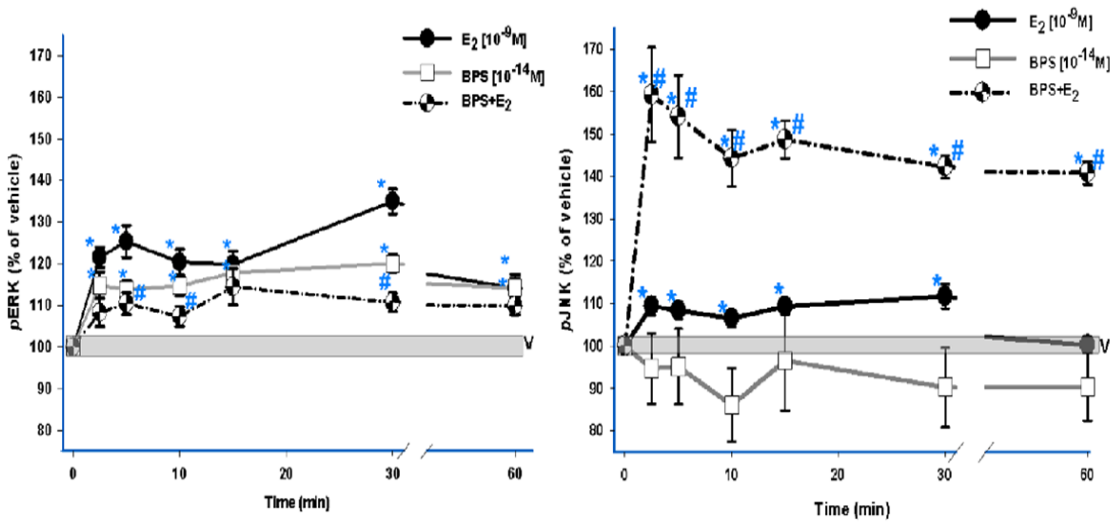

Figure 6. BPS disrupts E_2-induced time-dependent phosphorylations of (A) ERK and (B) JNK. A pNp signal for phosphorylated MAPKs was normalized to the CV value for cell number and expressed as a percentage of vehicle (V)-treated controls. Absolute absorbance values (normalized to cell number estimates) of the vehicle control were: ERK (mean =0.685); JNK (mean =0.395).The width of the vehicle bar represents the mean ± S.E. (n=24 over 3 experiments) * = p< 0.05 when compared to V. # =p< 0.05 when compared to 10^{-9}M E_2.

The combination of 10^{-14}M BPS and 10^{-9}M E_2 showed a slightly oscillating pattern, though differences between stimulated points were not significant. We have seen re-phasing of responses due to XE combinations with E_2 previously (Jeng et al. 2009; Jeng et al. 2010; Jeng and Watson 2011; Kochukov et al. 2009). Therefore, even at this very low concentration (10^{-14}M), BPS was capable of disrupting the timing of the response to a physiologic estrogen. Even though 10^{-14}M BPS could not by itself activate JNK at any time point tested, its combination with E_2 dramatically enhanced the early and sustained pJNK response to E_2 (Figure 6B).

A prototypic chemical inhibitor for ERα at its most selective concentration (10^{-8}M MMP) was the most effective antagonist of E_2 and BPS-induced responses (Figure 7). In comparison, inhibitors for ERβ (10^{-7}M) and GPER/GPR30 (10^{-7}M) were much less effective in reducing the phospho-activation of ERK by E_2 and BPS. Therefore, mERα is the predominant receptor that mediates this non-genomic response to BPS.

After a 3 day exposure, 10^{-9}M E_2 and BPS had similar effects on cell proliferation, causing a non-monotonic stimulation as we observed previously with E_2 (Jeng and Watson 2009; Kochukov et al. 2009). The combination of BPS and E_2 did not stimulate cell proliferation, but instead suppressed cell numbers far below those exposed to vehicle (Figure 8).

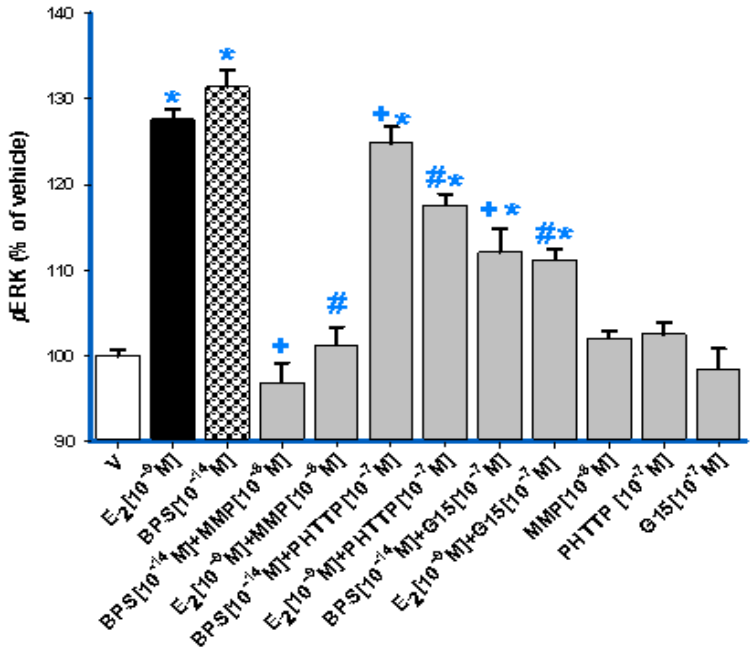

Figure 7. Receptor subtype-selective inhibition of BPS-induced ERK phospho-activation. Receptor selective inhibitors used were MMP (10^{-8}M) for ERα, PHTTP (10^{-7}M) for ERß, and G15 (10^{-7}M) for GPR30. BPS (10^{-14}M) and the positive control E_2 (10^{-9}M) were then applied to cells for 5 min, followed by plate immunoassay for ERK. Values are expressed as percentage of vehicle means ± S.E.; n=16 over two experiments; the vehicle control absorbance mean value for pNp product, normalized to cell number estimates, was 0.743. * = statistical significance compared to vehicle ($p<0.05$). # = significant change compared to estradiol. + = statistical significance compared to 10^{-14}M BPS.

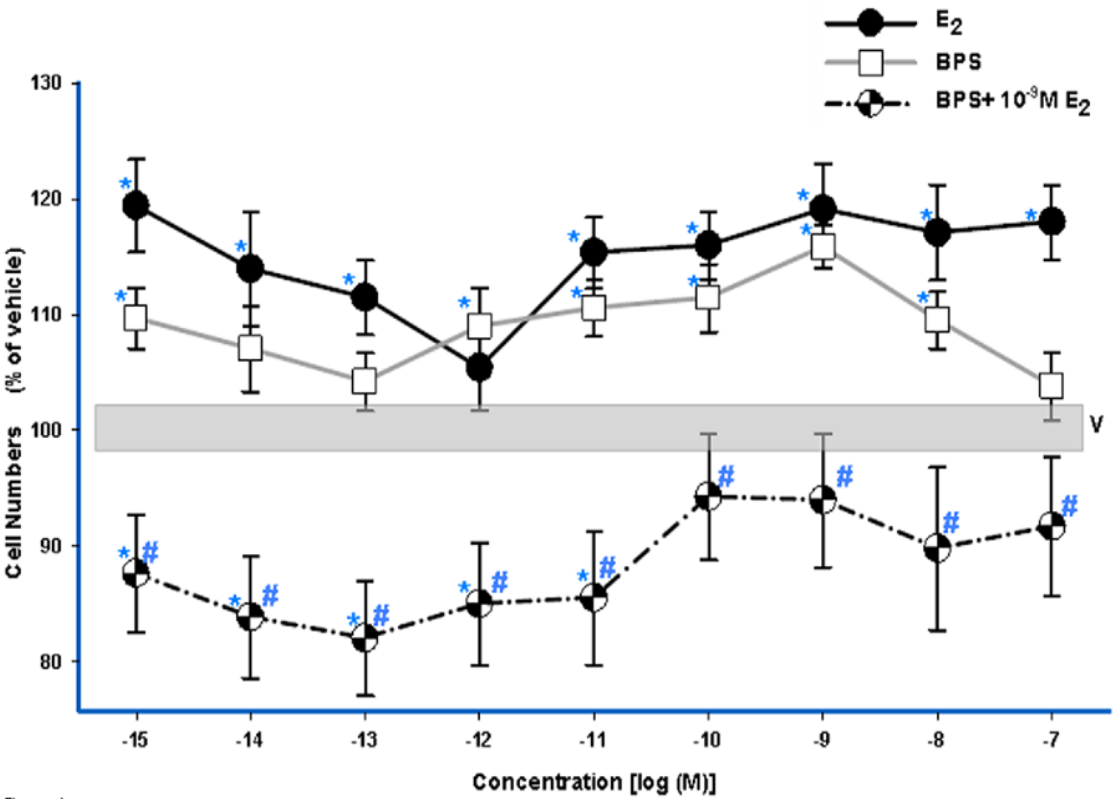

Figure 8. BPS induces cell proliferation. Increasing concentrations of BPS or E_2 alone, or BPS in combination with a physiologically relevant concentration of E_2 (10^{-9}M) were applied for a 3 day period, and cell number was estimated by the CV assay (n=24 over 3 experiments). Absolute absorbance values of the vehicle (V) control were mean = 0.299. The width of vehicle bar represents the means ± S.E.* = $p< 0.05$ when compared to vehicle. # = $p< 0.05$ when compared to 10^{-9}M E_2.

As decreases in cell number can be caused by apoptosis, we assayed caspase 8 and 9 to determine if the extrinsic or intrinsic apoptotic pathways were activated. Caspase 8 was activated by both BPS and its combination with E_2 [10^{-9}M] at all-time points tested (4 – 24hrs), regardless of the concentration used (Figure 9A). On the other hand, caspase 9 was significantly activated only at 24hrs, and by low concentrations of BPS (10^{-14}M) or its combination with E_2 (Figure 9B). The positive control (staurosporine) was active at all times and on all caspases, as expected. Interestingly, nM E_2 by itself suppressed caspase 9 activity below vehicle controls at all-time points, while inhibition below vehicle levels was only seen at the 8hr time point for caspase 8, as we had seen previously (Jeng and Watson 2009).

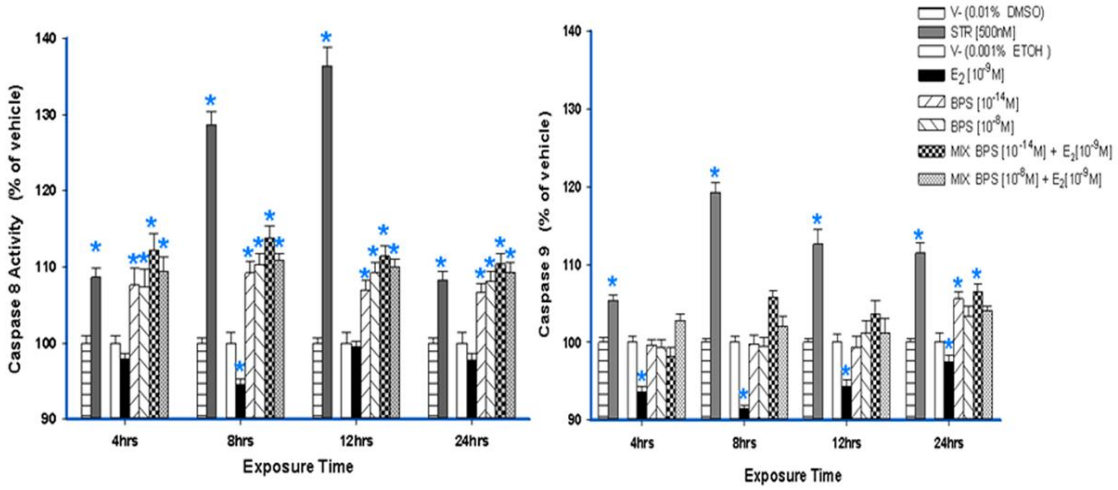

Figure 9. Activation of caspase 8 and 9 by BPS and E_2. The time dependence of caspase 8 (A) and 9 (B) activations.were measured by the release of fluorogenic substrates (AFC) expressed as percentage of vehicle (V)-treated controls. The absolute RFU values for V-ETOH were: Caspase 8 - 4hrs (63); 8hr (60); 12hrs (68); 24hrs (70); Caspase 9 – 4hrs (70); 8hr (78hrs); 12hrs (63); 24

hrs (76). MIX indicates a mixture of compounds. Staurosporine (STR) was used as a positive control for induction of caspase activities compared to its own DMSO V control (n=24 over 3 experiments). Error bars are means ± S.E. * =p<0.05 when compared to V.

The $GH_3/B_6/F_{10}$ cell line secretes PRL in response to E_2 and a variety of estrogenic compounds, thus making this model an excellent tool for evaluating functional responses to estrogens (Dufy et al. 1979; Jeng et al. 2009; Jeng et al. 2010; Kochukov et al. 2009; Pappas et al. 1995b; Wozniak et al. 2005). After a typical exposure time of 1 min, BPS could not significantly increase PRL secretion, as E_2 did (Figure 10).

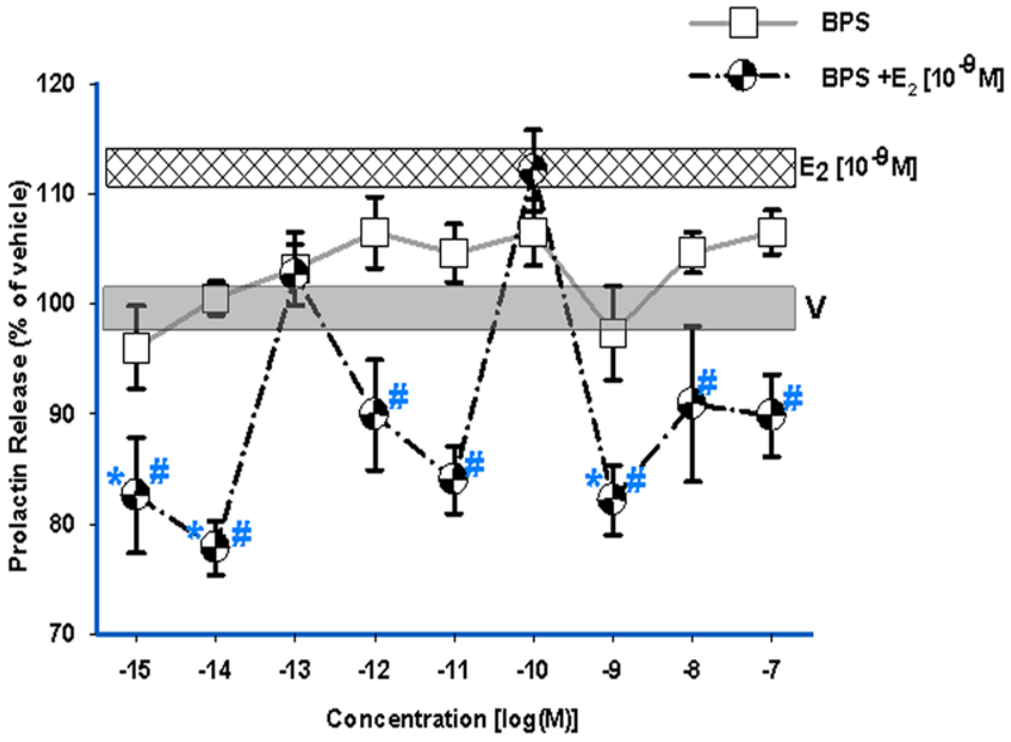

Figure 10. BPS alters E_2-induced PRL secretion. The amount of PRL secreted for each well (counts per minute) was normalized to the CV value for cell

number, and expressed as a percentage of vehicle (V)-treated controls. The absolute value (normalized to cell number estimates) of the vehicle control (V-ETOH) was 466. Error bars are means ± S.E. (n=24 over 3 experiments) * = p< 0.05 when compared to vehicle. # =p<0.05 when compared to 10^{-9}M E_2.

