ehp ENVIRONMENTAL HEALTH PERSPECTIVES

ehponline.org

Bisphenol S Disrupts Estradiol-Induced Nongenomic Signaling in a Rat Pituitary Cell Line: Effects on Cell Functions

René Viñas and Cheryl S. Watson

http://dx.doi.org/10.1289/ehp.1205826

Online 17 January 2013

NIEHS
National Institute of
Environmental Health Sciences

National Institutes of Health
U.S. Department of Health and Human Services

Bisphenol S Disrupts Estradiol-Induced Nongenomic Signaling in a Rat Pituitary Cell Line: Effects on Cell Functions

René Viñas and Cheryl S. Watson

Department of Biochemistry and Molecular Biology, University of Texas Medical Branch Galveston, Texas, USA

Corresponding author: Cheryl S. Watson, PhD, Professor, Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, Texas 77555-0645, USA. Telephone/fax: (409) 772-2383. Email: cswatson@utmb.edu

Running title: Bisphenol S Disrupts Physiologic Actions of Estradiol

Key words: Bisphenol S, ERα, ERK activation, JNK activation, membrane estrogen receptors, non-genomic effects, prolactinoma cell line, xenoestrogens

Acknowledgements and grant support: Passport Foundation, F31- Ruth L. Kirschstein National Research Service Award. We thank Dr. David Konkel for critically editing the manuscript.

The authors declare that they have no competing financial interests.

Abbreviations: (Ab) antibody, (BPA) Bisphenol A, (BPS) Bisphenol S, (JNK) c-Jun-N-terminal kinase, (DMEM) Dulbecco's modified Eagle medium, (ERKs) extracellular signal regulated kinases, (E_2) estradiol, (ER) estrogen receptor, (mERα) membrane estrogen receptor-α, (mERβ) membrane estrogen receptor-β, (MAPKs) mitogen-activated protein kinases, (pERK) phosphorylated ERK, (PRL) prolactin, (XE) xenoestrogen

ABSTRACT

Background: Bisphenol A (BPA) is a well-known endocrine disruptor that imperfectly mimics the effects of physiologic estrogens via membrane-bound estrogen receptors (mERα, mERβ, GPER/GPR30), thereby initiating non-genomic signaling. Bisphenol S (BPS) is an alternative to BPA in plastic consumer products and thermal paper.

Objective: To characterize the non-genomic activities of BPS, we examined signaling pathways it evoked in $GH_3/B_6/F_{10}$ rat pituitary cells, alone and together with the physiologic estrogen estradiol (E_2). Extracellular signal-regulated kinase (ERK)- and c-Jun-N-terminal kinase (JNK)-specific phosphorylations were examined for their correlation to three functional responses – proliferation, caspase activation, and prolactin (PRL) release.

Methods: We detected ERK and JNK phosphorylations by fixed-cell immunoassays, identified the predominant mER initiating the signaling with selective inhibitors, estimated cell numbers by crystal violet assays, measured caspase activity by cleavage of fluorescent caspase substrates, and measured PRL release by radioimmunoassay.

Results: BPS phospho-activated ERK within 2.5 min, in a non-monotonic dose-dependent manner (10^{-15}-10^{-7}M). When combined with 10^{-9}M E_2, the physiologic estrogen's ERK response was attenuated. BPS could not activate JNK, but greatly enhanced E_2-induced JNK activity. BPS caused cell proliferation at low concentrations (fM to nM), similar to E_2. Combinations of both estrogens reduced cell numbers below the vehicle control, and activated caspases. Earlier activation of caspase 8 vs. 9 demonstrated that BPS initiates apoptosis via the extrinsic pathway, consistent with activation via a membrane receptor. BPS also inhibited rapid (≤1 min) E_2-induced PRL release.

Conclusion: BPS, once considered a safe BPA substitute, disrupts membrane-initiated E_2-induced cell signaling, leading to altered cell proliferation, cell death, and PRL release.

INTRODUCTION

Xenoestrogens (XEs) are a diverse group of synthetic agents (e. g. pesticides, surfactants, and plastics monomers) that can mimic and disrupt the actions of physiologic estrogens (Colborn et al. 1993; Le and Belcher 2010; McLachlan 2001; Soto et al. 1994). Many XEs can remain in the environment for a long time, thus increasing the likelihood for human and wildlife exposure (Ahel et al. 1993; deJager C. et al. 1999; Dekant and Volkel 2008; Judson et al. 2010).

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).

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 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, commonly known as bisphenol S (BPS). 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 4 hr 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. 2005). Discrepancies between these studies were attributed to species (yeast vs. mammalian) differences (Kuruto-Niwa et al. 2005). 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.

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.

MATERIALS AND METHODS

Cell Culture

The clonal rat prolactinoma cell line $GH_3/B_6/F_{10}$ was selected on the basis of its naturally high expression of mERα (Pappas et al. 1994; Pappas et al. 1995a), enabling it to respond robustly in tests for non-genomic signaling and functional endpoints. 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.

Concentration Ranges Selected

All concentrations for time courses and dose-responses were chosen based on our previous studies (Jeng et al. 2009; Jeng et al. 2010; Jeng and Watson 2011; Kochukov et al. 2009) that demonstrated expected potencies, efficacies, and rapidity of the responses. The chosen concentrations of BPS reflect the range of concentrations likely to be found in the environment, centering on reported urinary levels in humans (0.299ng/ml or 1.2nM), found in Albany, NY residents (Liao et al. 2012a; Liao et al. 2012b). Lower concentrations are of interest to determine how sensitive biological systems are to presumably more widespread exposure concentrations. When we used such concentrations for other XEs, they were able to activate MAPKs and caspases, and disrupt PRL secretion.

