DOES BPS CAUSE LIPID SYNTHESIS AND ER STRESS IN HEPG2 CELLS?

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by

David M. Lollar

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ABSTRACT

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Bisphenol A (BPA) was originally designed as a synthetic estrogen but is now used as a monomer in the production of plastics. Recent research has linked exposure to low doses of BPA, below the reference dose (the safe long-term daily dose) of 50μg (kg-1 day-1), with a number of health problems including diminished fertility, insulin resistance, obesity, accumulation of triglycerides in the liver and the induction of endoplasmic reticulum (ER) stress with subsequent non-alcoholic fatty liver disease. Because of the problems with BPA, bisphenol S (BPS) has become a popular alternative in plastic production. However, emerging research has associated BPS with many of the same health problems as BPA. In this study, I examine the effects of low-dose exposure to BPS on HepG2 cells, an established *in vitro* model system of liver function in use since 1974. More specifically, I monitored the total amount of lipid droplets, and the amount of acetyl-CoA carboxylase (the catalyst of the first, and rate limiting step of fatty acid synthesis) to measure fatty acid synthesis in HepG2 cells exposed to low doses of BPS, and further I examine the levels of ER chaperone protein glucose-regulated protein 78 (GRP78/BiP) to study effects of BPS on ER stress. In short, I found no significant effect of BPS exposure on the cells in that there was no significant change in lipid levels, *p*-ACC, or GRP78/BiP levels.

KEY WORDS: Plastic, Bisphenol, Metabolism, Endoplasmic reticulum, Unfolded protein response

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CHAPTER I

Introduction

BISPHENOL A

BPA is currently used as a monomer in the production of polycarbonate plastics including food and beverage containers [5-8,10,11,13-15]. BPA leaching from plastic containers is the primary source of human exposure [5,6]. Based on high dose studies conducted on mice and rats in the 1980s, the US-EPA found 50mg (kg^{-1} day^{-1}) to be the lowest observed adverse effect level (LOAEL), i.e. the lowest dose at which adverse effects were observed, and used this value to calculate a reference dose (the safe long term daily dose) of 50µg (kg^{-1} day^{-1}) [8]. More recent studies have found a wide range of adverse effects of BPA at concentrations below the reference dose, because BPA has a nonmonotonic dose response curve [5-8,10-13] (Figure 1). The discovery of nonmonotonic dose responses has led to a paradigm shift in toxicology [6]. It is no longer sufficient to test high doses until a LOAEL is found. The possibility of physiological effects at levels below the LOAEL demands testing of a wide range of concentrations of a chemical at doses even below the LOAEL.

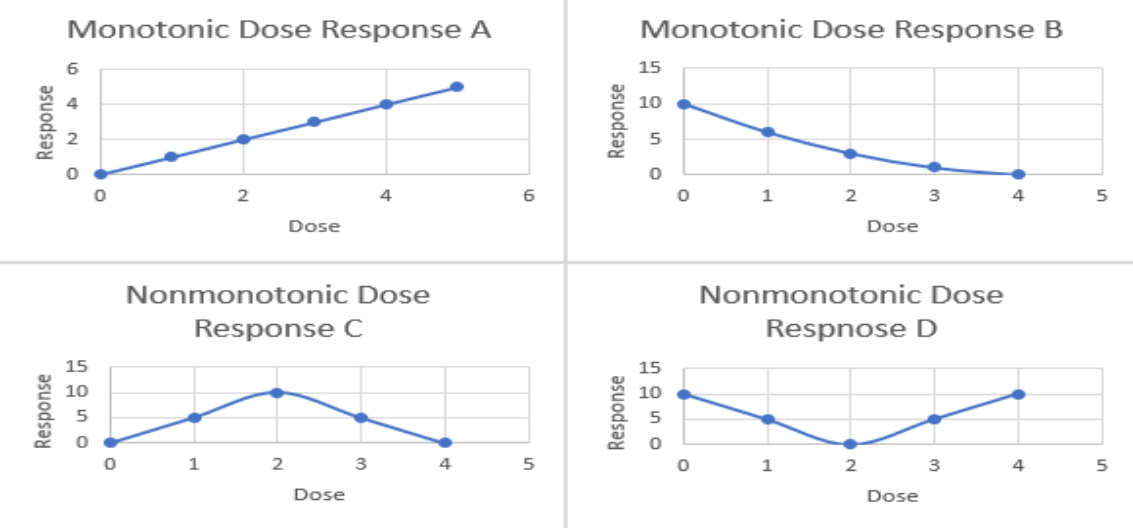

Fig. 1. Nonmonotonic dose response. A monotonic dose response curve is one in which the sign of the slope of the curve never changes (A and B above). Meanwhile, a nonmonotonic dose response dose have a change in the sign of the slope of the curve.

Biologically active levels of BPA, that is levels of BPA that have been observed to have adverse effects in animal and *in vitro* models, have been detected in the over 90% of people surveyed [5,6,8,11,13-15]. Studies have found an average BPA concentration of 1.4-2.4 ng/ml in human maternal sera, and 8.3 ng/ml in 15-18 week fetal amniotic fluid. Studies of human urine have found average levels of total (free and conjugated) BPA at 1.63 ng/ml in males and 1.12 ng/ml in females [16]. The ubiquity of BPA in human populations amplifies concerns about its potential toxicity.

Bisphenol A (BPA) was designed as a synthetic estrogen [5,6] and binds to nuclear estrogen receptors α and β with a 1000 to 2000 fold lower affinity than 17β-estradiol. On this basis, it is classified as a weak estrogen [5]. When nuclear estrogen receptors are bound by BPA they become differentially responsive to coactivators and corepressors, thereby leading to changes in gene expression leading to cellular proliferation [5].

More recent research has shown that BPA also has a number of non-genomic effects [5,8,9]. For example, BPA binds to estrogen receptors localized to the plasma membrane [5,6,7,9] and can act as an agonist to estrogen receptor α which promotes cellular proliferation. It also acts as an antagonist to estrogen receptor β which inhibits proliferation [5,8]. In this way, BPA is a proliferative agent [5].