When BPS was added together with nM E_2, E_2-induced PRL release was severely inhibited in a non-monotonic pattern, well below that with 10^{-9}M E_2, and at most concentrations well below that with vehicle. Though the PRL release caused by the mixture concentrations at 10^{-10}M were not statistically different from the level of release caused by nM E_2 alone, this response was also not statistically different from vehicle due to errors around that measurement.

3.3.0 Discussion

Increased scrutiny and concern by government agencies and environmental advocacy groups led to the development of potential chemical replacements for BPA, such as BPS. Though less likely to leach from plastic containers with heat and sunlight, it does still escape the polymer in small quantities under normal use (Kuruto-Niwa et al. 2005b; Simoneau et al. 2011; Vinas et al. 2010). Our results show that BPS is active at femtomolar to picomolar concentrations, and can alter a variety of E_2-induced non-genomic responses in pituitary cells, including pERK and pJNK signaling and functions (cell number, PRL release).

BPS had the same capability as E_2 for initiating the phospho-activation of ERK across concentrations and times (Jeng et al. 2009; Jeng et al. 2010; Jeng and Watson 2009; Jeng and Watson 2011; Kochukov et al. 2009; Wozniak et al. 2005) with the lower concentrations of BPS being the most effective. BPS was also found to be equipotent to BPA when examining the phospho-activation of ERK. Such non-monotonic dose-responses are controversial, and have been heavily examined lately (Vandenberg et al. 2012). The fluctuation of MAPK activities with concentration and time could involve several mechanisms (Conolly and Lutz 2004; Vandenberg et al. 2012; Watson et al. 2010; Weltje et al. 2005), including receptor desensitization due to overstimulation, activation of phosphatases, and simultaneous activation of multiple signaling pathways, thereby activating proteins at different rates (Vandenberg et al. 2012; Watson et al. 2011). MAPK down-regulation is critical for preventing adverse effects of extended pathway stimulation (Hunter 1995). In our mixture studies, attenuation of the ERK response perhaps protects the cell against unnecessary and perhaps dangerous estrogenic stimulation caused by the increased overall estrogenic concentration with two estrogenic compounds.

Non-genomic and functional actions initiated in this cell line were shown to be mediated predominantly by mERα. Previous studies using chemical inhibitors effective for both mERα and mERβ (ICI 187 634) also blocked ERK responses (Bulayeva et al. 2005; Bulayeva and Watson 2004). Additionally, in contrast to

the $GH_3/B_6/F_{10}$ cells used here, $GH_3/B_6/D_9$ pituitary cells expressing low mERα levels were unable to respond via E_2-induced activation of MAPK signaling (Bulayeva et al. 2005; Bulayeva and Watson 2004). Here our experiments with subtype-selective antagonists also demonstrated that mERα was the predominant membrane receptor mediating these responses, as we have seen previously (Alyea et al. 2008; Jeng and Watson 2011), though, as in our past studies, ERß and GPR30 also make contributions to this ERK response to estrogens.

Phospho-activation of ERK and JNK has been closely associated with opposing functional endpoints. For example, ERK signaling (RAF→MEK1,2→ERK1,2) is often associated with cell differentiation and growth, while JNK signaling is usually thought to accompany the initiation of apoptosis (Junttila et al. 2008; Meloche and Pouyssegur 2007; Nordstrom et al. 2009; Xia et al. 1995). Simultaneous phospho-activation of ERK and inactivation of JNK by BPS, as our data show, could simultaneously stimulate proliferation and inactivate cell death, magnifying the cell number increase (Junttila et al. 2008). Our BPS/E_2 mixture activated both ERK and JNK, perhaps correlating with a decline we saw in cell numbers if the balance of these two activities is important for the outcome. Earlier studies found that BPS alone is capable of inducing cell proliferation in the MCF-7 cell line (Hashimoto et al. 2001; Hashimoto and Nakamura 2000; Kuruto-Niwa et al. 2005a), but noted that BPS began to show cytotoxic effects at

concentrations above 10^{-4}M (well above the highest concentration we tested). Therefore, the proliferative/anti-proliferative responses caused by BPS can happen in multiple responsive tissues.

This is the first study that explores the ability of BPS to activate caspases. Early activation of caspase 8 (compared to 9) indicates that the extrinsic pathway, which involves extracellular stimuli acting on cell-surface receptors, is the primary apoptotic pathway. The reason for later and weaker activation of caspase 9 can be explained by crossover to that pathway via a lengthy process initiated by the cleavage of Bcl2-interacting protein (BID) in the caspase 8 pathway; this results in BID's translocation to mitochondria, where it causes later release of cytochrome *c* and subsequent activation of caspase 9 pathways (Kruidering and Evan 2000; Medema et al. 1997). We previously showed increased activation of caspase 8 in phytoestrogen-treated $GH_3/B_6/F_{10}$ cells after 24hr of treatment (Jeng and Watson 2009), but not activation of caspase 9.

Cell survival vs. death is determined by the balance of several cellular signaling responses, and the activation of capsases is only one of many factors. There are also discrepancies in the literature about the role of ERK and JNK activations in controlling cell numbers. Phospho-activation of ERK can, for example, lead to the activation of the anti-apoptotic protein Mcl-1 which binds to Bax protein, preventing its activation, and thus inhibiting apoptosis (McCubrey et al. 2007).

Activation of ERK has also been shown to inhibit caspase 9 upon phosphorylation (Allan et al. 2003; Allan and Clarke 2007; Allan and Clarke 2009), perhaps a mechanism promoting the protective effects we see with E_2 both here and in past studies (Jeng and Watson 2009). Phospho-activation of JNK can lead to activation of several pro-apoptotic proteins such Bax, caspase-3, cyclin D1, Fas, and interleukin 1 (Ip and Davis 1998). But JNK has also been linked to the activation of pro-survival pathways, with the final functional response dependent on the overall balance between ERK and JNK activities (Dhanasekaran and Reddy 2008; Sanchez-Perez et al. 1998). More examples of these conflicting outcomes will need to be studied to resolve the composite contributions of MAPKs to cell number control.

BPA and other XEs are potent inducers of PRL release (Jeng et al. 2009; Jeng et al. 2010; Kochukov et al. 2009; Wozniak et al. 2005); by contrast, BPS caused minimal PRL release on its own. However, BPS dramatically disrupted E_2-induced PRL release, as do other XEs. Disturbances in the timing or amount of PRL released can lead to a variety of physiologic complications including disruptions in electrolyte imbalance, growth and development, metabolic dysfunctions, behavioral disturbances, reproductive failure, or lactation failure. In all there are over 300 biological functions that PRL regulates (Bole-Feysot et al. 1998). The differences that we have observed between these two structurally

very similar bisphenol compounds warrant future examination of structure-activity relationships for these responses.

Using urine samples collected by the NHANES, total BPA concentrations across various demographic groups in the U.S. were reported with a geometric mean (GM) of 2.6µg/L (10nM) (Calafat et al. 2008). In comparison, a recent study determined the occurrence of BPS in humans in seven different countries, with the highest urinary GM concentrations in Japan, followed by the U.S. (Albany, NY) with 0.299ng/ml (1.2nM) (Liao et al. 2012a), a concentration still much higher than that used in our studies. Because past studies focused entirely on genomic mechanisms of BPS actions in which it was active only in the micro- to millimolar range, those effects would only be relevant to industrial accident types of exposures.

Our study is the first to demonstrate that the BPA-substitute BPS can induce rapid non-genomic signaling in estrogen-responsive pituitary cells at low (femtomolar-picomolar) concentrations. That BPS also interferes with physiologic E_2 signaling leading to several functional endpoints is a cause for concern. These findings highlight the need for efficient *in-vitro* screening methods to pretest possible substitutes for XEs before they are deployed in manufacturing. As more related compounds are tested, we can build an image of likely structural features

associated with risks in this class of chemicals, and perhaps guide future design away from these structures that can adversely affect human and animal health.

Chapter 4

Mixtures of Xenoestrogens Disrupt Estradiol-induced Non-Genomic Signaling and Functions in Pituitary Cells

4.1.0 Introduction

In the last chapter we demonstrated that BPS is potently estrogenic via non-genomic signaling pathways in the $GH_3/B_6/F_{10}$ rat pituitary cell line, including at the low-dose ranges likely to be present in food items and human fluids (Viñas and Watson 2012).This study also demonstrated that BPS can strongly interfere with the signaling actions of the endogenous estrogen, estradiol (E_2), at picomolar- and sub-picomolar concentrations, predominantly via membrane-bound estrogen receptor-α (mERα), leading to alterations in functional responses – cell proliferation and prolactin (PRL) release. Previous studies from our group also examined the estrogenic actions of BPA and NP via this rapid signaling pathway with similar consequences on functional endpoints (Alyea and Watson 2009; Jeng et al. 2010; Jeng and Watson 2011; Wozniak et al. 2005).

The aim of this study was to determine if mixtures of XE compounds could cause signaling alterations (i.e. antagonistic or additionally agonistic) on the E_2-induced non-genomic activity and correlated to functional cellular endpoints. To recreate likely real-world scenarios for exposure to mixtures, we compared each compound alone to a tertiary mixture (BPS +BPA + E_2), and a quaternary mixture

(BPS + BPA + NP + E_2) over wide concentration ranges of the XEs. We examined XE mixtures in combination with a physiologic level of E_2, as that is the way most organisms will experience them. In addition, we evaluated the effects of these mixtures on MAPK-associated downstream functional endpoints: cell proliferation; apoptosis (caspase 8 and 9 activity); and PRL secretion. Our medium-throughput quantitative plate assays make possible within-assay comparisons between responses to different compounds and their mixtures at a wide range of concentrations.

4.2.0 Results

4.2.1 Temporal changes in phospho-activation of MAPKs by BPS, BPA, and NP, and their combinations, during a 60 min exposure.

The time dependence of these responses was examined at optimal response concentrations (see Figure 11). E_2 produced a typical oscillating two-peak ERK response, with the first peak within 5 min, followed by a second peak at 30 min as we have observed previously (Bulayeva et al. 2004; Bulayeva and Watson 2004; Jeng et al. 2009; Jeng and Watson 2011). During the same 60 min time frame XEs generated temporal profiles different from E_2 (Figure 11A). The combination of 10^{-14}M XEs and 10^{-9}M E_2 (Figure 11A) caused a deviation from the E_2-induced temporal pattern, as well a decrease of the overall ERK response, as was also seen in the dose-dependent studies (Figure 11B). Similar deviations due to other XE combinations with E_2 have been previously documented (Jeng et

al. 2009; Jeng et al. 2010; Jeng and Watson 2011; Kochukov et al. 2009). Therefore, even at this very low concentration (10^{-14}M), XEs are capable of disrupting the timing of the response to a physiologic estrogen.

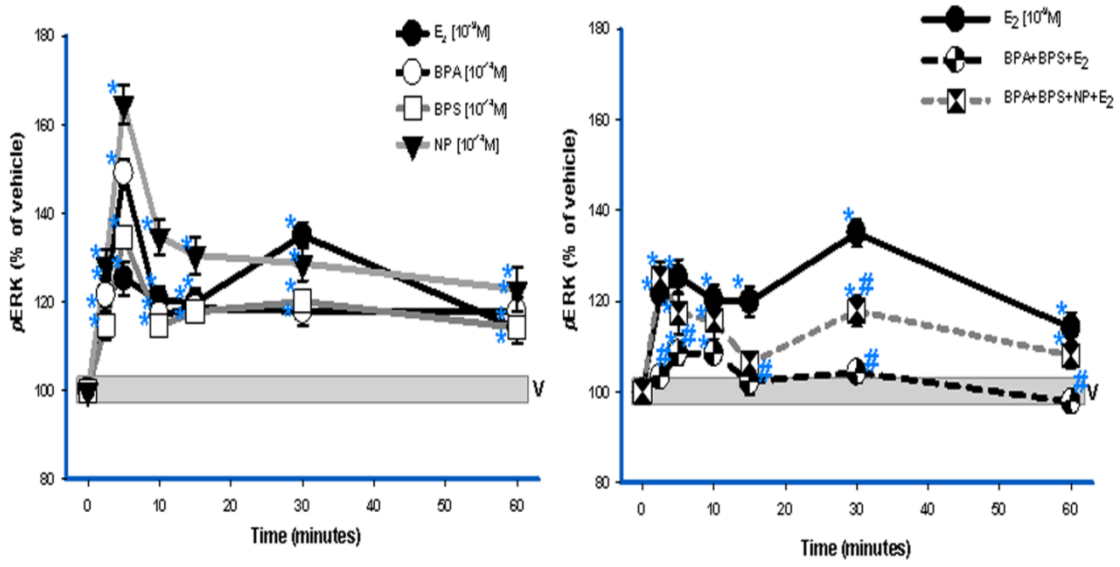

Figure 11. Timing of ERK activation by E_2, XEs, and XE/E_2 mixtures. Rat pituitary cells were exposed to BPS (10^{-14}M), BPA (10^{-14}M), NP (10^{-14}M) and/or E_2 (10^{-9}M) over a 60- min time course. Responses to individual XEs (A) and mixtures (B) were measured by plate immunoassay; the pNp signal generated for each well was normalized to cell number (measured by the CV assay). Values are expressed as percentage of vehicle (V)-treated controls. All error bars represent S.E M. The width of the vehicle bar represents a S.E. of ±1.2 (n=24 over 3 experiments). ***** = $p<0.05$ compared to vehicle (V); # =$p< 0.05$ compared to 10^{-9}M E_2.