Quantitative ERK and JNK Phosphorylation Assays

To quantify phospho-activation of ERK (pERK) and JNK (pJNK), a fixed cell-based immunoassay was employed, as previously developed and described in detail (Bulayeva and Watson 2004). Cells (10^4/well) were plated in 96-well plates (Corning Incorporated, Corning, NY) and allowed to attach for 24hrs. The original plating media was then replaced with DMEM containing 1% charcoal-stripped (4X) serum for 48hrs to deprive cells of serum hormones. The media were then removed and cells exposed to BPS [10^{-15}-10^{-7}M], BPA [10^{-15}M], or E_2 [10^{-9}M] (all from Sigma-Aldrich St. Louis, Mo) to assess time- (0-60min) and concentration-dependent changes. Test compounds were dissolved in ethanol then diluted in DMEM containing 1% charcoal-stripped serum. The vehicle control 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) at 4°C for 48 hrs. Once fixed, cells were incubated with phosphate-buffered saline (PBS) containing 0.2% fish gelatin and 0.1% Triton X-100 (Sigma-Aldrich) for 1 hr at room temperature (RT), then with primary antibodies (Abs) against pERK or pJNK (Cells Signaling Technology, Beverly, MA) (1:500 in PBS/0.2% fish gelatin/0.1% TritonX-100) overnight at 4°C. Cells were then washed 3X with PBS before a 1hr incubation at RT with a biotin-conjugated secondary Ab (Vector Labs, Burlingame, CA) (1:500 in PBS/0.2% fish gelatin), then again washed in PBS (3X) and incubated with Vectastain ABC-AP solution (Vector Labs) (50µL/well) for 1 hr at RT, followed by Vectastain alkaline phosphatase substrate (pNpp solution) (50µL/well). Plates were then incubated in the dark for 30min at 37°C, and the

signal for the product *para*-nitrophenol (pNp) read at A_{405} in a model 1420 Wallac microplate reader (Perkin Elmer, Boston, MA).

Crystal Violet (CV) Assays

The pNp signal was normalized to cell number, determined by the crystal violet assay (Campbell and Watson 2002). Alkaline phosphatase reaction reagents were removed with an H_2O wash (2X) and the plates dried at RT for 1hr. CV solution (0.1% in H_2O, filtered) was added (50µL/well), incubated for 1hr at RT, and washed 4X with H_2O. Dye was released from the cells with 50µL/well acetic acid (10% in H_2O) at RT for 30 min, and the A_{590} signal read in the Wallac microplate reader.

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]-1*H*-pyrazole dihydrochloride), ERβ ([PHTTP]- 4-[2-Phenyl-5,7-*bis*(trifluoromethyl) pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol), and GPER/GPR30 ([G15]-(3a*S**,4*R**,9b*R**)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3*H*-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^{-14}M] 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.

Determination of Cell Proliferation

As described in previous studies (Jeng and Watson 2009), sub-confluent cells were seeded into 96-well plates 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 48hrs, and finally with treatment medium containing increasing concentrations of BPS [10^{-15}-10^{-7}M] alone and in combination with E_2 [10^{-9}M]. After 3 days, cells were fixed (2% paraformaldehyde/0.1% glutaraldehyde in PBS; 50µl/well) for 20min at RT. Cell numbers were assessed by CV assay to compare the proliferative effects of BPS at different concentrations.

Determination of Caspase Activity

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 24hrs to the following treatments in DMEM-1% 4X charcoal-stripped serum-containing media: BPS [10^{-14}M]; BPS [10^{-8}M]; BPS [10^{-14}M] +E_2 [10^{-9}M]; BPS [10^{-8}M] + E_2 [10^{-9}M]. At designated times, 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. To perform

caspase assays, frozen plates were defrosted at 4˚C and assay buffer (50mM HEPES; 100mM NaCl; 0.1% CHAPS; 1mM EDTA; 10% glycerol) (50µl/well) was then added. Freshly prepared 10mM DTT and caspase 8 (Ac-IETD-AFC) or 9 (Ac-LEHD-AFC) substrates (Enzo Life Sciences, Farmingdale, NY) were added to the assay buffer at final concentrations of 50µM. Plates were then incubated in the dark (37˚C) for a period of 2hrs. The released fluorescent product 7-amino-4-trifluoromethylcoumarin (AFC) was read using a Flexstation 3 spectrofluorometer (Molecular Devices, Sunnyvale, CA; excitation wave-length: 400nm; emission wavelength, 505nm).

Prolactin Release

These study designs and conditions were based on previous studies from our lab (Kochukov et al. 2009;Wozniak et al. 2005). Cells (0.5–0.7× 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 incubated for 30 min in DMEM/0.1% BSA and exposed to different concentrations of BPS alone (10^{-15} -10^{-7}M) or in combination with E_2 [10^{-9}] for 1min, 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.

Statistical Analysis

Statistical analysis was performed using SigmaPlot version 12 (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 XE exposures. A Holm-Sidak comparison against vehicle control or against E_2 treatment was used after the ANOVA to evaluate significance. The overall α level selected for the statistical analysis was 0.05. We additionally ran a Student's T-test where the significance between some values was borderline by One-Way ANOVA, and this variation is noted by different significance symbols on the graph where that occurs.

RESULTS

Exposure to BPS caused ERK activation in $GH_3/B_6/F_{10}$ cells at 5 min (Figure 1A) 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 1B), but instead caused deactivation significantly below vehicle levels at the highest (10^{-7}M) concentration. 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 2A and 2B). 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. 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 2B).

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 3). 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 4).

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 5A). 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 5B). 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, with some timing differences from our previous studies (Jeng and Watson 2009).

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 6). 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.

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. 2005; 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 (Jeng and Watson 2011; Alyea et al. 2008), 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. 2005), 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 24 hr 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.