The most well-known consequence of BPA exposure is diminished fertility due to its effects at multiple physiological levels. For example, exposure of oocytes to BPA during the early stages of meiosis was shown to upregulate genes involved in double strand break, signaling, and repair [11]. This results in an increase in the number of crossing over events, as well as an increase in oocyte degeneration [11]. BPA exposure during later stages of meiosis was shown to correlate with an impaired cytoskeleton, lack of alignment of chromosomes, incomplete meiosis, and an increased rate of oocyte degeneration [11]. In addition, exposing pregnant mice to BPA causes a significant alteration in imprinted gene expression in the embryos, which impairs fetal, placental, and postnatal development [11]. Furthermore, researchers have seen that BPA exposure can disrupt blastocyst implantation either by mismatching the timing of the uterine receptivity window and blastocyst formation, or by directly altering uterine receptivity through BPA's estrogenic properties [11]. BPA may also increase the risk of polycystic ovary syndrome through its role as a xenoestrogen [14,18]. In male rats, BPA exposure was shown to decrease serum levels of testosterone [5,7]. Additionally, it was found to decrease sperm production, decrease the percentage of moving sperm, and increase the incidence of malformed sperm [7].

In addition to its well-known reproductive effects, BPA has been linked to metabolic dysfunction. Through its ability to impair thyroid function [5,17], BPA exposure has been associated with thyroid resistance syndrome, and attention deficit disorder [18]. In addition, BPA has been linked to insulin resistance, and the development of Type II diabetes [5,9,10,12,13,15,17], as well as increasing the size of atherosclerotic lesions, and increasing coronary stenosis [13], and cardiac arrhythmias by altering Ca^{2+} handling in cardiac myocytes [9,15,19]. It is particularly interesting that Machtinger et al. (2014) found an increase in the expression of genes involved in *de novo* fatty acid synthesis (ATP citrate lyase, Acetyl-CoA carboxylase 1, Acetyl-CoA carboxylase 2, and Fatty acid synthase) in the livers of adult mice exposed to low doses of BPA, but they did not establish a mechanism for the effects of BPA on lipid synthesis [11]. It has also been suggested that BPA exposure promotes inflammation and oxidative stress [14]. Importantly, exposure to BPA leads to the accumulation of triglycerides and the induction of ER stress in the livers of rabbits and mice and has been linked to in the development of non-alcoholic fatty liver disease [13]. Taken together, these findings suggest a mechanism linking BPA exposure to a variety of pathophysiological outcomes [2-4].

BISPHENOL S

Because of the problems with BPA, there has been great interest in finding an alternative. Currently, the leading choice is bisphenol S (BPS) one of many structural analogs of BPA collectively known as bisphenols. BPS is used as an alternative to BPA because of its similar chemical structure (Figure 2), because it is more heat stable, and because it is less prone to degradation from exposure to sunlight [37]. Even so, in 2012 a

study by Liao et al. found BPS in 78% of urine samples from 100 American adults, compared to BPA being found in 95%. BPS was found at concentrations up to 12.3 ng/mL, and BPA was found at up to 37.7 ng/mL in those urine samples [22]. The similarity means that BPS has the same physical properties that make BPA an attractive ingredient in plastic production but may also mean that BPS shares the same detrimental physiological effects as BPA. Indeed, emerging research shows that BPS is an endocrine disruptor with a potency comparable to that of BPA [20]. For example, one study found that both BPA and BPS can cause an increase in lipid content and a decrease in lipolysis in 3T3-L1 cells [10]. Postnatal exposure to BPS has been shown to induce uterine growth in rats [21]. BPS was also shown to disrupt reproduction in zebrafish, increase the female to male sex ratio, decrease body length, and disrupt testosterone and estrogen levels [21]. *In vitro* experiments have even shown BPS exposure to induce caspase 8 production [21]. BPS was shown to be even more potent than BPA at inhibiting testosterone secretion in human testis explants after three days of treatment [29]. BPS could also cause unique problems as evidenced by the same study showing BPS, but not BPA, increased glucose uptake and leptin production [10].

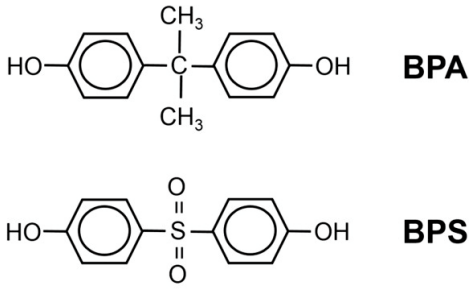

Fig. 2. Chemical Structures of BPA and BPS. Adapted from Kang et al. 2014.

ENDOPLASMIC RETICULUM (ER) STRESS

Most membrane bound or secreted proteins are first folded and modified in the ER. The environment of the ER is ideally suited to protein modification and folding because of the chaperone proteins, enzymes, and high levels of calcium contained within [30]. ER stress is caused by the accumulation of unfolded or improperly folded proteins in the ER. Persistent ER stress can cause a cell to undergo apoptosis, and lead to a variety of pathological outcomes. To alleviate ER stress, cells activate the unfolded protein response (UPR) (Figure 3). Under normal conditions the ER stress sensors protein kinase RNA-like ER kinase (PERK), inositol-requiring enzyme 1 α (IRE1α), and activating transcription factor 6 (ATF6) are bound to the chaperone protein glucose-regulated protein 78 (GRP78/BiP). However, during ER stress the protein folding capacity of the ER is exceeded, which causes GRP78/BiP to dissociate from these ER stress sensors. This dissociation of GRP78/BiP from PERK, IRE1α and ATF6 leads to the activation of the UPR. The ultimate goal of the UPR is to alleviate ER stress. This is accomplished by increasing the ER's protein folding capacity while concurrently decreasing protein synthesis. PERK inhibits protein synthesis by phosphorylating and inactivating eukaryotic initiation factor 2α (eIF2α). This results in a reduced protein load on the ER. IRE1α regulates the splicing of mRNA encoding X-box binding protein 1 (XBP1) thereby activating it. Active XBP1 stimulates transcription of additional ER chaperones thereby increasing the ER's protein folding capacity. IRE1α also induces degradation of mRNAs to reduce the protein load on the ER. In chronic ER stress IRE1α also activates pro-inflammatory and pro-apoptotic pathways [1-4]. ATF6 activates the transcription of several ER proteins including GRP78/BiP to increase the capacity of the

ER, and Derlin-3 which enhances ER associated degradation and reduces the amount of misfolded protein in the ER [1-4]. If normal protein folding is restored PERK, IRE1α, and ATF6 can again be bound by GRP78/BiP, the UPR can end, and the cell can resume normal function.