Neither BPA nor BPS could maintain activation of JNK by themselves during the 60-min time course (Figure 12A); however, their combination with E_2 (3-compound mixture) did activate JNK with a significant 60-min response, by which time the response to E_2 had declined to control values (Figure 12B). As a 4-compound mixture, E_2 plus all XEs inactivated JNK to below vehicle control values early in the time course, but then activated and sustained pJNK after 30 min. Overall these combinations with XEs markedly attenuated the E_2-induced JNK response.

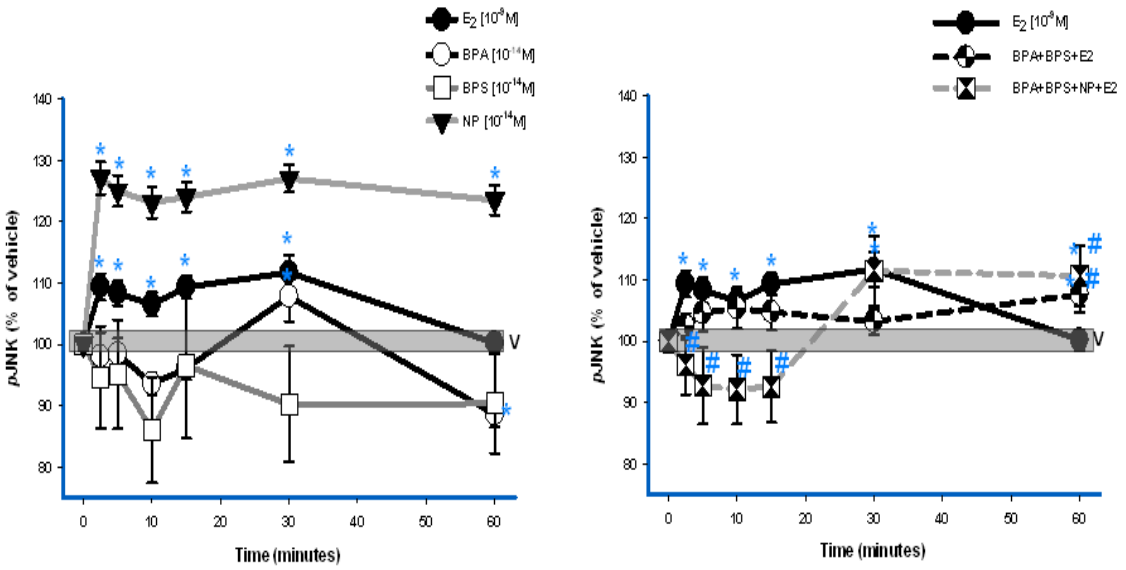

Figure 12. Timing of JNK activation by E_2, XEs, and XE/E_2 mixtures. Rat pituitary cells were exposed to BPS (10^{-14}M), BPA (10^{-14}M), NP (10^{-14}M) and/or E_2 (10^{-9}M) over a 60- min time course. Responses to individual XEs (A) and mixtures (B) were measured by plate immunoassay; the pNp signal generated for each well was normalized to cell number (measured by the CV assay). Values are expressed as percentage of vehicle (V)-treated controls. All error bars represent S.E M. The width of the vehicle bar represents a S.E. of ± 1.0 (n=24 over 3 experiments). ***** = $p<0.05$ compared to vehicle (V); # =$p< 0.05$ compared to 10^{-9}M E_2.

4.2.2 Concentration-dependent changes in phospho-activation of MAPKs by BPS, BPA, and NP, and their combinations, from a short exposure.

We have previously determined dose-response profiles for BPS (Vinas and Watson 2013), and are described here for comparison. Short exposures (5 min) to individual XEs (Figure 13A) caused ERK activation in $GH_3/B_6/F_{10}$ cells at concentrations similar to those elicited by E_2 (Jeng et al. 2010; Jeng and Watson 2011).

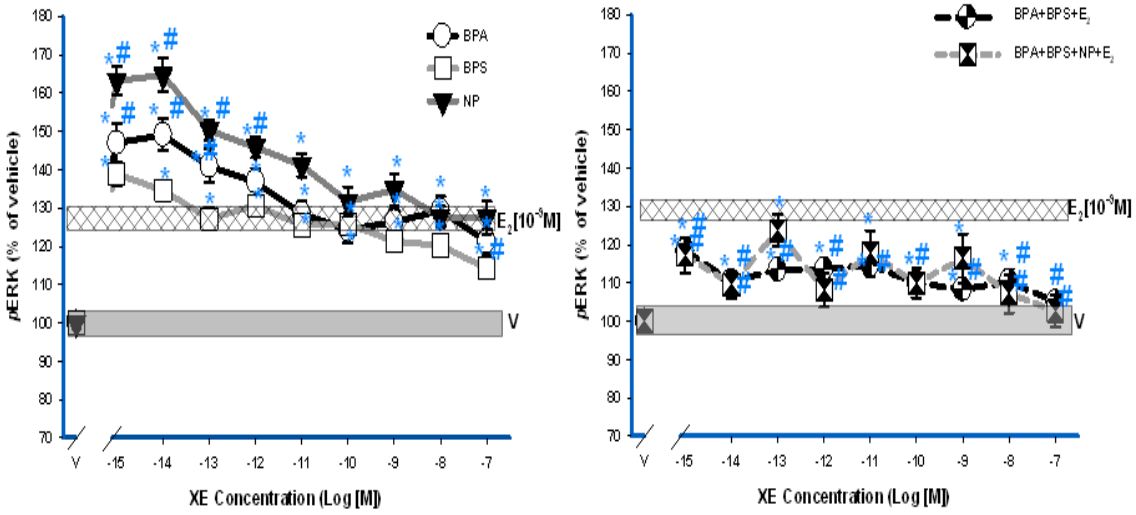

Figure 13. ERK activation dose-response analysis by E_2, XEs, and mixtures. $GH_3/B_6/F_{10}$ rat pituitary cells were exposed to increasing concentrations (10^{-15}M – 10^{-7}M) of BPS, BPA, and NP, compared to a single physiological level of E_2 (10^{-9}M). E_2 (10^{-9}M) is at a constant concentration throughout the XE dose-response range. Individual XEs (A) and XE mixture responses (B) were measured by plate immunoassay at a 5-min exposure time. All error bars represent S.E M. The widths of the vehicle and E_2 [10^{-9}M] bars represent a S.E. of ± 1.5 and ± 1.2 respectively, (n=24 over 3 experiments). ***** = $p<0.05$ compared to vehicle (V); # =$p< 0.05$ compared to 10^{-9}M E_2 S.E. ± 1.8. The E_2 (10^{-9}M) response is significantly different compared to the vehicle control.

The lowest tested XE concentrations evoked a higher pERK response than did 10^{-9}M E_2; the response steadily decreased with increasing XE concentrations. Responses to femtomolar concentrations of individual XEs were statistically different (by one-way ANOVA) from those in the nanomolar range and from the zero concentration point, indicating a non-monotonic dose-response (Vandenberg et al. 2012). Fentomolar concentrations of individual XEs were in general statistically different (One-way ANOVA) from those in the nanomolar range. The combination of increasing concentrations of XEs with constant 10^{-9}M E_2 (Figure 13B) reduced ERK activity below that of either E_2 or XEs alone.

Individual bisphenol compounds deactivated pJNK below vehicle levels (Figure 14A), unlike E_2 and NP that both activated JNK. However, when E_2 was administered together with both bisphenol compounds (Figure 14B), JNK was strongly activated, featuring a non-monotonic dose-response curve with the lowest concentrations evoking the largest responses; the 4-compund mixture evoked no activation of JNK and was consistently, though not statistically, below the level of the response to vehicle, thus erasing the response to 1nM E_2.

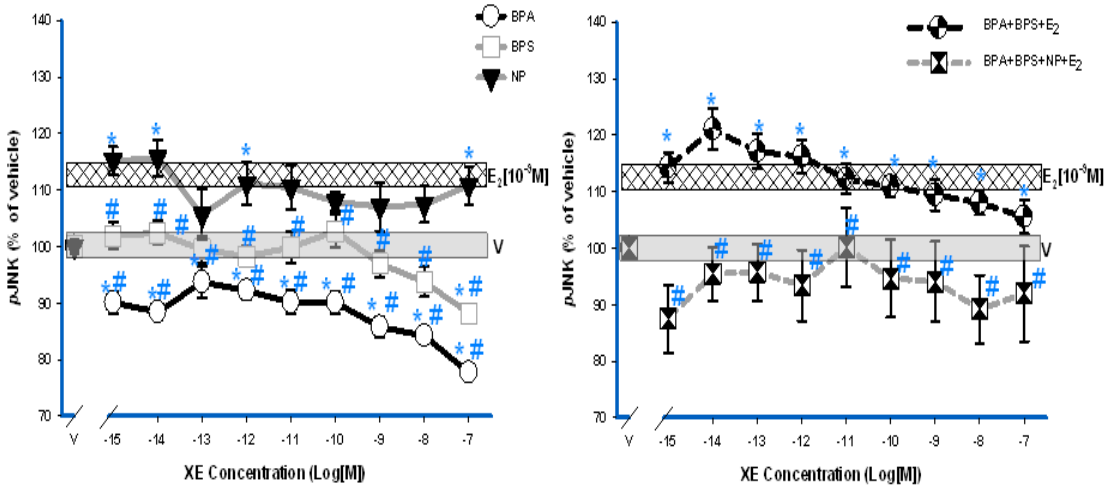

Figure 14. JNK activation dose-response analysis by E_2, XEs, and mixtures. $GH_3/B_6/F_{10}$ rat pituitary cells were exposed to increasing concentrations (10^{-15}M – 10^{-7}M) of BPS, BPA, and NP compared to a single physiological level of E_2 (10^{-9}M). E_2 (10^{-9}M) is at a constant concentration throughout the dose-response. Individual XEs (A) and XE mixtures (B) were measured by plate immunoassay at a 5-min exposure time. All error bars represent S.E M. The widths of the vehicle and E_2 [10^{-9}M] bars represent a S.E. of ± 1.3 and ± 1.2 respectively, (n=24 over 3 experiments). ***** = p<0.05 compared to vehicle (V); # =p< 0.05 compared to 10^{-9}M E_2. The E_2 (10^{-9}M) response is significantly different compared to the vehicle control.

4.2.3 XEs and mixtures affect cell proliferation

After a 3-day exposure, 10^{-9}M E_2 and BPS had similar effects on cell proliferation (Vinas and Watson, 2013). We looked at the dose responsiveness at this 3-day time point, demonstrating non-monotonic stimulations (Figure 15A), as we observed previously with E_2 and other XEs (Jeng and Watson 2009; Kochukov et al. 2009). NP did not increase cell numbers significantly until it reached 10^{-11}M, and BPA until it reached 10^{-7}M. Both XE mixtures with E_2 (Figure 15B) failed to

stimulate cell proliferation, but instead suppressed cell numbers far below those seen with vehicle, again showing these compounds' ability to disrupt a response to a physiologic estrogen.

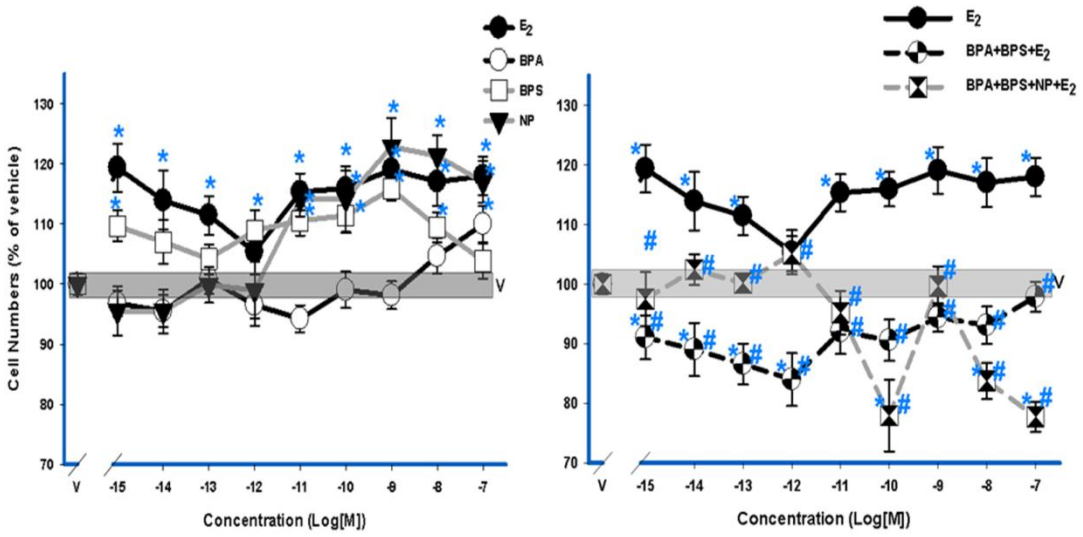

Figure 15. XEs cause cell proliferation, and XE mixtures disrupt cell proliferation evoked by E_2. Increasing concentrations of XEs (10^{-15}M – 10^{-7}M) compared to increasing concentrations of E_2 (10^{-15}M-10^{-7}M) alone (A) were assessed after a 3-day growth period. Mixtures of E_2 with XEs were assessed in B. Cell number was measured by the CV assay and compared to vehicle (V)-treated cells (n=24 over 3 experiments). All error bars represent S.E M. The width of the vehicle bar represents a S.E.of ± 1.3. *= p< 0.05 compared to vehicle; # = p< 0.05 compared to 10^{-9}M E_2.

4.2.4 Caspases activated and deactivated

Initiation of apoptosis is one of several factors that can influence cell numbers; we therefore assayed caspase 8 and 9 to determine if the extrinsic or intrinsic apoptotic pathways were activated over an 8-hr exposure period, the optimum time that was determined previously (Vinas and Watson, 2013). Caspase 8 was

significantly activated by BPS at both concentrations used (10^{-14}M and 10^{-8}M), while BPA, NP, and the mixture solutions at their respective concentrations did not result in significant activations (Figure 16A). Activations of caspase 9 were not detected with either individual XEs or mixtures, indicating that the extrinsic pathway (caspase 8) and not the intrinsic pathway (caspase 9) is the primary apoptotic pathway activated. However, both mixture combinations at the highest concentrations (10^{-8}M) resulted in a significant “deactivation” of caspase 9 activities (Figure 16B). Staurosporine, the positive control for activation, was active on both caspases, as expected. E_2 by itself suppressed caspase activity below vehicle controls for both apoptotic pathways, as we had seen previously (Vinas and Watson, 2013; Jeng and Watson 2009).