References

Ahel M, McEvoy J, Giger W. 1993. Bioaccumulation of the lipophilic metabolites of nonionic surfactants in freshwater organisms. Environ Pollut 79:243-248.

Allan LA, Clarke PR. 2007. Phosphorylation of caspase-9 by CDK1/cyclin B1 protects mitotic cells against apoptosis. Mol Cell 26:301-310.

-----. 2009. Apoptosis and autophagy: Regulation of caspase-9 by phosphorylation. FEBS J 276: 6063-6073.

Allan LA, Morrice N, Brady S, Magee G, Pathak S, Clarke PR. 2003. Inhibition of caspase-9 through phosphorylation at Thr 125 by ERK MAPK. Nat Cell Biol 5: 647-654.

Alonso-Magdalena P, Vieira E, Soriano S, Menes L, Burks D, Quesada I, et al. 2010. Bisphenol A exposure during pregnancy disrupts glucose homeostasis in mothers and adult male offspring. Environ Health Perspect 118:1243-1250.

Alyea RA, Laurence S E, Kim S H, Katzenellenbogen B S, Katzenellenbogen J A, and Watson, C. S. 2008. The roles of membrane estrogen receptor subtypes in modulating dopamine transporters in PC-12 cells. J Neurochem. 106(4):1525-1533.Alyea RA, Watson CS. 2009. Nongenomic mechanisms of physiological estrogen-mediated dopamine efflux. BMC Neurosci 16:59-68.

Bisphenol A Global Industry Group. 2002. Bisphenol A: Information Sheet. Available: http://bisphenol-a.org/pdf/DiscoveryandUseOctober2002.pdf. [accessed 5 September 2012].

Bole-Feysot C, Goffin V, Edery M, Binart N, Kelly PA. 1998. Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. Endocr Rev 19:225-268.

Bulayeva NN, Gametchu B, Watson CS. 2004. Quantitative measurement of estrogen-induced ERK 1 and 2 activation via multiple membrane-initiated signaling pathways. Steroids 69:181-192.

Bulayeva NN, Watson CS. 2004. Xenoestrogen-induced ERK-1 and ERK-2 activation via multiple membrane-initiated signaling pathways. Environ Health Perspect 112:1481-1487.

Bulayeva NN, Wozniak A, Lash LL, Watson CS. 2005. Mechanisms of membrane estrogen receptor-{alpha}-mediated rapid stimulation of Ca2+ levels and prolactin release in a pituitary cell line. Am J Physiol Endocrinol Metab 288:E388-E397.

Calafat AM, Ye X, Wong LY, Reidy JA, Needham LL. 2008. Exposure of the U.S. population to bisphenol A and 4-tertiary-octylphenol: 2003-2004. Environ Health Perspect 116:39-44.

Campbell CH, Watson CS. 2002. Regulation of the membrane estrogen receptor-α (mER-α) in the rat pituitary tumor cell line GH3/B6: The influence of cell density, passage number, serum starvation, and estradiol. FASEB J. 16(14):1917-1927.

Chen MY, Ike M, Fujita M. 2002. Acute toxicity, mutagenicity, and estrogenicity of bisphenol-A and other bisphenols. Environ Toxicol 17:80-86.

Colborn T, vom Saal FS, Soto AM. 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 101:378-384.

Conolly RB, Lutz WK. 2004. Nonmonotonic Dose-Response Relationships: Mechanistic Basis, Kinetic Modeling, and Implications for Risk Assessment. Toxicological Sciences 77:151-157.

deJager C., Bornman MS, Oosthuizen JM. 1999. The effect of p-nonylphenol on the fertility potential of male rats after gestational, lactational and direct exposure. Andrologia 31:107-113.

Dekant W, Volkel W. 2008. Human exposure to bisphenol A by biomonitoring: methods, results and assessment of environmental exposures. Toxicol Appl Pharmacol 228:114-134.

Dhanasekaran DN, Reddy EP. 2008. JNK signaling in apoptosis. Oncogene 27:6245-6251.

Dufy B, Vincent J-D, Fleury H, Pasquier PD, Gourdji D, Vidal AT. 1979. Membrane effects of thyrotropin-releasing hormone and estrogen shown by intracellular recording from pituitary cells. Science 204:509-511.

EPA. 1993. Integrated Risk Information System. Bisphenol A. (CASRN 80-05-7). Available online: http://www.epa.gov/ncea/iris/subst/0356.htm. [accessed 5 September 2012].

European Commission. 2011. Bisphenol A: EU ban on baby bottles to enter into force tomorrow. Available online: http://europa.eu/rapid/pressReleasesAction.do?reference=IP/11/664&format=HTML. [accessed 5 September 2012].

FDA. 2012. Indirect Food Additives: Polymers.Docket No. FDA-2012-F-0031. Available: http://www.gpo.gov/fdsys/pkg/FR-2012-07-17/pdf/2012-17366.pdf. [accessed 8 October 2012].

Gallar-Ayala H, Moyano E, Galceran MT. 2011. Analysis of bisphenols in soft drinks by on-line solid phase extraction fast liquid chromatography-tandem mass spectrometry. Analytica Chimica Acta 683:227-233.

Hashimoto Y, Moriguchi Y, Oshima H, Kawaguchi M, Miyazaki K, Nakamura M. 2001. Measurement of estrogenic activity of chemicals for the development of new dental polymers. Toxicol In Vitro 15:421-425.

Hashimoto Y, Nakamura M. 2000. Estrogenic activity of dental materials and bisphenol-A related chemicals in vitro. Dent Mater J 19:245-262.

Health Canada. 2009. Government of Canada Acts to Protect Newborns and Infants from Bisphenol A in Polycarbonate Plastic Baby Bottles. Available online: http://hc-sc.gc.ca/ahc-asc/media/nr-cp/_2009/2009_106-eng.php. [accessed 1 September2012].