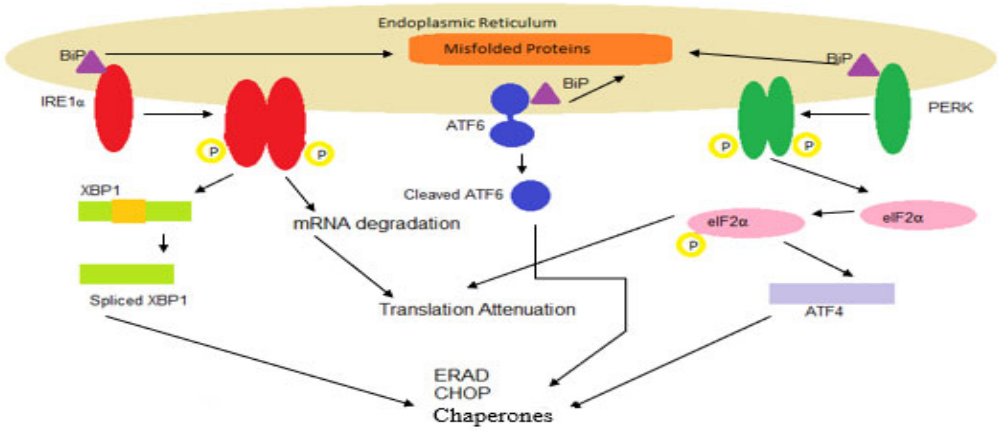

Fig. 3. ER stress and the unfolded protein response. Adapted from Mahli et al. 2011.

However, in cases where the UPR is unable to restore the natural order, an apoptotic response is triggered. In particular, the pro-apoptotic proteins Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist killer (Bak) will associate with IRE1α which activates c-Jun N-terminal kinase (JNK) to promote apoptosis. At the same time, PERK, through eIF2α, increases the level of CCAAT-enhancer-binding protein homologous protein (CHOP), which also promotes apoptosis [1] (Figure 4).

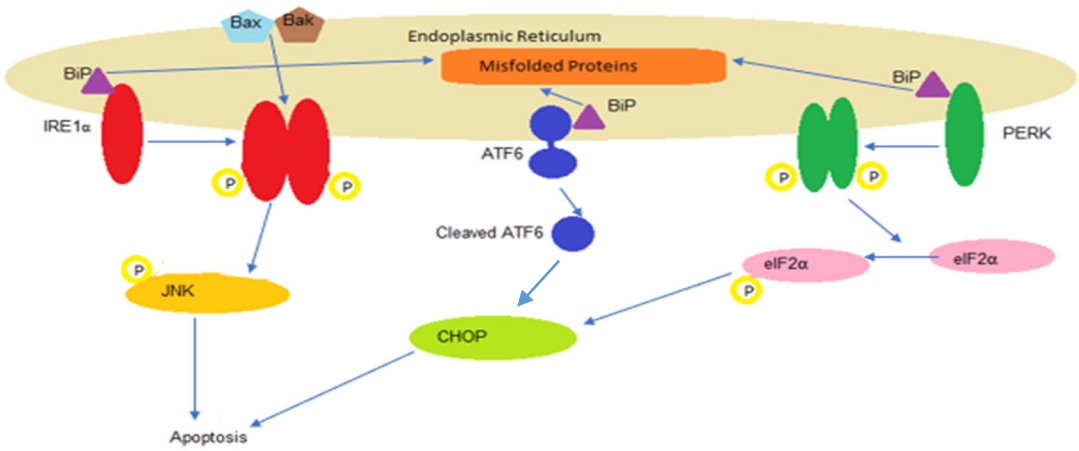

Fig. 4. The UPR and apoptosis. Adapted from Mahli et al. 2011.

ER STRESS AND LIPID METABOLISM

The mechanical relationship between lipids and ER stress remains unclear, but recent studies have shown that exposure to palmitate activates the Toll-like receptor 4 (TLR-4) pathway. They also demonstrated that palmitate exposure activated IRE1α, by activating TLR-4 and therefore could be responsible for lipid induced ER stress [32]. Importantly, exposure to BPA leads to the accumulation of triglycerides and the induction of ER stress in the livers of rabbits and mice [13] and has been associated with many pathologies, including the development of Type II diabetes [5,9,10,12,13,15,17], atherosclerosis [12], non-alcoholic fatty liver disease [13], inflammation and oxidative stress [14]. Therefore, it is possible that BPS exposure has many of the same effects.

FATTY ACID SYNTHESIS

Fatty acid synthesis occurs mainly in the liver and adipose tissue [30] (Figure 5). The first step of *de novo* fatty acid synthesis is the conversion of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (ACC). This is the rate-limiting step in fatty acid synthesis. Next fatty acid synthase (FAS) catalyzes a repeating process that generates

palmitate from acetyl-CoA and malonyl-CoA. FAS catalyzes the addition of malonyl-CoA to generate a palmitate, a 16-carbon fatty acid, as the final product. Palmitate can then translocate to the ER to be modified to make other fatty acids. Elongation of palmitate occurs by the addition of malonyl-CoA and is catalyzed by fatty acid elongases which are encoded by elongation of very long-chain fatty acid (ELOVL) genes [21]. Interestingly, an increase in lipid synthesis can cause ER stress through mechanisms that remain poorly understood, but it is noteworthy that ER stress is associated with a number of diseases including hepatitis, fatty liver disease, non-alcoholic fatty liver disease, insulin resistance, and inflammation [1-4].

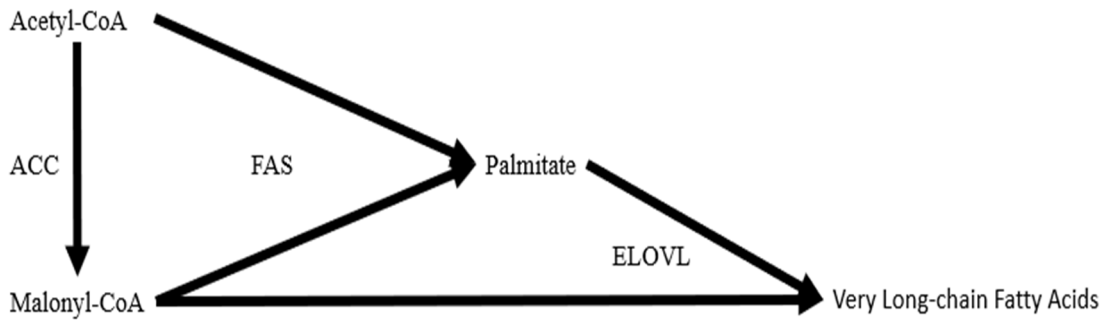

Fig. 5. Fatty acid synthesis.

In this study I used HepG2 cells as a model of liver function. HepG2 cells were derived from a human hepatoblastoma, have been used as a model for studying normal liver function since 1979 [4] and are known to express ERα, ERβ, GPR30 (a G-protein coupled estrogen receptor), as well as the insulin, and insulin-like receptors [23].