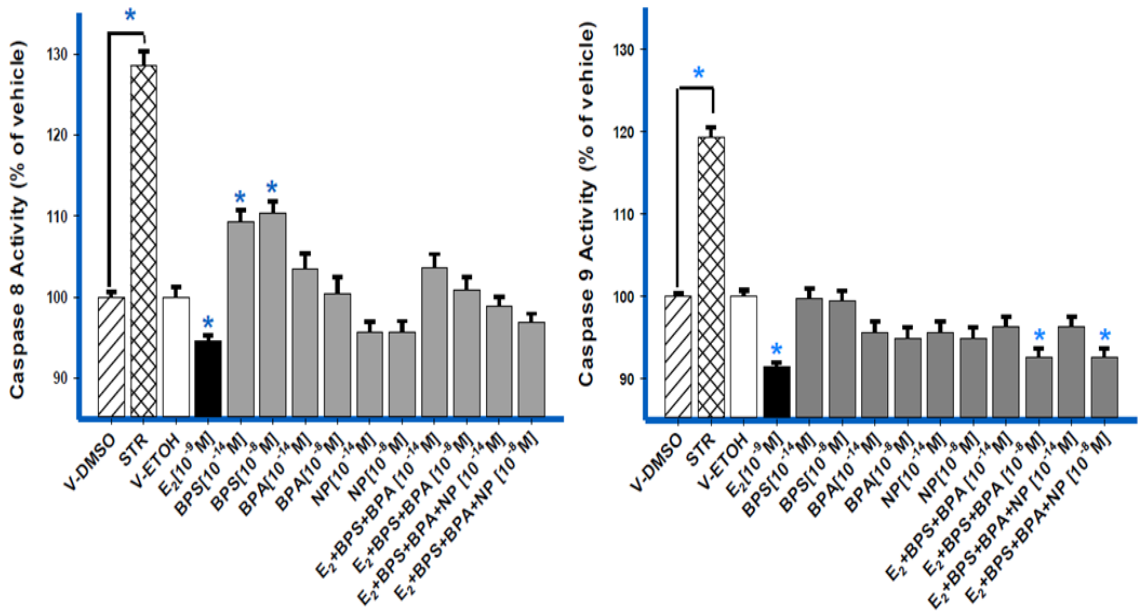

Figure 16. Activation or deactivation of caspases 8 and 9 by E_2, XEs, and mixtures. Over an 8-hr exposure period we measured caspase 8 (A) and 9 (B) activity evoked by two different concentrations of BPS, BPA, and NP (10^{-14}; 10^{-8}M) separately and together, with each other and with a physiological level of E_2 (10^{-9}M). E_2 (10^{-9}M) is at a constant concentration throughout. Caspase activity was measured by the release of a fluorogenic product (AFC) expressed as the percentage of vehicle (V)-treated controls. Staurosporine (STR, 500nM) was used as a positive control for induction of caspase activities compared to its own DMSO V control (n=24 over 3 experiments). Error bars are means ± S.E. (1.2). ***** =p<0.05 compared to V.

4.2.5 Mixtures of XEs disrupt E_2-induced PRL release

The rapid non-genomic secretion response for PRL caused by estrogenic exposure in $GH_3/B_6/F_{10}$ cells has become a standard tool in our lab for evaluating functional endpoints (Dufy et al. 1979; Jeng et al. 2010; Jeng and Watson 2011; Kochukov et al. 2009; Pappas et al. 1995; Wozniak et al. 2005). After a 1-min exposure, BPS could not increase PRL secretion as did E_2 [Figure 17 and (Viñas and Watson 2012)]. At certain concentrations, BPA and NP were able to

significantly increase PRL release, even above that caused by nM E_2 (Figure 17A); the shape of the dose-response curves are non-monotonic (as confirmed by determining that values at the peaks of activation were statistically different than those at other, usually higher, concentrations). As XE mixtures with 10^{-9}M E_2, the 3-compound mixture inhibited E_2-induced PRL release at low concentrations (significantly at 10^{-11}M). The 4-compound mixture caused more extreme inhibitions, even below the vehicle level at the lower concentrations (Figure 17B). Though the 4-compound mixture at 10^{-8}M appears to have resulted in PRL release, the errors in these mixture measurements did not allow this response to be distinguished as statistically different from vehicle, and the mixed signaling patterns caused by the multiple ligands may contribute to this variability.

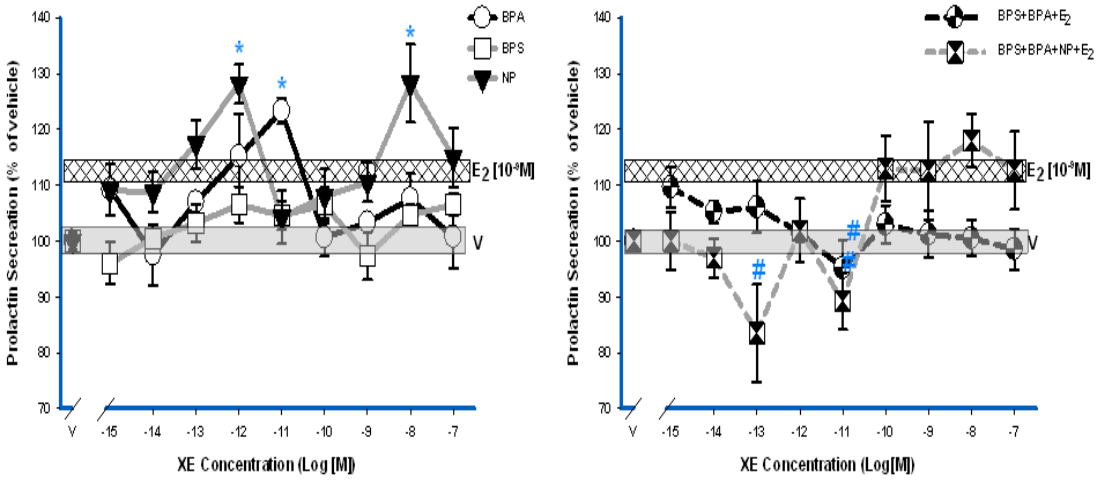

Figure 17. XEs cause PRL release, and XE mixtures alter these responses. We measured PRL release into the culture medium by RIA after a 1-min exposure to (A) individual XEs (10^{-15}M – 10^{-7}M) and also as (B) XE mixtures (10^{-15}M – 10^{-7}M) with a constant physiologic E_2 concentration (10^{-9}M) throughout the

dose-response range of the XEs. The amount of PRL secreted for each well was normalized to the CV value for cell number, and expressed as a percentage of vehicle (V)-treated controls. Error bars are means ± S.E. For positive (E_2) and negative (V) controls, the width of the bars indicate error ranges (V ±1.5; 10^{-9}M E_2 ± 1.6). n=24 over 3 experiments. ***** = p< 0.05 compared to vehicle; # =p<0.05 compared to 10^{-9}M E_2. The E_2 (10^{-9}M) response is significantly different compared to the vehicle control.

4.3.0 Discussion

Our study confirms that the novel BPA substitute, BPS, can initiate rapid non-genomic signaling in pituitary cells at environmentally relevant concentrations (as low as *femto*molar-*pico*molar), as do the more thoroughly tested BPA and NP. In combination these compounds altered endocrine responses differently, and more dramatically. Together, these compounds also interfered with the actions of the physiologic estrogen E_2 resulting in alterations to functional endpoints. These results not only highlight the need for stricter regulatory requirements for XEs, but also address the need to identify potentially adverse interactions of new chemicals with already existing chemicals in the environment. Such endocrine-disrupting effects should be identified during the initial phases of product development so that hazardous new combination exposures can be prevented (Schug et al. 2012).

We previously determined that BPS, BPA, and NP had similar high potencies, compared to E_2, for initiating the phospho-activation of ERK and JNK across a wide range of concentrations and times (Vinas and Watson, 2013; Jeng and

Watson, 2011; Wozniak et al. 2005; Jeng et al. 2009; Kochukov et al. 2009; Jeng and Watson, 2009; Jeng, et al. 2010.). Non-monotonic dose-response curves were seen, as low concentrations of individual XEs produced high MAPK activation, decreasing as concentrations increased. The occurrence of non-monotonic responses is the source of much debate. In regards to our system, such occurrences could be due to: 1) negative feedback regulation of MAPKs as concentrations increase, thereby preventing unnecessary pathway activation; 2) receptor down-regulation or desensitization; 3) the presence of multiple receptor subtypes that bind to the same ligand yet initiate interactions with different signaling partners, thereby eliciting different response patterns (stimulatory or inhibitory); and/or 4) the activation of multiple pathways from the same receptor, where signaling can be redundant or divergent (Conolly and Lutz 2004; Hunter 1995; Vandenberg et al. 2012; Watson et al. 2010; Weltje et al. 2005). The more detailed mechanisms responsible for these non-monotonic responses are still largely unexplored at the cellular level.

The phospho-activation of ERK and JNK is often associated with opposing functional endpoints. ERK signaling promotes cell growth and differentiation by activating pro-survival enzymes (McCubrey et al. 2007) and inhibiting apoptotic enzymes such as caspases (Allan et al. 2003; Allan and Clarke 2007; Allan and Clarke 2009). Conversely, JNK signaling is often associated with inflammation or the initiation of cell death, activating pro-apoptotic proteins including Bax,

caspase-3, and Fas, cyclin D1 under some circumstances, and interleukin 1 (Ip and Davis 1998; Junttila et al. 2008; Meloche and Pouyssegur 2007; Nordstrom et al. 2009; Xia et al. 1995). Our data have shown a correlation between the increase in cell numbers and ERK activation by BPS, as we discussed previously (Vinas and Watson, 2013). However, BPA and NP were slightly more efficacious than BPS at activating ERK, yet were unable to increase cell numbers as effectively, suggesting that pro-apoptotic proteins may also be involved in regulating final cell number outcomes. Dramatic decreases in cell numbers, in particular with the 4-compound mixture, could be due to the simultaneous attenuation of the E_2-induced ERK activation, as ERK activation are usually linked to cell proliferation responses. Our present data therefore present another example of how the final cell number outcome is dependent on the overall balance between ERK and JNK related activities (Dhanasekaran and Reddy 2008; Sanchez-Perez et al. 1998).

Caspase activation by XE mixtures was also examined to determine whether activation occurred via an external stimulus (caspase 8) or through an internal stimulus (caspase 9), and to correlate caspase activity to changes in cell numbers. We previously reported that BPS at two concentrations (10^{-14}M and 10^{-8}M) throughout a 4-24 hr time course resulted in activation of caspase 8, with a delayed and probably secondary activation of caspase 9 (Vinas and Watson, 2013). However, here we show that BPA, NP, and their mixtures did not activate,

and in some cases (as when combined with 10^{-9}M of E_2), deactivated caspases. Deactivation of caspase activity protects cells from death and contributes to E_2's well-known proliferative effect on the GH_3 cell lines (Jeng and Watson 2009; Kochukov et al. 2009; Rhode and Gorski 1991) and other cancer and normal cells. Caspases contribute to the inhibition of cell proliferation by XE mixtures, but cell number is clearly not controlled by caspases alone. The balance of multiple factors – including the actions of several pro-apoptotic or anti-apoptotic enzymes and other proteins – may contribute, along with numerous other proteins that control the cell cycle.

However, it is clear that XEs can alter such responses. BPA and NP (10^{-15} – 10^{-7}M) induced significant PRL secretion with each compound producing non-monotonic dose responses, agreeing with previous studies from our lab (Kochukov et al. 2009; Wozniak et al. 2005). Mixtures of BPS with BPA, and additionally NP, disrupted E_2-induced PRL release, causing an overall attenuation of secretion compared to individual XE compounds. Such inhibitory actions could be part of a negative feedback mechanism protecting against excess stimulation by multiple estrogens causing unnecessary PRL release. Because PRL regulates over 300 biological functions directly and indirectly (Bole-Feysot et al. 1998), alteration of its secretion (either enhancement or inhibition) can cause many different physiologic complications, including such medical

problems as metabolic dysfunctions, behavioral disturbances, or reproductive and offspring-rearing failures.

Adverse actions from chemicals introduced to the environment should be suspected whenever they can disrupt the actions of a physiologic hormone like E_2. In addition, compounds acting as estrogens on their own and possibly causing estrogenic responses in an organism at inappropriate life stages have been shown in many studies to be unfavorable. As growing concern over the safety of BPA has led to stricter regulatory action, we are likely to see other chemical replacements offered. The increased presence of BPS in an environment already contaminated with BPA, NP, and a variety of other prevalent and persistent environmental pollutants now requires increasing scrutiny of their potential hazards as chemical mixtures, and especially those that act via ERs (Bulayeva et al. 2005; Bulayeva and Watson 2004; Jeng and Watson 2011). Our tissue-relevant responses, such as the ones we have demonstrated with these medium-throughput quantitative assays in pituitary cells, offer efficient test systems that could be used to monitor pollutant mixtures at the cellular level. In addition, altered pituitary responses are very likely to have consequences for all other endocrine tissues. However, it is important to establish effective pre-screening of the endocrine-disruptive potential of any new chemicals whose structures make them candidates for these endocrine-disruptive activities (Schug et al. 2012).

Chapter 5

Non-genomic Signaling Activities of Modified (Chlorinated, Sulfonated, and Glucuronidated) Bisphenol A

5.1.0 Introduction

A major health concern is the presence of BPA in drinking water. Release of BPA into aquatic environments, particularly to waste water treatment plants, occurs primarily from plastic-to-water migrations such as when water runs through poly-vinyl chloride (PVC) pipes in water supply systems, or from discarded plastic in the environment (Yamamoto and Yasuhara 1998). Once in the drinking water, BPA is rapidly biodegraded by microorganisms (e.g. *Pseudomonas*), with a half-life of 2.5-4 days (Gibson 1984; Kang and Kondo 2002; Dorn et al. 1987; Staples et al. 1998).

The addition of chlorine to water supply systems as a means of preventing water borne diseases, along with BPA's constant replenishment from environment can result in its reaction with free chlorine giving rise to a variety of chlorinated forms (Figure 18) (Kuruto-Niwa et al. 2005; Gallard et al. 2004; Yamamoto and Yasuhara 2002). Depending on the amount of chlorine present in an aqueous medium, it can rapidly consume BPA within 4hrs leading to formation of these chlorinated congeners with half-lives anywhere from 10 to 20hrs (in a controlled

environment) (Gallard et al. 2004). While an abundance of evidence does exist about other poly-chlorinated biphenols having endocrine-disrupting capabilities, little is known about the estrogenic potential of poly-chlorinated BPA compounds (Fig. 18, right panels), and nothing about their influence via non-genomic signaling pathways. Our present study therefore uses the $GH_3/B_6/F_{10}$ rat prolactinoma cell line as our in-vitro model to examine the ability of these chlorinated congeners to initiate non-genomic kinase signaling.