Hunter T. 1995. Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. Cell 80:225-236.

Ip YT, Davis RJ. 1998. Signal transduction by the c-Jun N-terminal kinase (JNK)--from inflammation to development. Curr Opin Cell Biol 10:205-219.

Jeng YJ, Kochukov M, Watson CS. 2010. Combinations of physiologic estrogens with xenoestrogens alter calcium and kinase responses, prolactin release, and membrane estrogen receptor trafficking in rat pituitary cells. Environ Health 9:61-74.

Jeng YJ, Kochukov MY, Watson CS. 2009. Membrane estrogen receptor-alpha-mediated nongenomic actions of phytoestrogens in GH3/B6/F10 pituitary tumor cells. J Mol Signal 4:2-13.

Jeng YJ, Watson CS. 2009. Proliferative and anti-proliferative effects of dietary levels of phytoestrogens in rat pituitary GH3/B6/F10 cells - the involvement of rapidly activated kinases and caspases. BMC Cancer 9:334-351.

-----. 2011. Combinations of physiologic estrogens with xenoestrogens alter ERK phosphorylation profiles in rat pituitary cells. Environ Health Perspect 119:104-112.

Judson RS, Martin MT, Reif DM, Houck KA, Knudsen TB, Rotroff DM, et al. 2010. Analysis of eight oil spill dispersants using rapid, in vitro tests for endocrine and other biological activity. Environ Sci Technol 44:5979-5985.

Junttila MR, Li SP, Westermarck J. 2008. Phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival. FASEB J 22:954-965.

Kochukov MY, Jeng Y-J, Watson CS. 2009. Alkylphenol xenoestrogens with varying carbon chain lengths differentially and potently activate signaling and functional responses in GH_3/B_6/F10 somatomammotropes. Env Health Perspect 117:723-730.

Kruidering M, Evan GI. 2000. Caspase-8 in apoptosis: the beginning of "the end"? IUBMB Life 50:85-90.

Kubwabo C, Kosarac I, Stewart B, Gauthier BR, Lalonde K, Lalonde PJ. 2009. Migration of bisphenol A from plastic baby bottles, baby bottle liners and reusable polycarbonate drinking bottles. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 26:928-937.

Kuruto-Niwa R, Nozawa R, Miyakoshi T, Shiozawa T, Terao Y. 2005a. Estrogenic activity of alkylphenols, bisphenol S, and their chlorinated derivatives using a GFP expression system. Environ Toxicol Pharmacol 19:121-130.

Le HH, Belcher SM. 2010. Rapid Signaling Actions of Environmental Estrogens in Developing Granule Cell Neurons Are Mediated by Estrogen Receptor. Endocr 151:5689-5699.

Li DK, Zhou Z, Miao M, He Y, Wang J, Ferber J, et al. 2011. Urine bisphenol-A (BPA) level in relation to semen quality. Fertil Steril 95:625-630.

Liao C, Liu F, Alomirah H, Loi VD, Mohd MA, Moon HB, et al. 2012a. Bisphenol s in urine from the United States and seven asian countries: occurrence and human exposures. Environ Sci Technol 46:6860-6866.

Liao C, Liu F, Kannan K. 2012b. Bisphenol s, a new bisphenol analogue, in paper products and currency bills and its association with bisphenol a residues. Environ Sci Technol 46:6515-6522.

McCubrey JA, Steelman LS, Chappell WH, Abrams SL, Wong EW, Chang F, et al. 2007. Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. Biochim Biophys Acta 1773:1263-1284.

McLachlan JA. 2001. Environmental signaling: what embryos and evolution teach us about endocrine disrupting chemicals. Endocr Rev 22:319-341.

Medema JP, Scaffidi C, Kischkel FC, Shevchenko A, Mann M, Krammer PH, et al. 1997. FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). EMBO J 16:2794-2804.

Meloche S, Pouyssegur J. 2007. The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition. Oncogene 26:3227-3239.

Midoro-Horiuti T, Tiwari R, Watson CS, Goldblum RM. 2010. Maternal bisphenol a exposure promotes the development of experimental asthma in mouse pups. Environ Health Perspect 118: 273-277.

Nordstrom E, Fisone G, Kristensson K. 2009. Opposing effects of ERK and p38-JNK MAP kinase pathways on formation of prions in GT1-1 cells. FASEB J 23:613-622.

Oehlmann J, Schulte-Oehlmann U, Kloas W, Jagnytsch O, Lutz I, Kusk KO, et al. 2009. A critical analysis of the biological impacts of plasticizers on wildlife. Philos Trans R Soc Lond B Biol Sci 364:2047-2062.

Pappas TC, Gametchu B, Watson CS. 1995a. Membrane estrogen receptor-enriched GH_3/B6 cells have an enhanced non-genomic response to estrogen. Endocrine 3:743-749.

-----. 1995b. Membrane estrogen receptors identified by multiple antibody labeling and impeded-ligand binding. FASEB J 9:404-410.

Pappas TC, Gametchu B, Yannariello-Brown J, Collins TJ, Watson CS. 1994. Membrane estrogen receptors in GH3/B6 cells are associated with rapid estrogen-induced release of prolactin. Endocrine 2:813-822.

Sanchez-Perez I, Murguia JR, Perona R. 1998. Cisplatin induces a persistent activation of JNK that is related to cell death. Oncogene 16:533-540.

Simoneau C, Valzacchi S, Morkunas V, Van den Eede L. 2011. Comparison of migration from polyethersulphone and polycarbonate baby bottles. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 28:1763-1768.

Sohoni P, Tyler CR, Hurd K, Caunter J, Hetheridge M, Williams T, et al. 2001. Reproductive effects of long-term exposure to Bisphenol A in the fathead minnow (Pimephales promelas). Environ Sci Technol 35:2917-2925.