OBJECTIVES

The objective of this study was to determine if low dose exposure to BPS induces an ER stress response as a result of increased lipid synthesis in a cell culture model of

hepatic function, namely the HepG2 cell line. For this study low dose of BPS will be defined as below the LOAEL of BPA, because the LOAEL of BPS is not yet established.

HYPOTHESIS

My null hypothesis is that exposure to low doses of BPS will have no effect on lipid synthesis and induction of ER stress in HepG2 cells. My specific hypotheses are that low dose BPS exposure will increase lipid synthesis in HepG2 cells, and that low dose BPS exposure will lead to ER stress in HepG2 cells.

I tested these hypotheses by measuring global lipid synthesis in intact HepG2 cells using Oil Red O stain, as well as measuring the activity of acetyl-CoA carboxylase 1 (ACC1) and inactive *p*-ACC1 via Enzyme Linked Immunosorbent Assay (ELISA). In addition, I will measure the induction of ER stress using the expression of GRP78/BiP as an indicator via ELISA.

CHAPTER II

Materials and Methods

CELL LINES

In this study the human HepG2 cell line was used. HepG2 cells were derived from a human hepatoblastoma and have been used as a model for studying liver function since 1979 [4]. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 2mM of stable glutamine, 1mM sodium pyruvate, 0.5% penicillin/streptomycin, and 10% serum in a humidified atmosphere at 37 °C.

Serum free DMEM was made from DMEM with 2mM of stable glutamine, 1mM sodium pyruvate, and 2% BSA.

CELL CULTURE

Despite its utility as a pH indicator in cell culture media, phenol red has been demonstrated to have estrogenic properties [24]. Consequently, cells were grown in phenol red free media to avoid this potential confound. Culture flasks were seeded with one million cells per flask and grown for two weeks changing the media every three days. Then cells were moved to 12-well plates, and after the cells attached to the plates, the media was changed to fresh media containing BPS at 0, 10^{-9},10^{-8},10^{-7},10^{-6}, 10^{-5} and 10^{-4} mol/L; concentrations of BPA at 10^{-5} mol/L and lower are considered low doses [5]. The concentration range for low dose BPA was used because BPS is currently unregulated and can be used freely [27]. Cells were treated under these concentrations for 6, 48, and 96 hours.

ASSESSMENT OF EFFECTS OF BPA ON LIPID METABOLISM AND ER STRESS

In order to assess the effect of BPS on hepatic lipid metabolism, replicate cultures of cells were grown in flasks, then moved to 12-well plates and treated with BPS as above. Immediately afterwards, cells were lysed in lysis buffer containing protease and phosphatase inhibitors, and total protein extracted using standard techniques. Prior to ELISA, the total protein content of individual lysates was determined using a BCA assay. Samples were then diluted to a concentration of 300ug/ml and then 30 micrograms of total protein were loaded into the ELISA wells. Protein was loaded into antibody coated wells of PathScan® sandwich ELISA kits (Cell Signaling Technologies) for ACC1 and p-ACC1 according to the manufacturer's instructions. In brief, a 96 well plate was coated with a capture antibody specific to the kit's target. Thirty micrograms of protein were added to each well, and then bound to the capture antibodies. Unbound protein was then washed out before a detection antibody was added to bind to the target protein. The plate was then washed again before a secondary horseradish peroxidase (HRP) linked antibody was added and bound to the detection antibody. Finally, HRP substrate was added, broken down into a colored product, and quantified using spectrophotometry. The color was proportional to the amounts of ACC and *p*-ACC in the protein samples.

ACC1 catalyzes the conversion of acetyl-CoA to malonyl-CoA, which is the rate determining step in the synthesis of fatty acids and is inactivated by phosphorylation by cAMP-dependent kinase, and by AMP-activated kinase [25]. By comparing levels of active ACC1 to inactive p-ACC1 lipid metabolism will be estimated *in vitro.*

In addition, global lipid synthesis of wells of cells was quantified using an Oil Red O stain kit, from Sigma-Aldrich, on three replicate cultures tested in parallel. In brief, cells were removed from the incubator and placed in the biosafety cabinet followed by the aspiration of the media from each well. The plates were rinsed with 1 ml of sterile DPBS dispersed along the side of each well to avoid disturbing the monolayer. The DPBS was then aspirated, and 1 ml of 10% formalin was added along the sides of each well to fix the cells after incubating for 60 minutes at room temperature. Formalin was then removed from the sides of each well with a pipettor and discarded. Then, each well was rinsed twice with 1ml of sterile water followed by the addition of 1 ml of 60% reagent alcohol for 5 minutes. Finally, the alcohol was aspirated, and 1 ml of Oil Red O solution was added to each well to completely cover the cells. The dishes were rotated to spread the stain evenly and allowed to stand for 30 minutes. Each well was rinsed with water until the excess stain was removed. One milliliter of hematoxylin counterstain was then pipetted into each well to completely cover the cells and allowed to stand for one minute. Hematoxylin was aspirated, and the plates were rinsed with tap water as above. Plates were kept wet with water until ready to be viewed on a phase contrast microscope. Cells grown in DMEM mixed with vegetable oil in a 1:1 ratio were used as a positive control.

After observing the cells under a phase contrast microscope, the red coloring was extracted and quantified using spectrophotometry. Cells were washed with 500 µl of 100% isopropanol for 5 minutes to extract the red stain. The solution was then transferred to a new plate and absorbance at 520nm was read on the spectrophotometer.

MEASUREMENT OF ER STRESS

The ER stress in response to BPS treatment was estimated using GRP78/BiP as an indicator via ELISA using the lysates prepared above. GRP78/BiP was measured using an Enzo Life Sciences competitive ELISA Kit. In brief, samples were added to a microwell plate coated with a donkey anti-sheep IgG antibody. Then protein samples were added followed by a solution of anti GRP78/BiP antibody. Next, a solution of GRP78/BiP conjugated with HRP was added. The GRP78/BiP antibody competitively bound to the GRP78/BiP in the sample, or the GRP78/BiP that was conjugated to HRP. HRP substrate was added and generated a colored product. The resulting color was quantified by spectrophotometry and was inversely proportional to the amount of GRP78/BiP in the protein sample.