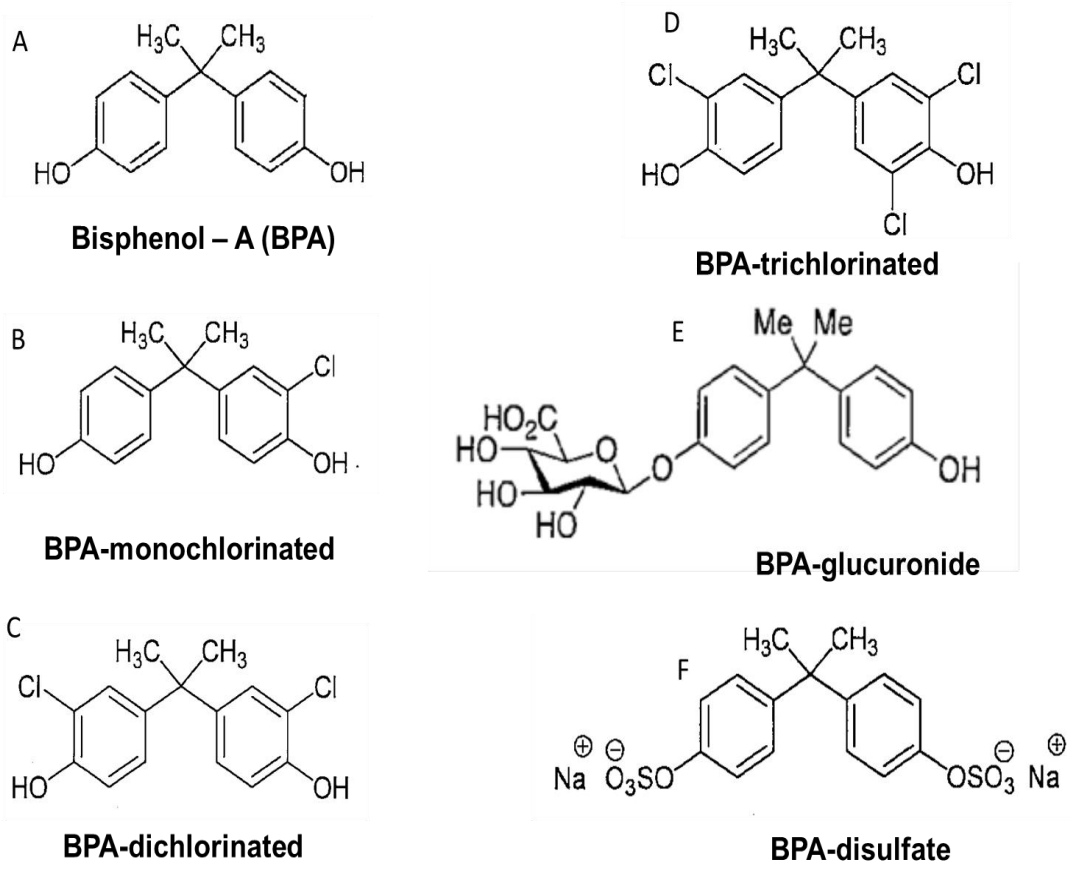

Figure 18. Structure of BPA, its chlorinated congeners, and its phase II metabolites. (A) Bisphenol-A; (B) Mono-, (C) Di-, and (D) Tri-chlorobisphenol-A; (E) Bisphenol-A β-d-glucuroide; (F) Bisphenol-A disulfate

In addition, we also assess the ability of phase II glucuronidated and sulfated metabolites of BPA (see Fig. 18, left panels) to induce non-genomic signaling. Phase II conjugations occur in many tissues and increase the water solubility of such lipophilic compounds, therefore facilitating their excretion (Reed et al. 2005; Zhu and Conney 1998). These conjugations prevent endogenous estrogens (Zhu and Conney 1998) and exogenous estrogens like BPA (Matthews et al. 2001; Shimizu et al. 2002; Snyder et al. 2000) from binding to iERs, thus rendering the compounds biologically inactive in the genomic signaling pathway. Previous studies from our group (Campbell and Watson 2001; Norfleet et al. 2000; Norfleet et al. 1999a), and others (Powell et al. 2001; Razandi et al. 2003) have documented that iERα is closely related to mERα. However, we hypothesized mER's access to extracellular compounds that may not even enter cells. In addition, mER's residence in lipid membranes (as opposed to iERs' aqueous environment in the cytosol or nucleus) may allow differences in the steroid and steroid mimic binding between the alternatively localized receptors, thus perhaps permitting conjugated metabolites of BPA to initiate non-genomic signaling.

As in our previous studies, we used a fixed cell-based 96-well plate immunoassay to evaluate protein phosphorylation and dephosphorylation of the mitogen-activated protein kinases (MAPKs) (Bulayeva et al. 2004; Campbell et al. 2002; Jeng et al. 2010a; Jeng et al. 2009; Jeng and Watson 2011; Kochukov et al. 2009; Wozniak et al. 2005). While this method has yielded excellent results,

it can be a very time-consuming process. To increase efficiency and reproducibility, we designed an automated medium-throughput screening system with the use of the BIOMEK FX^P workstation for liquid handling.

5.2.0 Materials and Methods

5.2.1 Cell Culture Conditions and Experimental Compounds

The $GH_3/B_6/F_{10}$ rat prolactinoma cell line was selected on the basis of its naturally high expression of mERα (Bulayeva et al. 2004; Pappas et al. 1995; Pappas et al. 1994). Cells were routinely sub-cultured with phenol red-free Dulbecco's Modification of Eagle's Medium (DMEM, high glucose; Mediatech, Herdon,VA) containing 12.5% horse serum (Gibco BRL, Grand Island, NY) and defined supplemented calf and fetal serum (Thermo Fisher, Waltham, MA) at 2.5% and 1.5%, respectively. Cells of passages 10-20 were used for these experiments.

BPA chloride congeners and conjugates (glucuronidated and sulfated) were kindly provided by the National Institute for Environmental Health Sciences (NIEHS). Before use on cells they were dissolved in ethanol, and then diluted in DMEM containing 1% charcoal-stripped serum.

5.2.2 Automated ERK and JNK Phosphorylation Assays

A fixed cell-based immunoassay was used to quantify phospho-activation of extracellular-regulated kinase (ERK/pERK) and c-jun N-terminal kinases (JNK/pJNK), which we previously developed and optimized, as detailed in (Bulayeva et al. 2004; Campbell et al. 2002).

5.2.3 Chlorinated BPA Congeners

Cells (10^4/well) were plated in 96-well plates (Corning Incorporated, Corning, NY) and allowed to attach for 24hrs. The cells were then incubated with DMEM containing 1% charcoal-stripped (4X) serum for 48hrs to deprive them of serum hormones. The medium was then removed and cells were exposed to increasing concentrations (10^{-15}-10^{-7}M) of individual mono-, di-, and tri- chlorinated BPA congeners for 5 min. The short time point of 5 min was chosen to observe non-genomic phospho-activations without interference from later genomic responses. The concentration range was chosen to bracket environmentally relevant concentrations and test very low concentrations that we have shown to activate these responses. Test compounds were dissolved in ethanol and then diluted in DMEM containing 1% charcoal-stripped serum. The vehicle control (V) was 0.001% ethanol in DMEM. To stop mER-initiated signaling, cells were fixed with a 2% paraformaldehyde/0.2% picric acid solution (Fisher Scientific, Pittsburgh, PA) and incubated at 4°C for 48hrs. The cells were then incubated in phosphate–buffered saline (PBS) containing 0.2% fish gelatin and 0.1% Triton X-100

(Sigma-Aldrich) for 1hr at room temperature (RT), followed by overnight incubation at 4°C with primary Abs against pERK or pJNK (Cell Signaling Technology, Beverly, MA) (1:500 in PBS/0.2% fish gelatin/0.1% TritonX-100). The cells were then washed with PBS (3X) before incubation with biotin-conjugated Ab (Vector Labs, Burlingame, CA) (1:500 in PBS/0.2% fish gelatin) for 1hr at RT, then washed again in PBS (3X) and incubated with 50µL/well Vectastain ABC-AP solution (Vector Labs) for 1hr at RT, followed by addition of 50µL/well alkaline phosphatase substrate (pNpp solution, Thermo Scientific, Rockford, IL). The plates were incubated in the dark for 30 min at 37°C and the A_{405} signal due to the *para*-nitrophenol (pNp) product was measured. The pNp signal was then normalized to the cell number, estimated by the crystal violet (CV) assay (Campbell et al. 2002) and measured at $A_{590.}$ Absorbance signals for both were read in a model 1420 Wallac microplate reader (Perkin Elmer, Boston, MA).

5.2.4. Phase II BPA Metabolites

The anterior pituitary is known to contain β-glucuronidase (Demarest et al. 1984) and sulfatase (Connolly and Resko 1989; Reed et al. 2005), which can cause enzymatic hydrolysis and deconjugate glucuronic acid and sulfate from the parent compound, respectively. To maintain the integrity of our conjugated compounds during the analysis, we pre-incubated our cells with 20mM D-glucaric acid-1,4-lactone (Carbosynth, UK), a potent inhibitor of β-glucuronidase (Oleson

and Court MH 2008) or 10nM 667 Coumate/STX-64 (Sigma-Aldrich), a sulfatase inhibitor (Reed et al. 2005), each for 1hr at 37°C (50µl/well). Conjugates were added to wells (50µl/well) at final concentrations of10^{-15}-10^{-7}M, incubated for 5min, and stopped with 2% paraformaldehyde/0.2% picric acid solution as above. Automated immunoassay methods proceeded as above for the chlorinated compounds.

5.2.5 Statistical Analysis

Statistical analysis was performed using Sigmaplot version 12.3 (Systat Software Inc). One-way analysis of variance (ANOVA) was applied to the dose-dependent and timed studies to assess the statistical significance of mean values produced by varying exposures. A Holm-Sidak comparison against vehicle control or against 10^{-9}M E_2 treatment was used to evaluate significance. The overall α level selected for the statistical analysis was 0.05.

5.3.0 Results

5.3.1. Concentration-dependent changes in phospho-activation of MAPKs by BPA and its chlorinated congeners.

BPA caused ERK activation (Figure 19A) in $GH_3/B_6/F_{10}$ cells within 5 min, with a non-monotonic response pattern, as expected (Jeng et al. 2010b; Jeng and Watson 2009; Kochukov et al. 2009; Wozniak et al. 2005). Exposure to mono-

and di-chlorinated compounds caused ERK activation at most concentrations, and at some concentrations the responses were equivalent to that of 10^{-9}M E_2 (Figure 19, B and C). The dose-response curves did not gradually rise and plateau at the highest concentrations, as is seen with classical responses, but instead showed non-monotonic patterns (Vandenberg et al. 2012). The tri-chlorinated BPA congener did not cause phospho-activation of ERK, but instead led to a dramatic dephosphorylation below vehicle levels (Figure 19D). With the exception of the unconjugated (parent) and mono-chlorinated BPA, which had no significant effects (Figures 20, A and B, respectively), all other compounds caused a significant dephosphorylation of JNK (Figure 20, C and D). Thus chlorination to different degrees had different effects, with the more extensively modified compounds disrupting mER's actions on MAPKs.

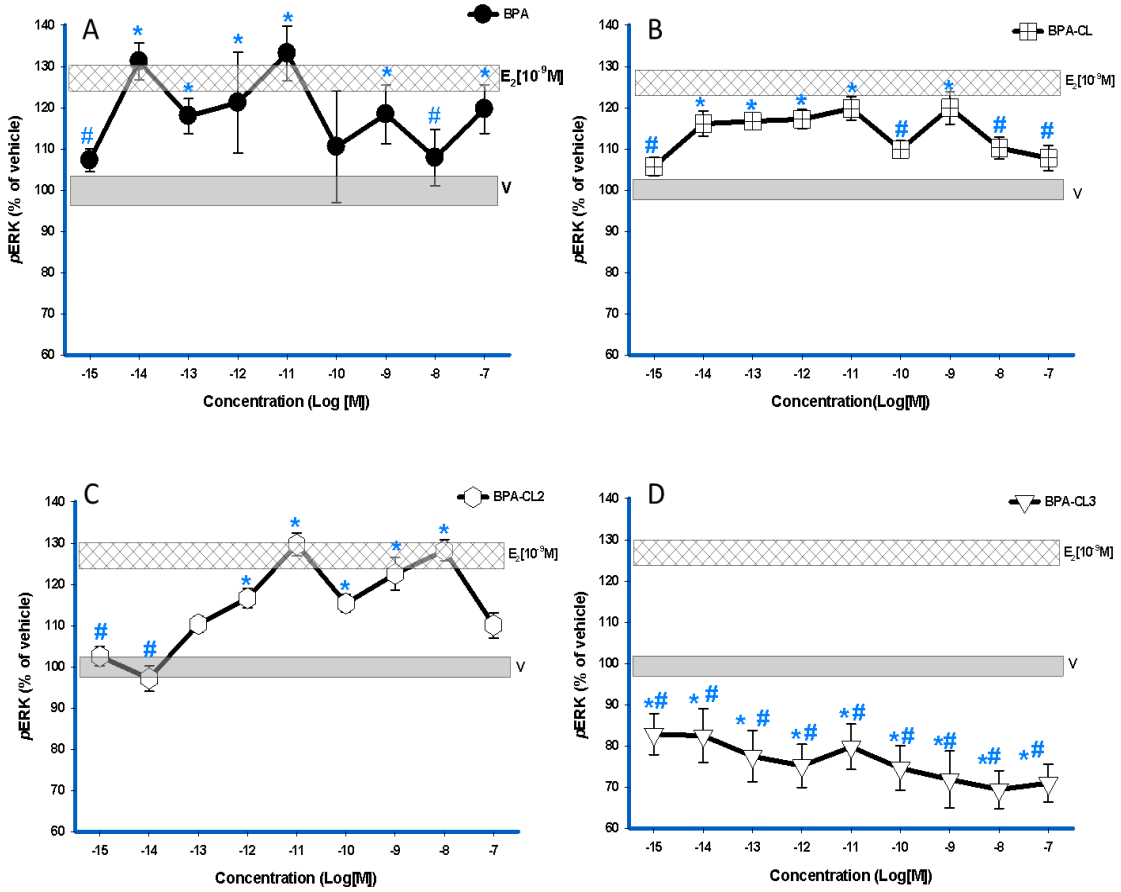

Figure 19. Dose-response analysis of the phosphorylation of ERK (to pERK) upon exposure to BPA and its chlorinated congeners. $GH_3/B_6/F_{10}$ cells were exposed to increasing concentrations (in log increments) of (A) BPA, (B) Mono-, (C) Di-, and (D) Tri-chlorinated BPA. pERK was measured by plate immunoassay at a 5 min exposure time. The widths of the vehicle and E_2 [10^{-9}M] bars represent the means ± S.E. (n=24 over 3 experiments) * = $p<0.05$ when compared to vehicle (V). # =$p< 0.05$ when compared to 10^{-9}M E_2. E_2 (10^{-9}M) is significantly different from vehicle.