Soto AM, Chung KL, Sonnenschein C. 1994. The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen-sensitive cells. Environ Health Perspect 102:380-383.

Vandenberg LN, Colborn T, Hayes TB, Heindel JJ, Jacobs DR, Jr., Lee DH, et al. 2012. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. Endocr Rev 33:378-455.

Vinas P, Campillo N, Martinez-Castillo N, Hernandez-Cordoba M. 2010. Comparison of two derivatization-based methods for solid-phase microextraction-gas chromatography-mass spectrometric determination of bisphenol A, bisphenol S and biphenol migrated from food cans. Anal Bioanal Chem 397:115-125.

Watson CS, Jeng YJ, Hu G, Wozniak A, Bulayeva N, Guptarak J. 2011. Estrogen- and xenoestrogen-induced ERK signaling in pituitary tumor cells involves estrogen receptor-alpha interactions with G protein-alphai and caveolin I. Steroids 77:424-432.

Watson CS, Jeng YJ, Kochukov MY. 2010. Nongenomic signaling pathways of estrogen toxicity. Toxicol Sci 115:1-11.

Weltje L, vom Saal FS, Oehlmann J. 2005. Reproductive stimulation by low doses of xenoestrogens contrasts with the view of hormesis as an adaptive response. Hum Exp Toxicol 24:431-437.

Wozniak AL, Bulayeva NN, Watson CS. 2005. Xenoestrogens at picomolar to nanomolar concentrations trigger membrane estrogen receptor-alpha-mediated Ca2+ fluxes and prolactin release in GH3/B6 pituitary tumor cells. Environ Health Perspect 113:431-439.

Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. 1995. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270:1326-1331.

Zalko D, Jacques C, Duplan H, Bruel S, Perdu E. 2011. Viable skin efficiently absorbs and metabolizes bisphenol A. Chemosphere 82:424-430.

Zhou J, Zhu XS, Cai ZH. 2011. The impacts of bisphenol A (BPA) on abalone (Haliotis diversicolor supertexta) embryonic development. Chemosphere 82:443-450.

FIGURE LEGENDS

Figure 1. 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.

Figure 2. 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.

Figure 3. 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 4. 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.

Figure 5. 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.

Figure 6. 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.

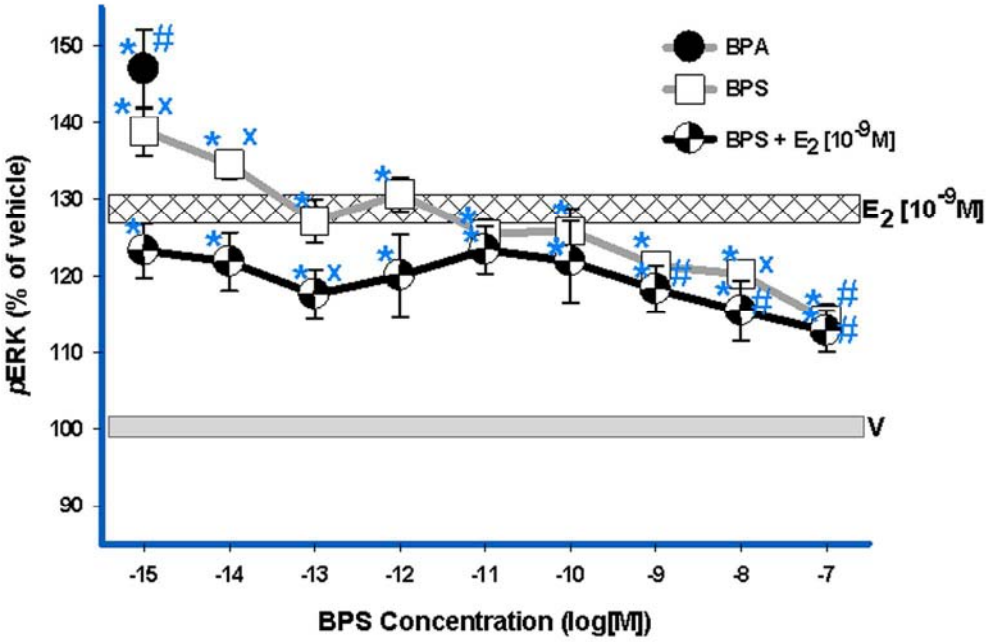

Figure 1A

201x155mm (300 x 300 DPI)