GRP78/BiP is a chaperone protein found in the ER. In response to ER stress, GRP78/BiP dissociates from IRE1α, and PERK thereby activating the unfolded protein response. One consequence of the unfolded protein response is an increase in the amount of GRP78/BiP in the ER. GRP78/BiP was used as an indicator of ER stress, because it is the chaperone protein that binds the activating proteins for all three arms of the UPR. Cells were treated with 5µg/ml tunicamycin as a positive control, because tunicamycin induces the UPR by inhibiting N-linked glycosylation.

STATISTICAL ANALYSIS

This experiment consists of three replicate cultures done in parallel. Results (e.g. GRP78/BiP expression levels, degree of Oil Red O staining) were subjected to a two-way ANOVA using an F-distribution and a post-hoc pairwise Tukey test to determine statistical significance.

CHAPTER III

Results and Discussion

OIL RED O STAIN

Qualitative Analysis

Lipid droplets were evenly distributed between cells, but there is no qualitative difference between the doses at 96 hours (Figure 6), 48 hours (Figure 7), or at 6 hours (Figure 8).

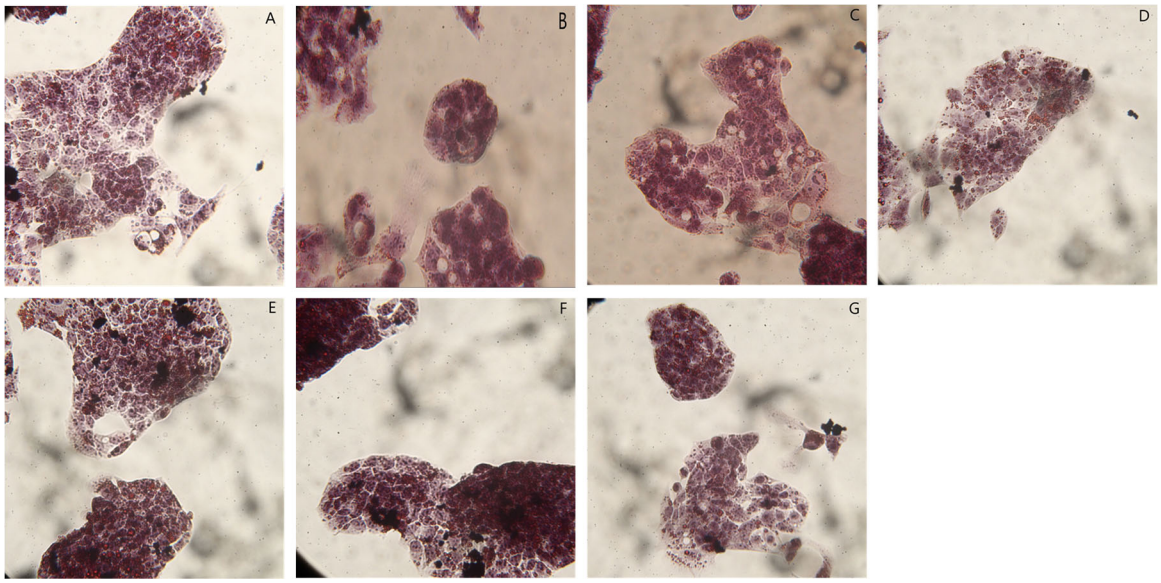

Fig. 6. Oil Red O stain 96 hours. Lipid droplets were evenly distributed between cells. Cells were treated with 0 (A), 10^{-9} (B),10^{-8} (C),10^{-7} (D),10^{-6} (E), 10^{-5} (F) and 10^{-4} (G) mol/L BPS

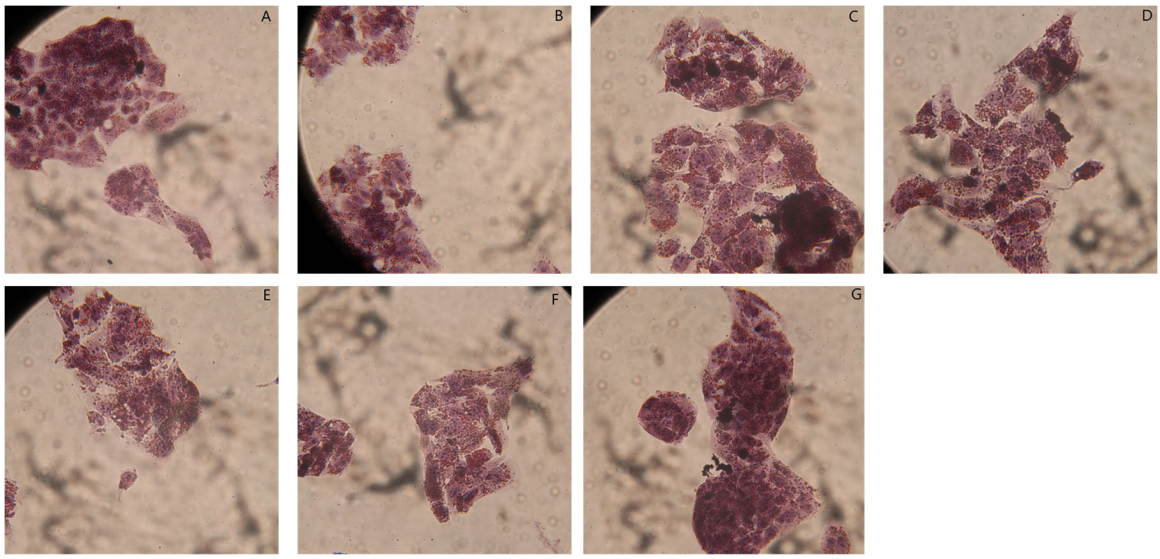

Fig. 7. Oil Red O stain 48 hours. Lipid droplets were evenly distributed between cells. Cells were treated with 0 (A), 10^{-9} (B),10^{-8} (C),10^{-7} (D),10^{-6} (E), 10^{-5} (F) and 10^{-4} (G) mol/L BPS