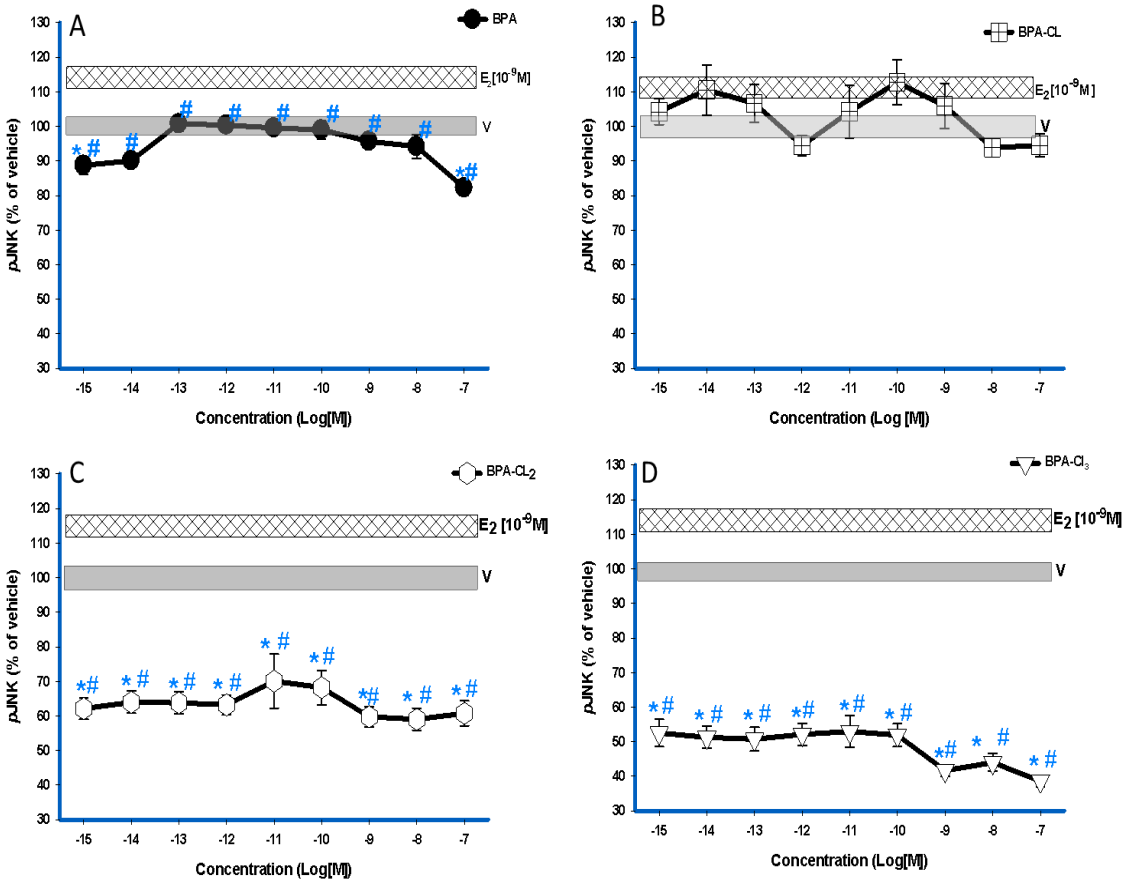

Figure 20. Dose-response analysis of JNK phospho-activation (to pJNK) upon exposure to BPA and it chlorinated congeners. $GH_3/B_6/F_{10}$ cells were exposed to increasing concentrations (in log increments) of (A) BPA, (B) Mono-, (C) Di-, and (D) Tri-chlorinated BPA. pJNK was measured by plate immunoassay at a 5 min exposure time. The widths of the vehicle and E_2 [10^{-9}M] bars represent the means ± S.E. (n=24 over 3 experiments) * = $p<0.05$ when compared to vehicle (V). # =$p< 0.05$ when compared to 10^{-9}M E_2. E_2 (10^{-9}M) is significantly different from vehicle.

5.3.2 Concentration-dependent changes in phospho-activation of MAPKs by BPA phase II metabolites.

Cells were exposed to BPA phase II metabolites ± β-glucuronidase or sulfatases inhibitors. In the presence of the inhibitor STX-64 sulfated-BPA was only able to

activate ERK at 10^{-7}M (Figure 21A). However, in the absence of STX-64, the sulfated compound phospho-activated ERK with a non-monotonic dose-response, indicating some conversion of the compound to the unconjugated form. In the presence of the inhibitor D-glucaric acid-1, 4-lactone, glucuronidated BPA did not activate ERK, but in its absence it activated ERK at the lowest concentration (Figure 21B), where we have previously found BPA to be the most active (Jeng et al. 2010b; Jeng and Watson 2009; Kochukov et al. 2009; Wozniak et al. 2005).

In the presence of the inhibitor, stably conjugated BPA-glucuronide inactivated ERK significantly below the pERK level caused by treatment with vehicle. Variability in the uninhibited activation profiles of these two forms of conjugated compounds may suggest differences in the relative rates of bond cleavage by sulfatases vs. glucuronidases. Both conjugated compounds in either the presence or absence of their respective deconjugation inhibitors were unable to activate JNK signaling above vehicle-treated levels, but instead deactivated JNK at some concentrations in a non-monotonic pattern (Figure 22, A and B). The disulfated compound was far more consistently effective in this deactivation. Thus phase II enzymatic conjugation of sulfate and glucuronic acid to BPA disrupts its actions at mERs.

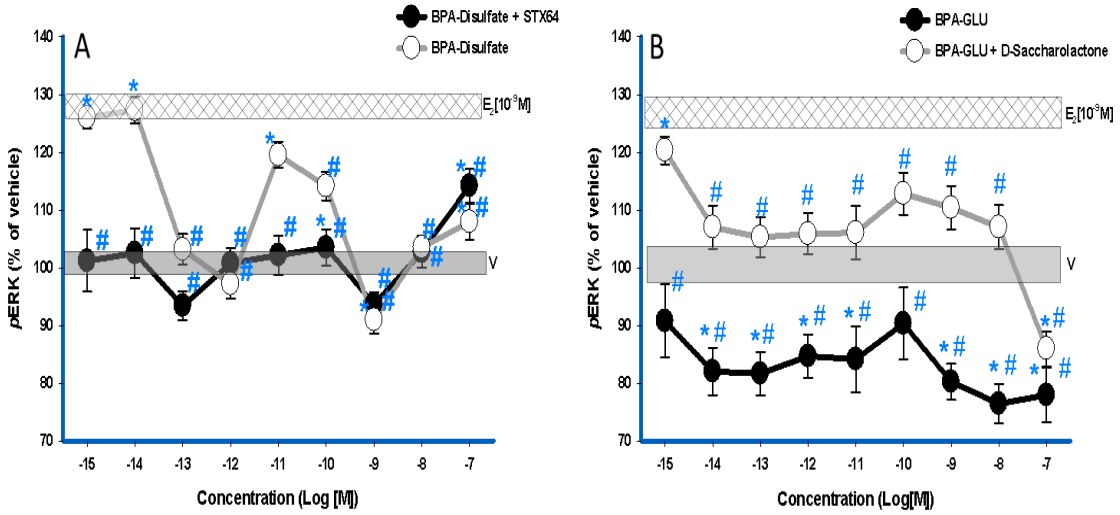

Figure 21. Dose-response analysis of ERK phospho-activation by BPA phase II metabolites. $GH_3/B_6/F_{10}$ cells were exposed to increasing concentrations (in log increments) of (A) Bisphenol-A β-d-glucuronide and (B) Bisphenol-A disulfate. The cells were pre-incubated ± D-glucaric acid-1,4-lactone or STX-64 to inhibit β-glucoronidase and sulfatase, respectively. pERK was measured by plate immunoassay at a 5 min exposure time. The widths of the vehicle and E_2 [10^{-9}M] bars represent the means ± S.E. (n=24 over 3 experiments) * = $p<0.05$ when compared to vehicle (V). # =$p< 0.05$ when compared to 10^{-9}M E_2. E_2 (10^{-9}M) is significantly different from vehicle.

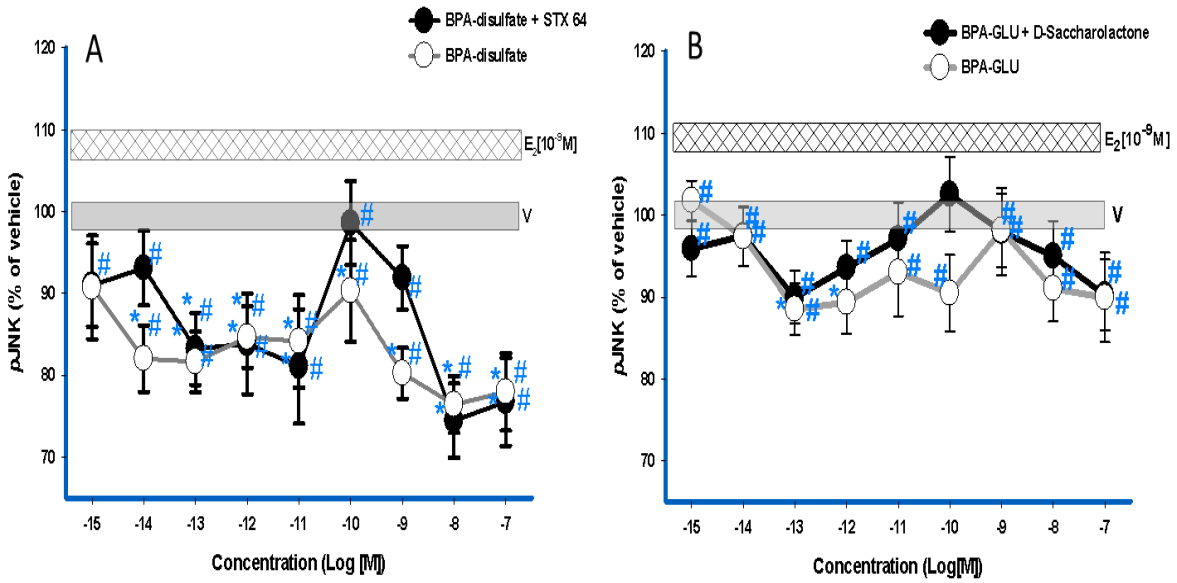

Figure 22. Dose-response analysis of JNK phospho-activation by BPA phase II metabolites.$GH_3/B_6/F_{10}$ cells were exposed to increasing concentrations (in log increments) of (A) Bisphenol-A β-d-glucuronide and (B) Bisphenol-A disulfate. The cells were pre-incubated ± D-glucaric acid-1,4-lactone or STX-64 to inhibit β-glucoronidase and sulfatase, respectively, prior to analysis. pJNK was measured by plate immunoassay at a 5 min exposure time. The widths of the vehicle and E_2 [10^{-9}M] bars represent the means ± S.E. (n=24 over 3 experiments) * = $p<0.05$ when compared to vehicle (V). # =$p< 0.05$ when compared to 10^{-9}M E_2. E_2 (10^{-9}M) is significantly different from vehicle.

5.3.3 Comparison of automated plate assay results with prior manual assays

In designing an automated program for these assays using the BIOMEK software, many adjustments were necessary to match the automated results to our previous manual results (Bulayeva et al. 2004; Bulayeva and Watson 2004; Jeng et al. 2010b; Jeng et al. 2009; Jeng and Watson 2011; Kochukov et al. 2009; Wozniak et al. 2005). Liquid- handling parameters were optimized, such as

the speed of aspiration and dispensing, and determining the adequate depth of tip immersion into the liquid reservoirs. Such adjustments decreased the amount of unwanted liquid carryover that could ultimately build up and increase the final liquid volume in each well, thereby causing added variability. Time adjustments were necessary, taking into consideration the elapsed time from the moment the probe grippers moved or replaced tip boxes to the point at which the multi-channel probe picked up pipette tips and then finally dispensed the liquids into the wells of the plates. These changes made it possible to meet our target times for exposure to estrogens and precise termination of the response by fixation. In general, the results of these optimized automated assays agreed very well with previously published MAPK activation/deactivation measurements for both E_2 and BPA (Bulayeva et al. 2004; Bulayeva and Watson 2004; Jeng et al. 2010b; Jeng et al. 2009; Jeng and Watson 2011; Kochukov et al. 2009; Wozniak et al. 2005).

5.4.0. Discussion

Drinking water systems that rely on the addition of chlorine as an inexpensive disinfectant are also abundant sources of EDCs, including BPA. BPA is rapidly chlorinated to form BPA congeners (Gallard et al. 2004). Our study demonstrates that environmentally relevant concentrations (femtomolar-picomolar) of chlorinated derivatives of BPA can initiate or alter non-genomic actions via mERs in pituitary cells. We determined that glucuronidated and sulfated phase II

conjugates of BPA often depressed MAPK activities instead of activating them. Our results thus provide further insights into these metabolites' biological activities, which differed from those of their parent compounds (Matthews et al. 2001; Shimizu et al. 2002; Snyder et al. 2000; Zhu and Conney 1998). Such actions on MAPKs, as previously shown, have the potential to cause inappropriate cellular signaling in humans and other animals, and also to interfere with the actions of natural estrogens (like E_2 and other physiologic estrogenic compounds). Altering these pathways may lead to health changes at downstream functional endpoints such as cell proliferation, and the release of other hormones like prolactin (Jeng et al. 2010b; Jeng and Watson 2009; Kochukov et al. 2009; Wozniak et al. 2005). Finally, we developed an automated protocol to increase assay efficiency and reproducibility in examining the large number of environmental compounds, and their chemically modified forms, that can disrupt estrogenic actions such as these kinase activations.