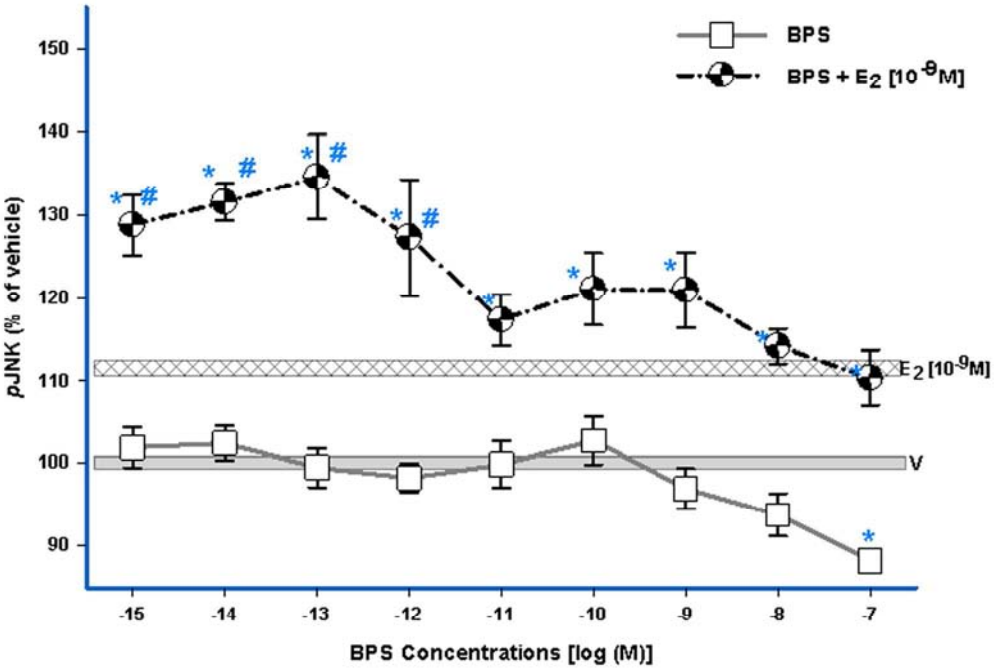

Figure 1B

174x130mm (300 x 300 DPI)

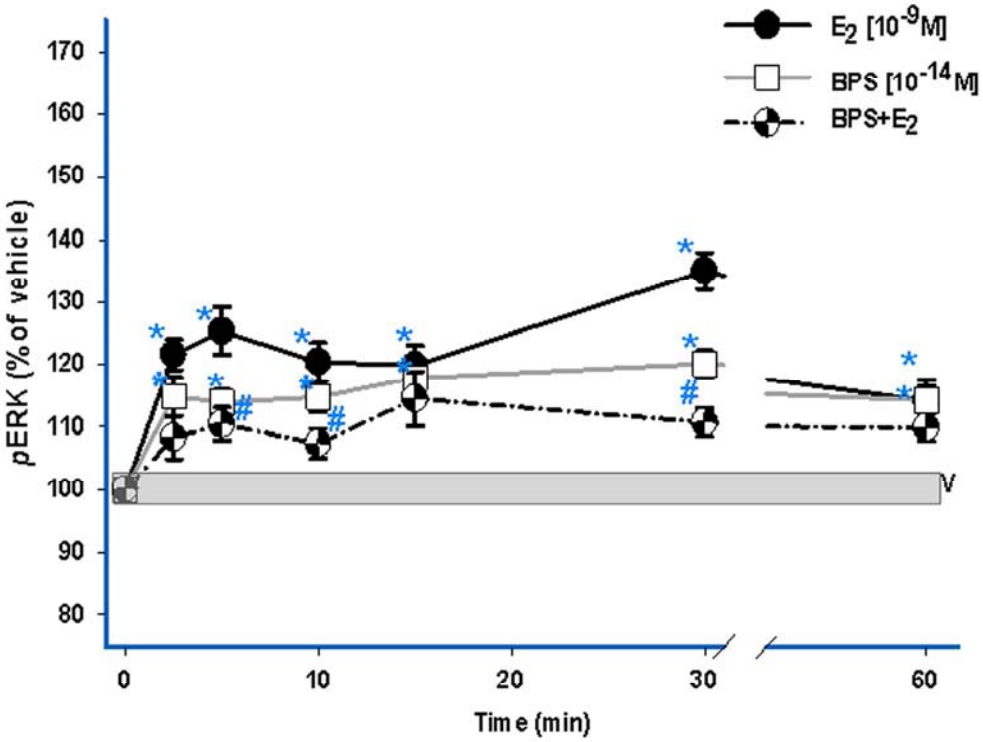

Figure 2A

178x144mm (300 x 300 DPI)

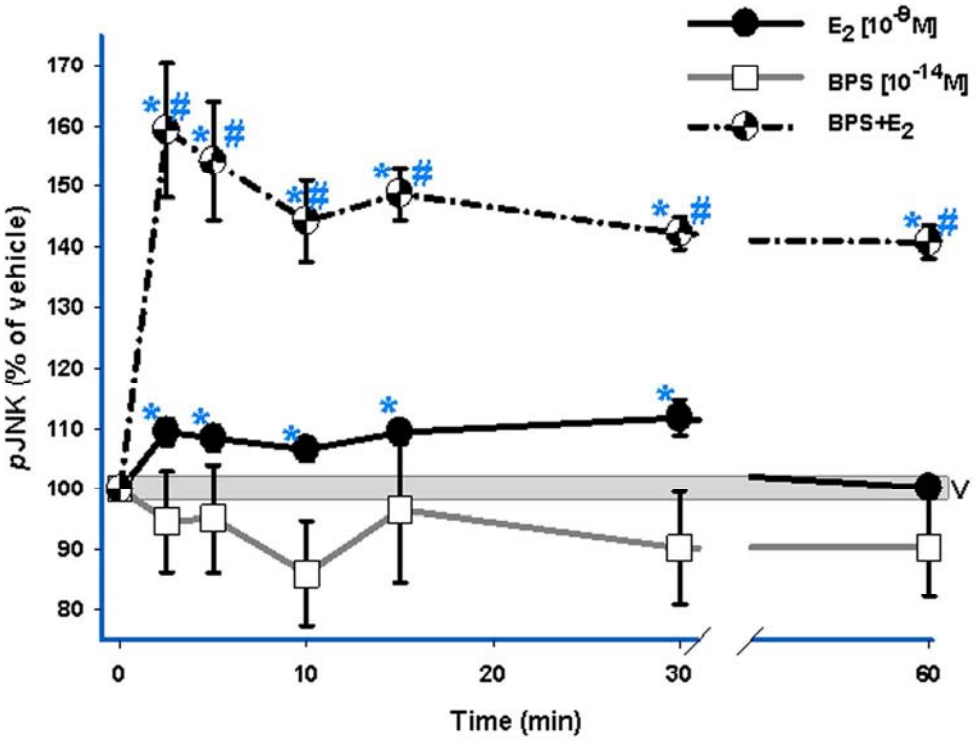

Figure 2B

180x151mm (300 x 300 DPI)