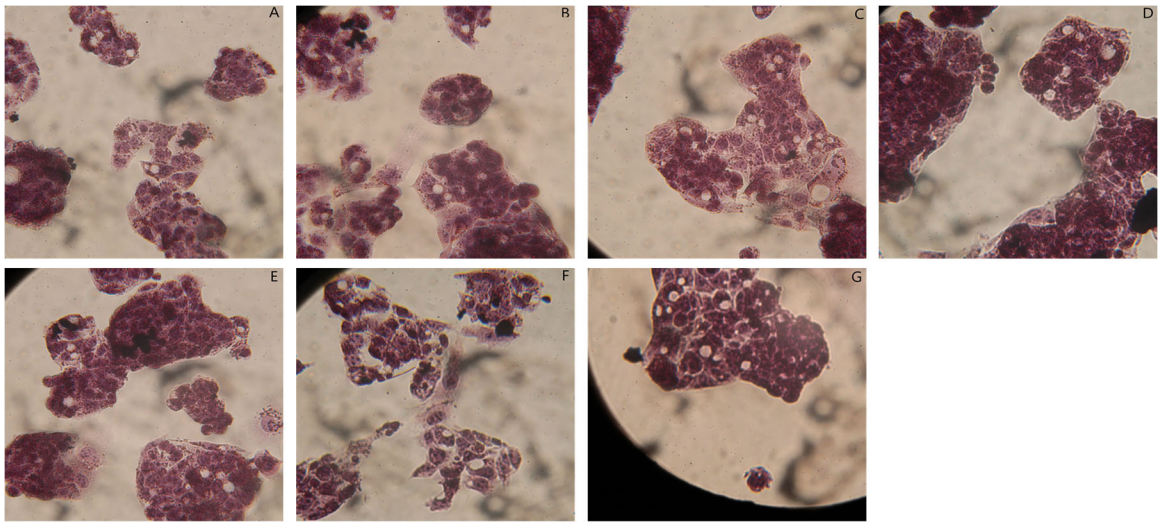

Fig. 8. Oil Red O stain 6 hours. Lipid droplets were evenly distributed between cells. Cells were treated with 0 (A), 10^{-9} (B),10^{-8} (C),10^{-7} (D),10^{-6} (E), 10^{-5} (F) and 10^{-4} (G) mol/L BPS

Quantitative Analysis

After 48 hours, all absorbance readings dropped compared to 6 hours. By 96 hours however, all absorbance readings were back up to, or slightly higher than, the readings at 6 hours. Overall, variations in dose response were very slight. However,

every concentration of BPS tested showed a higher absorbance at 96 hours than serum free did (Figure 9) The results of the Oil Red O staining showed a significant effect of time F (2, 55) = 4.13, p = 0.025, but there was no significant effect of dose F (6, 55) = 0.06, p = 0.999. There was no significant interaction between dose and time F (12, 55) = 0.04, p = 1.000. A post hoc Tukey test found that the 96 and 48 hour sets were significantly different from one another, but neither was significantly different from the 6 hour set.

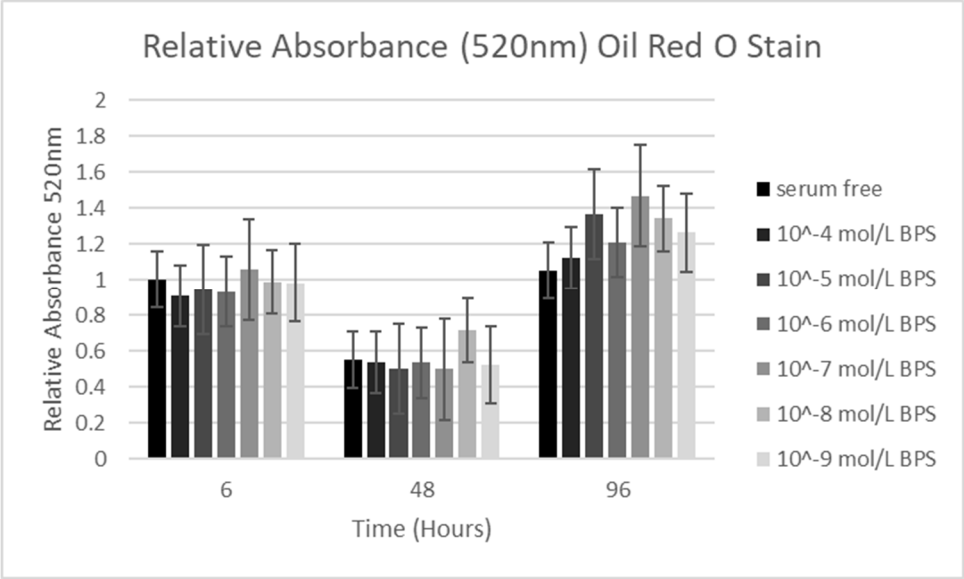

Fig. 9. Relative absorbance of Oil Red O stain. The results of the Oil Red O staining showed a significant effect of time F (2, 55) = 4.13, p = .025, but there was no significant effect of dose F (6, 55) = .06, p = .999. There was no significant interaction between dose and time F (12, 55) = .04, p = 1.000.

PROTEIN QUANTIFICATION

Total protein from each sample was quantified using a BCA assay (Figure 10) so that exactly 30ug of protein could be loaded into each well of the ELISA. In general, the 48 and 96 hour timepoints had a higher amount of total protein than the 6 hour timepoint.

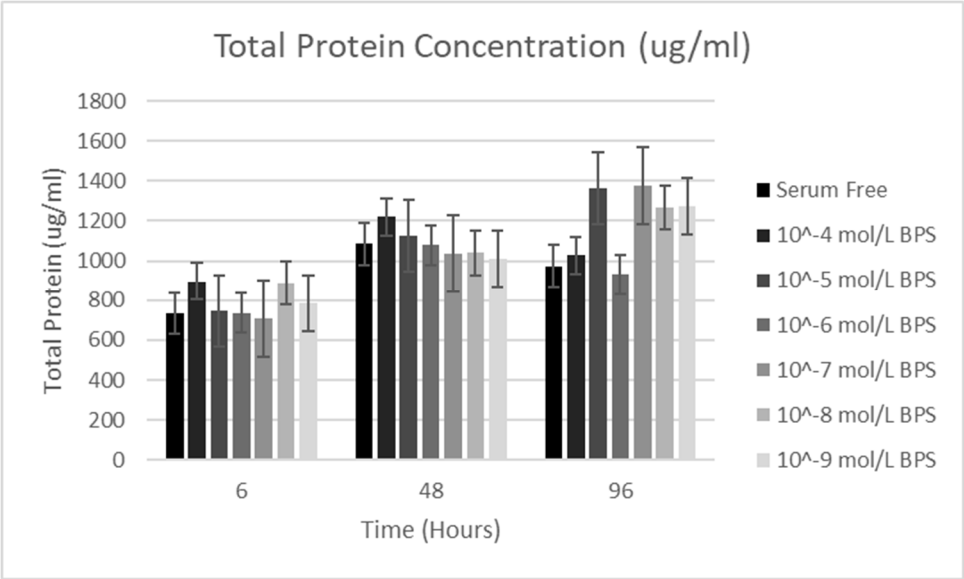

Fig. 10. BCA Assay. There was little variation in protein concentration between doses of BPS.