Dose-response studies of BPA and its chlorinated conjugates generated atypical curve shapes (Figures 19 and 20), now often recognized as a consequence of examining low concentrations of EDCs or endogenous hormones (Jeng et al. 2010b; Jeng and Watson 2009; Kochukov et al. 2009; Wozniak et al. 2005). These non-monotonic dose relationships are now more widely recognized (Calabrese 2010; Vandenberg et al. 2012), and are commonly seen when the range of concentrations examined is extended to femtomolar to picomolar levels

in sensitive assays. However, the molecular bases for non-monotonicity are largely unknown, are still a source of much controversy, and could involve protective down-regulation of MAPK activities to eliminate unnecessary pathway stimulation; the presence of multiple receptor subtypes that bind the same ligand, each generating a different response pattern (stimulatory or inhibitory); concentration-dependent receptor down-regulation or desensitization; and/or stimulation of multiple pathways from the same receptor where signaling can subsequently be redundant, divergent, and convergent (Conolly and Lutz 2004; Hunter 1995; Vandenberg et al. 2012; Watson et al. 2010; Weltje et al. 2005).

Phospho-activation of ERK and JNK is often associated with opposing responses. For instance, ERK signaling promotes cell growth and differentiation by activating pro-survival enzymes (McCubrey et al. 2007) and inhibiting apoptotic enzymes (Allan and Clarke 2007; Allan and Clarke 2009; Allan et al. 2003). Conversely, JNK signaling is often associated with inflammation or the initiation of cell death by activating pro-apoptotic proteins, including Bax, Fas, or caspases (Ip and Davis 1998; Junttila et al. 2008; Meloche and Pouyssegur 2007; Nordstrom et al. 2009; Xia et al. 1995). Opposing responses of ERK and JNK can be appreciated in our data by comparing MAPK responses to di-chlorinated BPA where ERK is activated above the vehicle-treated level, and JNK is inactivated. Simultaneous activation of ERK and deactivation of JNK by EDCs have previously been correlated to severe stimulatory effects on

downstream functional endpoints such as cell proliferation (Viñas and Watson 2012). In contrast, tri-chlorinated BPA suppressed the activities of both MAPKs below vehicle-treated levels. Suppression of multiple kinases has been seen with another polychlorinated compound, 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) (Lu et al. 2011; North et al. 2010), where 0.03 and 0.3nM treatments inhibited phospho-activation of ERK and JNK in CH12.LX B lymphocytes within 15 min. Dual suppression of ERK and JNK may also suggest that tri-chlorinated BPA affects a common target upstream of ERK and JNK, thus impairing their activation, and would probably lead to cancellation of many downstream actions for which these kinases are responsible, dramatically disrupting endocrine function. Therefore, the final functional outcomes may depend on the overall balance between ERK and JNK related activities (Dhanasekaran and Reddy 2008; Sanchez-Perez et al. 1998).

Early structure-activity studies noted that the 17β-hydroxyl group of E_2 was necessary for binding within the ligand-binding pocket of iER (Brzozowski et al. 1997; Tabira et al. 1999). Therefore, any chemical mimicking this feature of E_2, such as in BPA, has the possibility of binding to an ER and inducing an estrogenic response (Nakai et al. 1999). The addition of multiple chlorine atoms at these sites might then disrupt this interaction and block or alter downstream responses. Such a trend was shown using a green fluorescent protein expression reporter system (Kuruto-Niwa et al. 2002; Kuruto-Niwa et al. 2005b);

when the number of chlorinated congeners added to BPA or nonylphenol increased, their estrogenic activity decreased. Increasing brominated substitutions on BPA also cause attenuation in estrogenic cell proliferation (Samuelsen et al. 2001).

However, our observations are not consistent with merely blocking of ligand binding to the receptor, because these modified BPAs do have effects in some cases (e.g. inactivation of MAPKs). Phase II conjugation of both endogenous and exogenous chemicals changes them from hydrophobic to hydrophilic molecules, which while facilitating their elimination from the body, also likely changes their ability to bind certain ER conformations or to alter the receptor shape once bound. The addition of chlorine, sulfate, or glucuronide moieties surrounding the phenolic-hydroxyl groups of BPA could direct different conformations of the ER with resulting changes in the ability to partner with other proteins. In such cases, interacting phosphatases affecting ERK and JNK could be activated instead (Canesi et al. 2004; Liu et al. 2012; Song et al. 2002; Xu et al. 2011).

We and others have explored the protein identities of mERα and iERα (Campbell and Watson 2001; Powell et al. 2001). A close protein similarity was established when nine iERα-specific Abs recognized mERα epitopes (Campbell and Watson 2001; Watson et al. 1999), the iERα-specific Ab H151 elicited or blocked non-genomic responses to estrogenic ligands (Norfleet et al. 2000; Watson et al.

1999), and disrupting the ERα mRNA (via antisense or siRNA methods) resulted in decreased expression of both the membrane and intracellular versions of ERα (Norfleet et al. 1999b; Pedram et al. 2006). Phase II conjugates failed to activate non-genomic responses in our study, another similarity between mER and iER (Reed et al. 2005; Zhu and Conney 1998), though the conjugates in our studies instead inactivated kinases, suggesting an alternative signaling partner. Though iERs and mERs are modified forms of the same protein, they may differ in conformation, due to their posttranslational modifications (Pedram et al. 2012) and their different chemical environments (soluble vs. membrane), and so may accommodate a wider variety of estrogen-like molecules (Watson and Gametchu 1999) and different signaling partner proteins.

The studies presented here demonstrate that some chlorinated conjugates of BPA can induce rapid non-genomic signaling in estrogen-responsive pituitary cells at low (femtomolar-picomolar) concentrations, but that more extensively altered forms (tri-chlorinated, glucuronidated, sulfated) inactivate the same kinases. Further structure-activity studies would be needed to gain additional insights and to identify the endocrine-disruptive potential of any new chemicals or their modifications (Schug et al. 2012). With our implementation of the BIOMEK FX^P Laboratory Automation Workstation protocol, we have developed a much more efficient and reliable automated screening tool for testing and identifying potential EDCs, and facilitating large-scale structure-activity studies.

Chapter 6

Future Directions

6.1.0 In Vivo Studies

Our studies indicate that endocrine disruptors as single entities and as mixtures are indeed a serious concern due to their ability to mimic or disrupt a physiologic hormone's response. The next logical step would be to translate the performed cell-based non-genomic studies onto animal-based models. In-vivo pituitary imaging has thus far been limited only to non-mammalian models, such as zebrafish (Liu NA, et al. 2008). Currently, there are no simple applicable technical approaches for monitoring mammalian (in-vivo) real-time functions of an endocrine gland unless through surgical externalization or ex-vivo methods such as by organ explant cultures or using tissue slice preparations (Nyman LR, 2008; Alim et al. 2012; Foradori et al. 2012). Furthermore, ex-vivo methodology cannot directly make a real-time physiological response measurement to a chemical exposure because of changes in blood flow or changes in partial oxygen pressure (P_{tissue}, O_2) (Lafont, et al. 2010) in the preparation prevent it. Direct measurements would be essential for understanding the relationship between rapid non-genomic signaling and functional responses.

A study by Chrystel Lafont and others have provided one of the few, if not the only, means of measuring non-genomic activity from the pituitary gland in-vivo

(Lafont et al. 2010). By using male mice expressing green fluorescent protein (GFP)–tagged GH cells (parent cell line of our $GH_3/B_6/F_{10}$ cells) and female mice expressing DsRedExpress-tagged PRL protein, they developed an optical imaging system with which to directly measure the in-vivo relationship between the blood vasculature and the GH cell network. Their methods and results are briefly summarized here:

To image the pituitary of terminally anesthetized mice, a “long working lens” attached to a fluorescent stereomicroscope was inserted via blunt dissection through the animal’s palate (See Figure 23); respiration was monitored throughout these experiments. Once in place, lens resolution, light excitation, and emission intensities could be adjusted for observing cellular organization within the pituitary and thus making it possible to distinguish different colored fluorescent reporters including PRL (DsRed, cytoplasmic) in female mice and growth hormone (GH) (eGFP, secretory vesicles) in male mice (Figure 23). An i.v. bolus of growth hormone releasing hormone (GHRH), a specific GH secretagogue, via the jugular vein catheter was given to each anesthetized animal in order to measure a variety of physiologic effects (blood flow rate, oxygen supply and consumption, and hormone output). Changes in blood flow upon GHRH treatment were monitored by measuring the velocity or flow of red blood cells (RBCs) in individual capillaries, which recorded changes as early as 5min when compared to injections of saline (Figure 24). Figure 24(B) shows a

correlation between RBC velocities (left axis) and plasma GH (right axis, vertical gray bars) that were measured in parallel over a 60min time period via RIA using mouse reagents provided by the NIDDK (Bethesda, MD) (*exact protocol as ours; see chapter 2*) before, during, and after i.v. injections of saline and GHRH, respectively. Blood collections for RIA processing were made via the jugular venous catheter. We too could apply this methodology and correlate changes in RBC velocities to plasma PRL levels within the short non-genomic time-frame upon single XE or mixture exposure if the equipment were available.

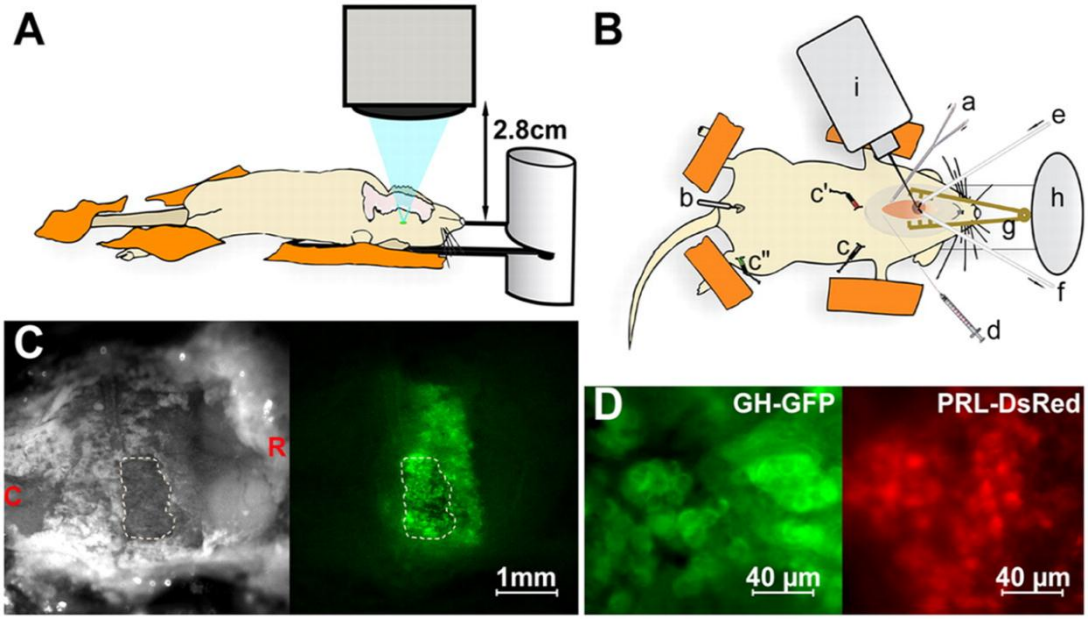

Figure 23. Cellular in vivo imaging of the pituitary gland in fluorescent protein-tagged transgenic mice with long-range microscopy.
(A&B) Experimental schematics. (B) a, Tracheal catheter for motorized ventilation and oxygen and anesthetics supply; b, rectal temperature control; c, heart rate monitor; d, jugular catheter for i.v. injection and blood sampling; e and f, via a small hole in the palate bone, the pituitary surface is continuously irrigated with saline through inlet and outlet tubes, respectively; g, retractor, h, the skull is glued onto a glass slide; i, micropipette holder. (C Left) Palate bone of GH-eGFP anesthetized mouse (C,Right) Corresponding fluorescence image. (D) superficial cell layers of pituitaries from anesthetized GH-eGFP (Left) and PRL-DsRed (Right) mice. **Taken from Lafont C et al. PNAS 2010;107:4465-4470**

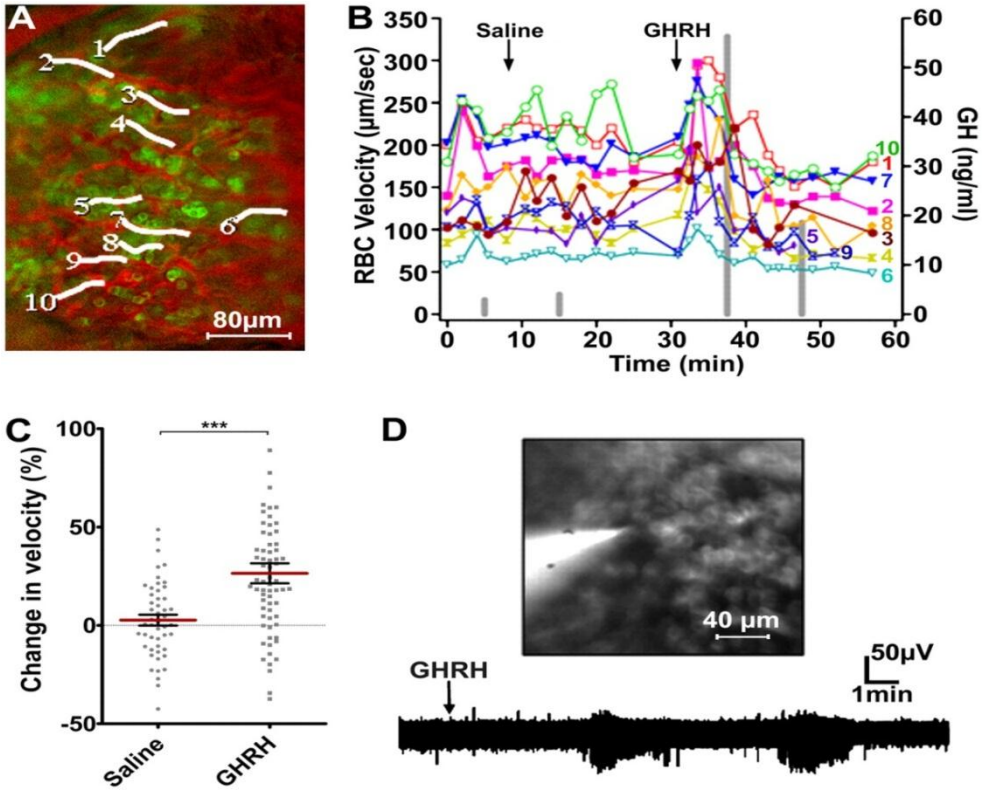

Figure 24. In vivo GHRH-induced changes in blood flow correlated with GH cell function. (A) In vivo imaging of blood flow in a GH-eGFP mouse pituitary. White lines represent vessel branches in which RBC velocities were measured (B). (B) RBC velocities (left axis) and plasma GH (right axis, vertical gray bars) were sequentially measured before, during, after i.v. injections of saline and GHRH, respectively. (C) Distributions of change in velocity (percentage of control RBC velocity) within 5-min periods following i.v. injections of either saline or GHRH. Also represented are means ± SEM for saline and GHRH conditions, respectively (***P < 0.001, n = 8 animals). (D Upper) Extracellular electrical recording of GH-eGFP cells. The patch pipette was filled with a saline solution and Lucifer yellow. (Lower) Jugular injection of GHRH (1 µg) triggered recurrent bursts of spikes. **Taken from Lafont C et al. PNAS 2010;107:4465-4470**

Changes in P_{tissue} O_2 in response to GHRH were achieved by the use of oxygen microsenors inserted into a cell cluster of GH cells. Injections of GHRH triggered downward deflections in P_{tissue} O_2 levels indicative of O_2 consumption (Figure 25 B), and associated with Ca^{2+}-spiking activity in GH cells (Figure 25 I). An attempt to correlated blood flow with O_2 supply was not possible due to the differences in

detection times, that is individual RBC velocities were measured every 15-30s, while P_{tissue} O_2 levels were measured every 0.1s.