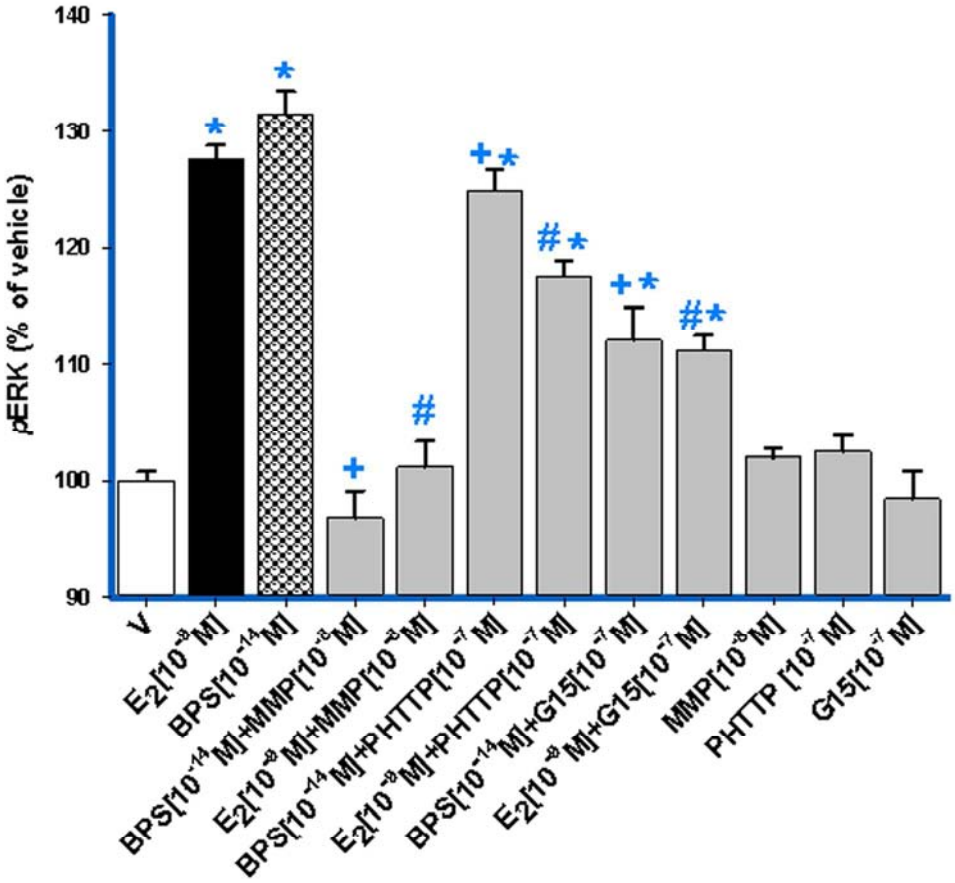

Figure 3
191x194mm (300 x 300 DPI)

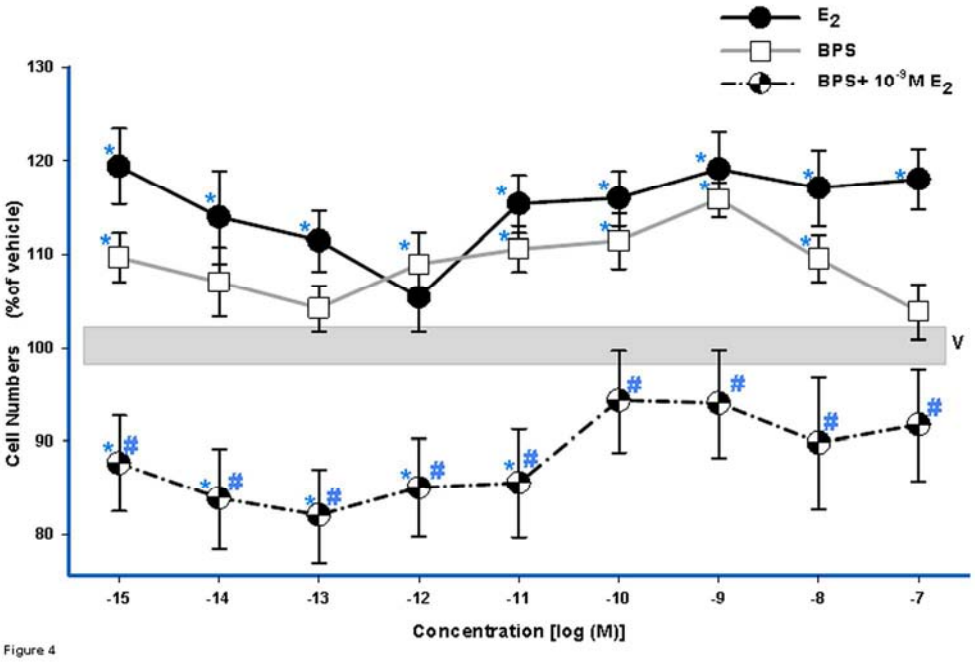

Figure 4

192x135mm (300 x 300 DPI)