ACC and p-ACC ELISA

At 6 hours, the ratio of *p*-ACC to ACC was low. It rose up at 48 hours, and then fell back down at 96 hours, but remained higher than at 6 hours. Although there was no statistically significant effect of dose on the ratio of *p*-ACC to ACC, the ratio was higher for almost every concentration of BPS tested than for the serum free control at the same timepoint. This suggests that there could be an effect of dose on the ratio (Figure 11). According to the manufacturer's instructions, 10 minute treatment with 10mM H_2O_2 phosphorylates ACC to be detected by the Phospho-Acetyl-CoA ELISA kit, without affecting the total ACC.

The ELISA's for ACC and p-ACC showed a significant effect of time F (2, 57) = 32.52, $p < 0.01$, but not dose F (6, 57) = 1.92, p = .104. There was no significant interaction between time and dose F (12, 57) = 1.54, p = 0,155 (Figure 9). A post hoc Tukey test showed that the 48 hour set was significantly different from the 96 and 6 hour sets, and that the 96 and 6 hour sets were not significantly different from one another. The positive H_2O_2 control was 43% higher than the negative serum free control.

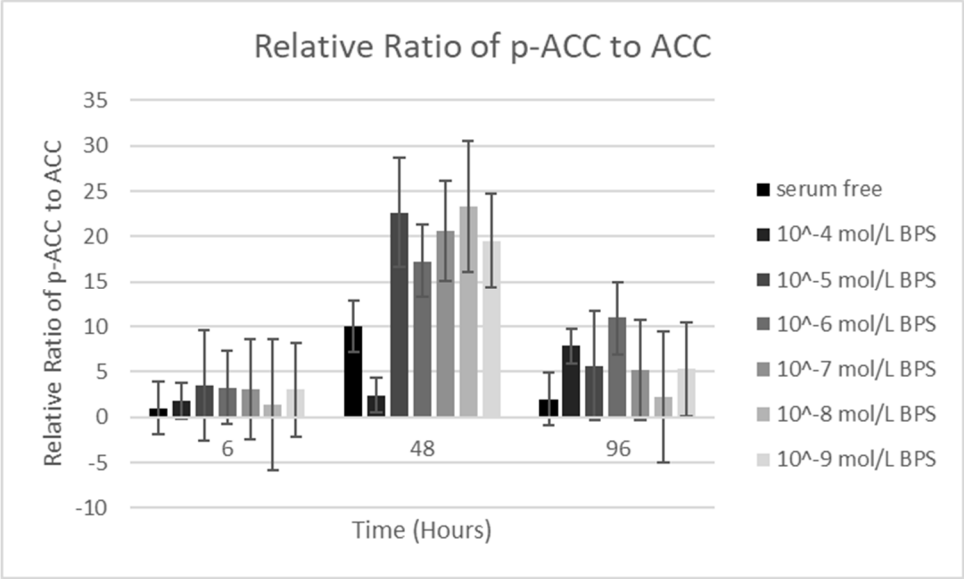

Fig. 11. Relative ratio of p-ACC to ACC. The ELISA's for ACC and p-ACC showed a significant effect of time F (2, 57) = 32.52, $p < 0.01$, but not dose F (6, 57) = 1.92, p = .104. There was no significant interaction between time and dose F (12, 57) = 1.54, p = 0,155

GRP78/BIP ELISA

There was little variation between doses at the 6 hour timepoint. At 48 hours, all of the low doses of BPS showed a higher amount of GRP78/BiP than the serum free control. At 96 hours BPS at 10^{-4}, 10^{-5}, and 10^{-6}mol/L were higher than the serum free control. The GRP78/BiP ELISA showed no significant effect of time F (2, 62) = .53, p =

0.591, or dose F (6, 62) = 0.46, p = 0.835. There was also no significant interaction between dose and time F (12, 62) = 0.47, p = 0.919 (Figure 12).

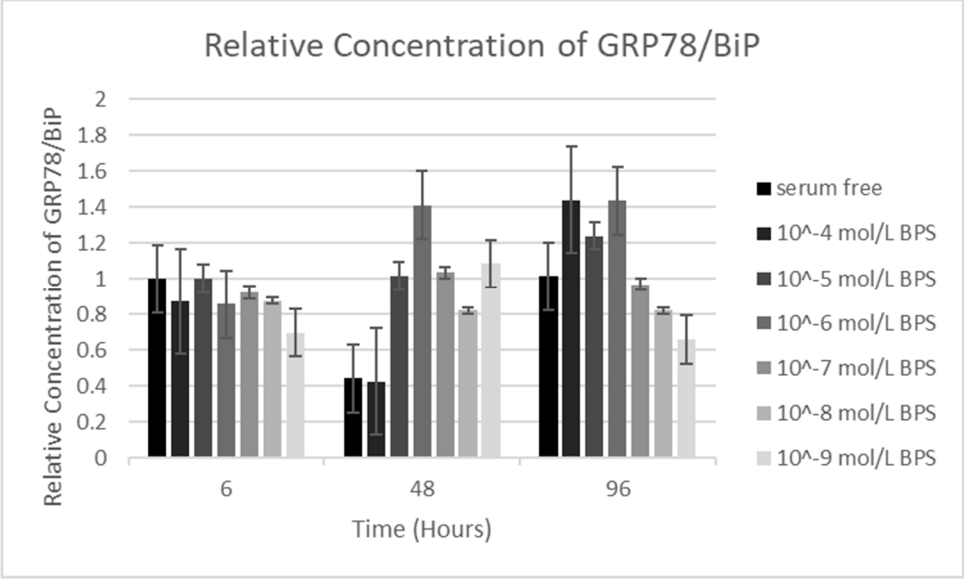

Fig. 12. Relative Concentration of GRP78/BiP. The GRP78/BiP ELISA showed no significant effect of time F (2, 62) = .53, p = 0.591, or dose F (6, 62) = 0.46, p = 0.835. There was also no significant interaction between dose and time F (12, 62) = 0.47, p = 0.919

DISCUSSION

Although BPS had no significant effect on measures of lipid biology and ER stress in HepG2 cells, there are still interesting patterns. For example, after 48 hours of exposure to BPS at all doses tested below 10^{-4}mol/L, there was an increase in GRP78/BiP relative to serum free media. This suggests that BPS exposure may affect ER stress. After 48 hours of exposure at all concentrations of BPS, there was an increase in the ratio of *p*-ACC to ACC, relative to serum free media, but then at 96 hours of exposure there was an increase in absorbance measured by the ORO stain, relative to serum free media. This suggests that the increase in lipid levels was not caused by *de novo* lipid synthesis. The ORO stain shows that there was an increase in lipid levels, but at the same time, the

increased ratio of inactive *p*-ACC to active ACC actually suggests a decrease in *de novo* lipid synthesis. This trend of increasing lipid levels is simmilar to a study by Héliès-Toussaint et. all (2014). Taken together, these findings suggest that BPS exposure may affect both ER stress and lipid accumulation, but more research is needed to confirm.