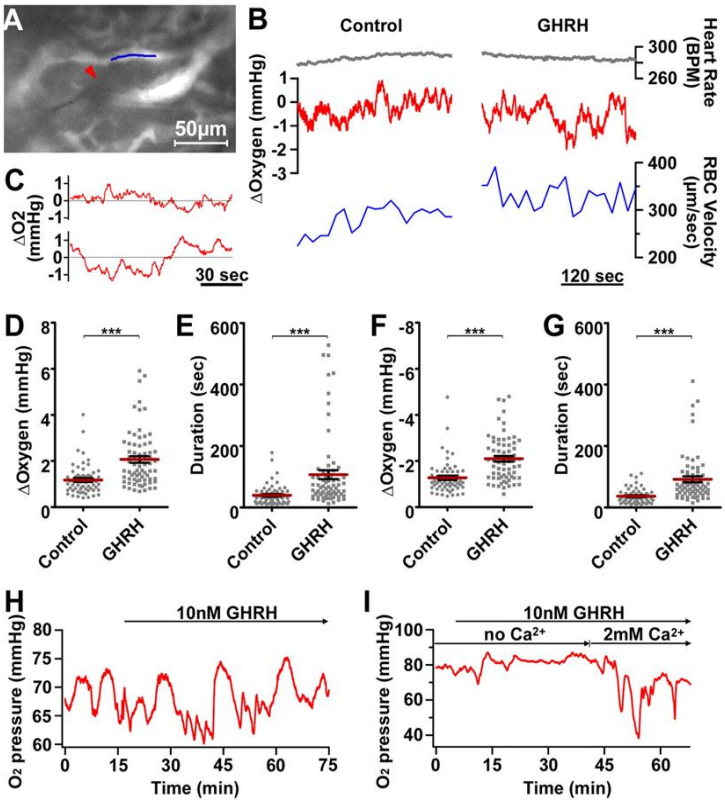

Figure 25. In situ Ptiss, O_2 responses to GHRH. (A–G) In vivo measurements of pituitary Ptiss,O2 levels with oxygen microsensors in anesthetized GH-eGFP mice. (A) The tip (5 µm) of an oxygen sensor (red arrowhead) was inserted into a GH cell cluster (not illustrated). (B) In the same experiment, heart rate (top traces), relative changes in Ptiss,O2 levels (middle traces), and RBC velocity (bottom traces) were monitored before (Left) and after i.v. (Right) GHRH injection. (C) In another experiment, expanded time-lapse recordings of relative changes in Ptiss,O2 levels before (top trace) and after (bottom trace) i.v. GHRH injection. (D and E) Distributions of both amplitude (D) and duration (E) of upward Ptiss,O2 deflections measured before (control) and after (GHRH) secretagogue injection. (F and G) Distributions of both amplitude (F) and duration (G) of downward Ptiss,O2 deflections measured before (control) and after (GHRH) secretagogue injection. (H and I) Monitoring of pituitary Ptiss,O2 levels with oxygen microsensors in acute GH-eGFP pituitary slices perfused with saline solution. **Taken from Lafont C et al. PNAS 2010;107:4465-4470**

As mentioned in previous chapters, ion fluxes are a common signaling response to estrogens and XEs, which can lead to changes in cell mobility, downstream signaling processes, and secretion of peptide hormones (Watson and Gametchu 1999). The authors in this study loaded cells with fluorescent dye fura-2 acetoxymethyl (AM) ester which allowed for real-time measurements (within seconds) of cytosolic Ca^{2+} activities after a bolus injection of GHRH (Figure 26). Spikes of Ca^{2+} activity were displayed across the measured field of interest, demonstrating spontaneous electrical activity of individual cells. Interestingly, this type of methodology is in congruence with in-vitro Ca^{2+} influx studies performed in the Watson lab (Kochukov et al. 2009; Wozniak et al. 2005), and can therefore be used to measure in-vivo Ca^{2+} activities after XE treatment.

“Secretory bursts” of PRL and GH were not directly measured but were modeled by monitoring the uptake, clearance, and flow of fluorescent markers (4kDa or 20kDa rhodamine-labeled dextran) of a similar size to pituitary hormones (4kDa ACTH; 20kDa GH, PRL) from the parenchyma into vessels upon GHRH stimulation. The overall clearance and flow of the fluorescent markers was rapid with larger markers trailing behind smaller ones. All parameters measured occurred within seconds of the GHRH stimulation (Figure 27).Use of model markers as opposed to the already fluorescently-labeled PRL (DsRed) and GH (eGFP) for measuring hormone output is not explained by the authors. I suspect

that direct hormone secretion from secretory vesicles could have been measured by changes in fluorescence intensities upon GHRH treatment.

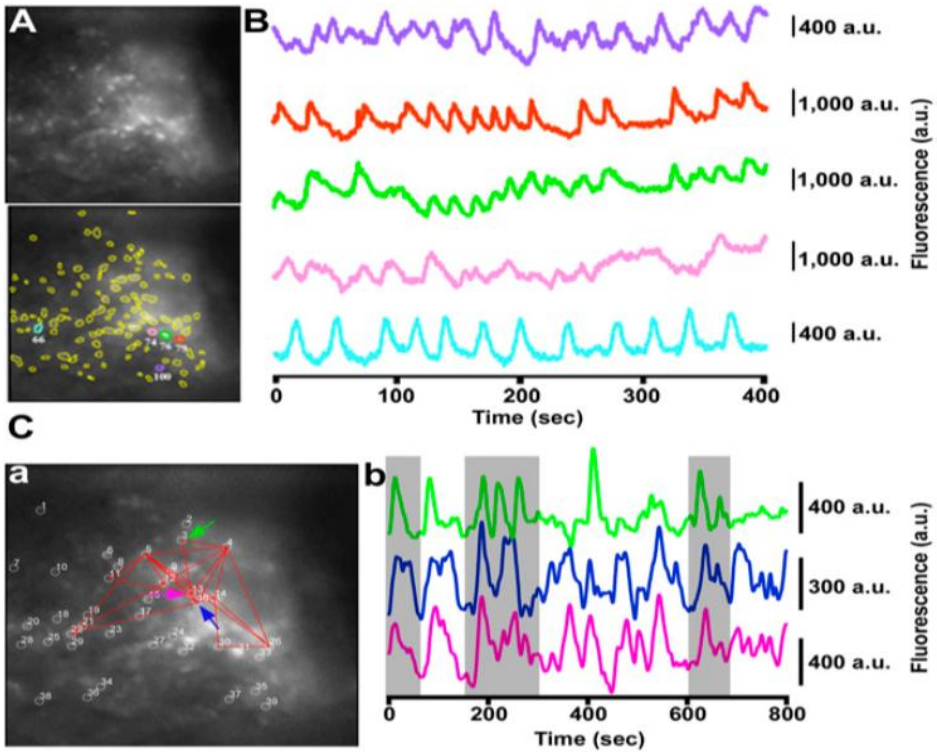

Figure 26. **In vivo calcium imaging of pituitary cells.** (A Top) Overview of a field of the ventral pituitary following fura-2/AM loading with the multibolus cell loading protocol. (Bottom) Same pituitary field with regions of interest (ROIs) corresponding to fura-2-labeled cells. (B) Time-lapse recording of cytosolic calcium activities in representative cells delimited with colored ROIs. (C a and b) Cross-correlogram analysis between calcium recordings among spontaneously active cells. Red lines link cell pairs with R > 0.5. Colored arrows indicate cells from which calcium recordings are illustrated in b. Vertical gray bands point out some episodes of highly correlated calcium activities between these three cells. **Taken from Lafont C et al. PNAS 2010;107:4465-4470**

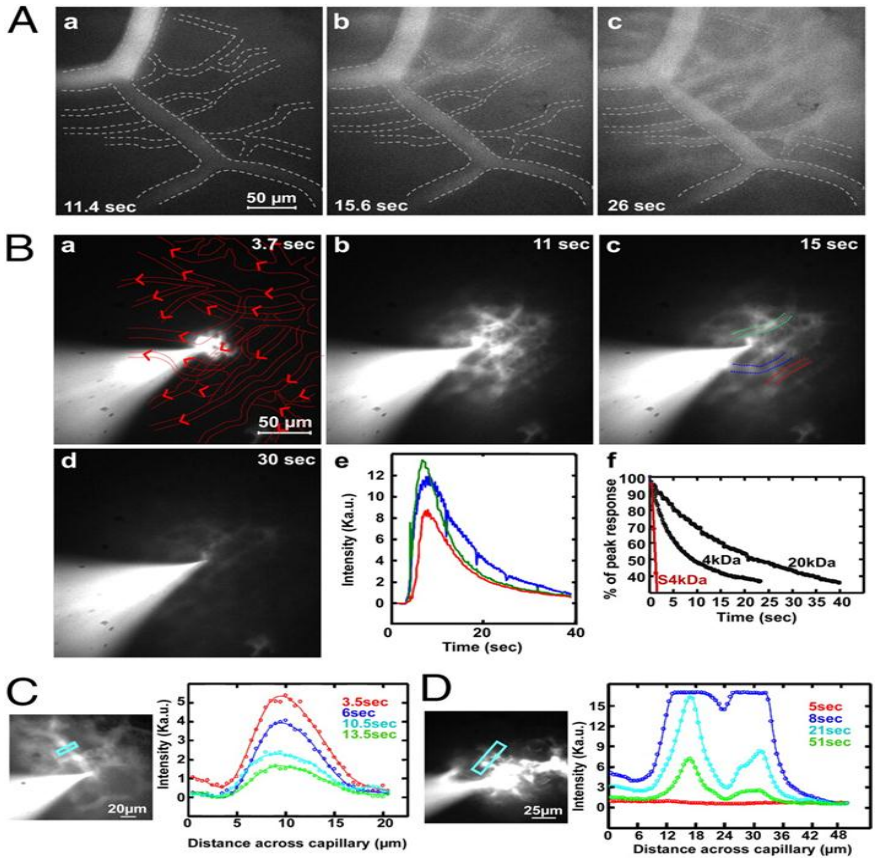

Figure 27. In vivo imaging of incoming molecules through the micro vasculature, fate of products released into the extracellular space, and product clearance at the vascular level. (A) Time-lapse imaging of 4-kDa rhodamine dextran in pituitary vessels at high magnification. Values indicate the time delay after i.v. dye injection. Right-left direction shows the rostrocaudal orientation of the gland. Note that fluorescence signal in the parenchyma was detectable only after small vessels became fluorescent (b and c). Focal release of products was mimicked by iontophoretic injection of 4 kDa rhodamine-labeled dextran (Movie S6). In a, maps of microcirculation and blood flow directions (arrowheads) are marked in red. Values indicate the time delay after dye injection. (e) Fluorescence intensity as a function of time in three vessels close to the site of dye injection (see vessel locations in c). (f) Decay times for fluorescence clearance in blood vessels following iontophoretic injections of 4-kDa and 20-kDa fluorescent dyes, respectively. The red line represents a simulation of the 4-kDa dye clearance from the vessel as its measured blood flow. (C and D) Fluorescence intensity profiles across vessels and nearby perivascular spaces (Left, blue windows) after iontophoretic injections of 4-kDa (C) or 20-kDa (D) rhodamine-labeled dextrans, respectively. **Taken from Lafont C et al. PNAS 2010; 107: 4465-4470**

In summary, the adaption/translation of our in-vitro non-genomic experiments to animal-based models has been a long wished for method of inquiry for our lab, and the topic of much discussion at any seminar presented. Methodologies such as the one summarized here (from Lafont et al.) may indeed offer an option for directly determining how rapid non-genomic actions stimulated by XEs may influence physiologic functions.

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VITA

Mr. René Viñas was born December 21, 1980 in Eagle Pass, Texas, to René Gilberto Viñas Pineda, M.D. and Mrs. Leonor Viñas-Trillo, R.N., B.S.Ed. Mr. Vinas received his formal education through the Eagle Pass Independent School District and graduated from C.C. Winn High School in May 1999. He received his Bachelor of Science in Cell and Molecular Biology from Texas Tech University in May, 2004. In September of the same year, he began work on his Masters of Science degree in Environmental Toxicology at The Institute of Environmental and Human Health (Texas Tech University) under the direction of Ernest Smith, PhD, where he graduated in May 2007 focusing on development of toxicological *in-vitro* screening assays for Latin American herbal remedies. In September 2007, he entered the Graduate School of Biomedical Sciences at UTMB in the Department of Pharmacology and Toxicology joining the lab of Cheryl S. Watson, PhD, in October 2010. His PhD work focused on the *in-vitro* toxicological assessment of chemical mixtures of endocrine disrupting chemicals.

Mr. Viñas married Mrs. Carolina Máynez-Viñas, B.A. on August 29, 2009, in Santo Domingo Cathedral in the city of Oaxaca, Mexico. Mr. Viñas plans to follow a career in regulatory toxicology.

EDUCATION

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Pharmacology & Toxicology, University of Texas Medical Branch, Galveston, TX

2007- Master of Science
Environmental Toxicology, Texas Tech University, Lubbock, TX

2004- Bachelor of Science
Cell & Molecular Biology, Texas Tech University, Lubbock, TX

PEER REVIEWED SCIENTIFIC PUBLICATIONS

Zhu Jinqiu; **Viñas, René**; Smith, Ernest E. 2008. In vitro evaluation of human liver cancer cells and the potential cytotoxicity of Tecoma stans (Bignoniaceae) and Brickellia cavanillesi (Asteraceae) both single and in combination. Toxicological & Environmental Chemistry, 90:4,801-808.

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ABSTRACTS & PRESENTATIONS

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