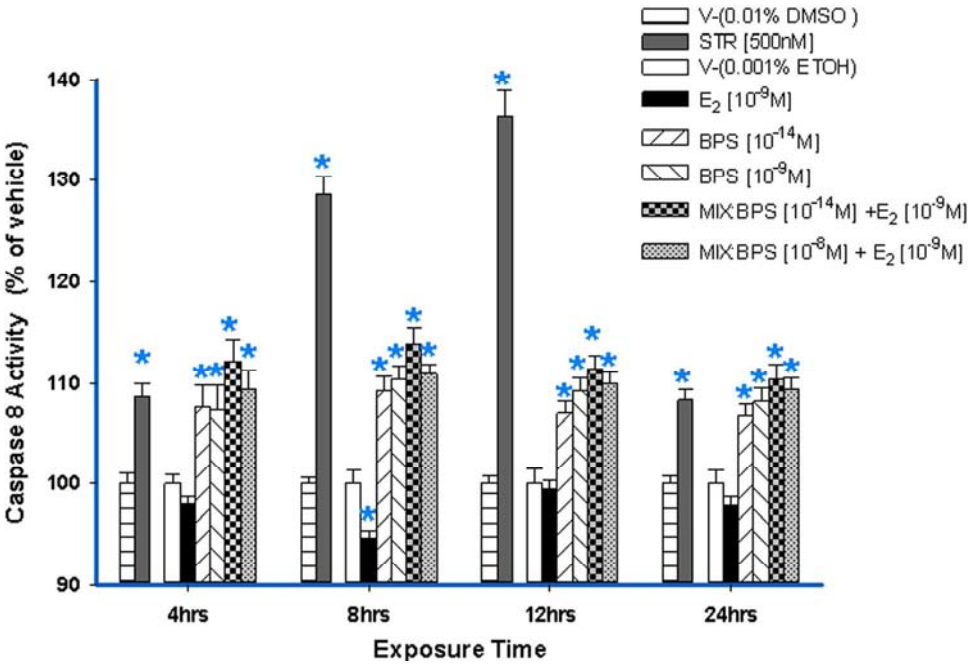

Figure 5a

184x142mm (300 x 300 DPI)

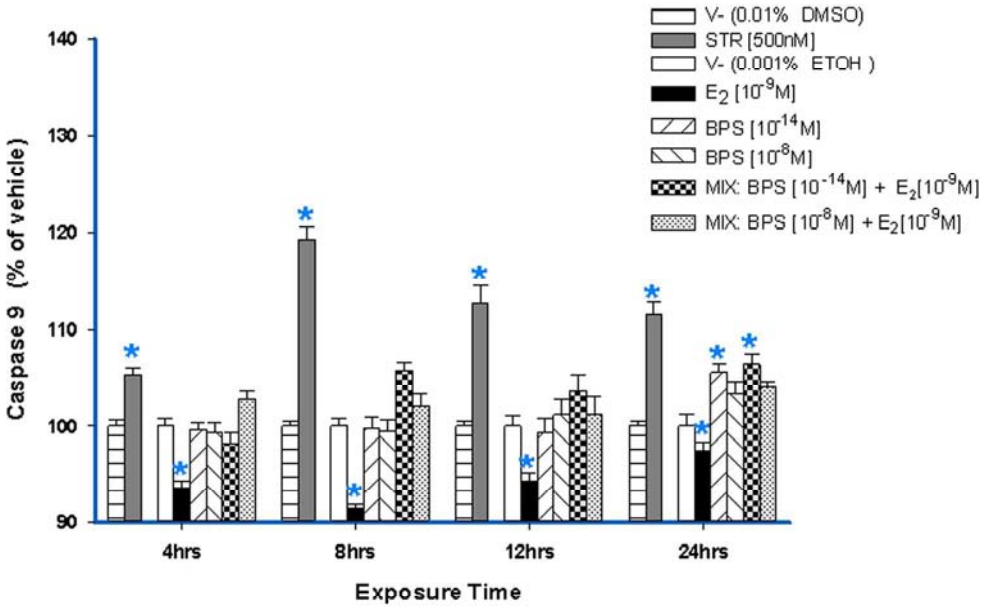

Figure 5b

187x138mm (300 x 300 DPI)

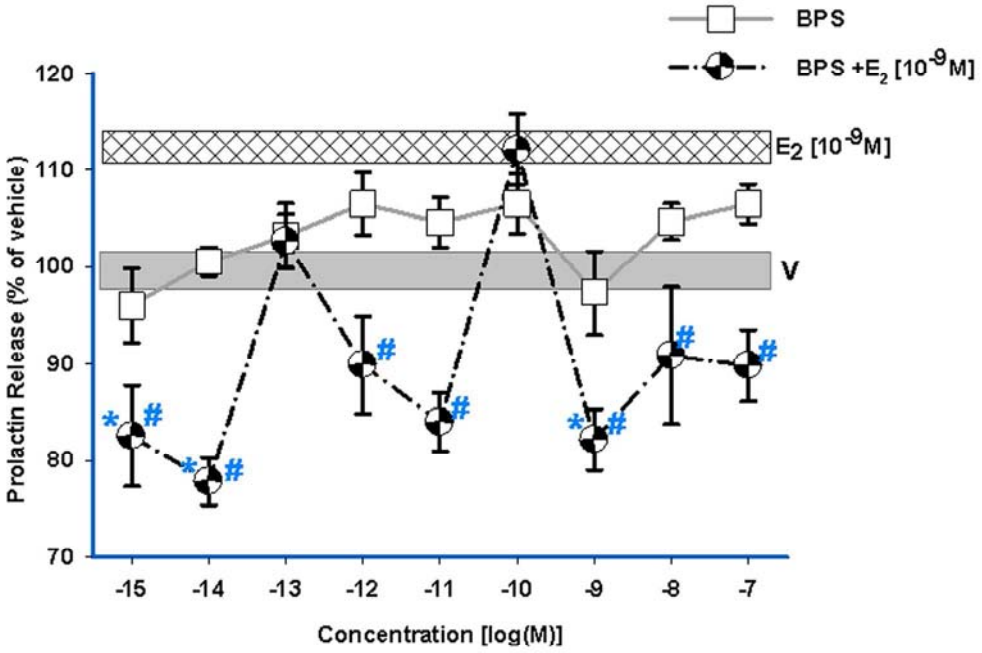

Figure 6

192x146mm (300 x 300 DPI)