Regardless of treatment, the amount of lipid staining observed was lower at 48 hours of exposure than after 6 hours of exposure, but it did increase again at 96 hours. In fact, the levels seen at 96 hours tended to be higher in the BPS-treated cells relative to 6 hours of exposure whilethe level of ORO staining in the serum free control was relatively unchanged. This suggests that any changes in ORO staining are a result of BPS exposure

The changes in lipid levels across all treatments at each timepoint could instead be a result of the cells adapting to the serum free conditions. At the 48 hour timepoint every condition tested saw a spike in the ratio of *p*-ACC to ACC, compared to 6 hours, and at 96 hours the ratio fell back down. By every measure, all conditions tested were similar at 6 hours, there was more varriation at the 48 and 96 hour timepoints. Interestingly, the results for GRP78/BiP show a similar pattern to the lipid data. There is little variation at 6 hours, but at 48 hours GRP78/BiP is higher than serum free for every concentration of BPS other than 10^{-4} mol/L. Taken together these results tell a story of cells becoming stressed after spending 48 hours in serum free conditions, with cells that have been exposed to BPS responding more strongly to the stressful conditions.

While more research is needed, the results paint a picture of one mode of BPS toxicity. Previous studies have linked BPA to the accumulation of triglycerides, and induction of ER stress mouse and rabbit livers [13]. Fatty acid accumulation, and ER stress can lead to non alchoholic fatty liver disease (NAFLD), wich is linked to obesity

and type II diabetes, some of the greatest health challenges of our time [2-4]. The structural similarity between BPA and BPS suggests that they could have many of the same effects, and indeed *in vitro* studies have found a small increase in lipid content in HepG2 cells exposed to BPS although they did not identify a mechanism for the increse in lipid content [10]. Concerns about the effects of BPS are especially relevant as a 2012 study by Liao et al. found BPS in 78% of urine samples from 100 American adults, a number that will likely rise as BPS becomes more frequently used to replace BPA.

The next step of this project could be to repeat the study with larger sample sizes. The effects of endocrine disruptors like BPA and BPS tend to be of low magnitude [5], therfore it could be helpful to use a larger sample size for more statistical power. Additionally, it would be interesting to repeat the study with primary hepatocytes. HepG2 cells are an imortalized cell line and therefore could have differences from normal hepatocytes. Primary hepatocytes, howerver, are difficult to derive, do not proliferate well, and are only good for about a week in culture. HepG2 cells remain one of the best *in vitro* models of liver function [25]. One other option would be to use whole mice to do this study *in vivo*.

Additionally using a broader range of markers would be informative. Observing the three arms of the UPR independently by measuring IRE1α, PERK, and ATF6; could give a more detailed picture than GRP78/BiP alone. The UPR is a process that alleviates stress on the ER. Durring ER stress GRP78/BiP dissociates from IRE1α, PERK, and ATF6, causing them to be activated. PERK inhibits protein synthesis by phosphorylating and inactivating eukaryotic initiation factor 2α (eIF2α). This results in a reduced protein load on the ER. IRE1α regulates the splicing of mRNA encoding X-box binding protein

1(XBP1) thereby activating it, and transcribing additional chaperones to increase the ER's protein folding capacity [1-4]. ATF6 activates the transcription of several ER proteins including GRP78/BiP to increase the capacity of the ER, and Derlin-3 which enhances ER associated degradation and reduces the amount of misfolded protein in the ER [1-4]. If normal protein folding is restored, PERK, IRE1α, and ATF6 can again be bound by GRP78/BiP, the UPR can end, and the cell can resume normal function.

A study by Asahi *et al.* (2010), found an increase in GRP78/BiP and in CHOP after exposing NCTC Clone 1469, non-parenchymal hepatocytes derrived from mice, to 100μM BPA. They also saw an increase in reactive oxygen species (ROS). Taken together these results indicate that BPS could also cause ER stress. Yin *et al.* (2017) found the UPR to be generally upregulated in mouse spermatocytes, and that knocking down PERK protected against BPA induced apoptosis. This shows that the PERK arm of the UPR may be the most useful target for a future experiment.

A broader look at lipid metabolism would also be helpful. Muramugi *et al.* (2011) observed the livers of mice exposed to BPA, and saw an increase in genes involved in *de novo* fatty acid synthesis including ACCα, ACCβ, FASN and ATP citrate lyase (Acly). A 2015 study by Feng *et al.* also saw an upreglation of genes involved in fatty acid synthesis *in vivo* in rabbits. Both of these studies suggest that BPS exposure could cause an increase in fatty acid synthesis, and indeed, a later study by Héliès-Toussaint *et al.* (2014) found a small increase in lipid content in HepG2 cells, consistent with the results of this study. This contrasts with an earlier study by Peyre *et al.* (2014) which found a modest increase in lipid content in hepatocytes exposed to BPA, but no increase in hepatocytes exposed to BPS. Furthermore, they saw no increase in expression

of fatty acid synthase (FASN) or perilipin (PLIN). These conflicting studies demand a more thurough examination of the effects of BPS exposure on lipid synthesis.

Finally, a broad transcriptomic and proteomic approach would be helpful. BPS and other endocrine disruptors can exert their effects through a variety receptors and mechanisms, therefore it is difficult to assess their effects using only a few markers. With a larger budget, one could do large scale proteomic and transcriptomic analyses of multiple tissue types in an animal model. Searching for relationships between the differentally expressed genes could elucide mechanisms of action formed from many small changes working in concert.

While the results of this study did not rise to the level of statistical significance, they still hint at an interesting story. There does seem to be a pattern of increased lipid content and ER stress as a result of exposure to low doses of BPS. This study and another by Peyre *et al.* (2014) found no significant of BPS exposure on lipid levels in HepG2 cells, while another study by Héliès-Toussaint *et al.* (2014) found only a small increase compared to BPA. Taken together, these studies suggest that BPS is a safer alternative to BPA in terms of liver function, however, the prevelance of BPS and its association with such diseases as obesity, diabetes, NAFLD continues to make BPS worthy of investigation.

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