HHS Public Access

Author manuscript

Environ Mol Mutagen. Author manuscript; available in PMC 2018 March 01.

Published in final edited form as:

Environ Mol Mutagen. 2017 March ; 58(2): 60–71. doi:10.1002/em.22072.

Induction of oxidative stress by bisphenol A and its pleiotropic effects

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Abstract

Bisphenol A (BPA) has become a target of intense public scrutiny since concerns about its association with human diseases such as obesity, diabetes, reproductive disorders, and cancer have emerged. BPA is a highly prevalent chemical in consumer products, and human exposure is thought to be ubiquitous. Numerous studies have demonstrated its endocrine disrupting properties and attributed exposure with cytotoxic, genotoxic, and carcinogenic effects; however, the results of these studies are still highly debated and a consensus about BPA's safety and its role in human disease has not been reached. One of the contributing factors is a lack of molecular mechanisms or modes of action that explain the diverse and pleiotropic effects observed after BPA exposure. The increase in BPA research seen over the last ten years has resulted in more studies that examine molecular mechanisms and revealed links between BPA-induced oxidative stress and human disease.

Here, a review of the current literature examining BPA exposure and the induction of reactive oxygen species (ROS) or oxidative stress will be provided to examine the landscape of the current BPA literature and provide a framework for understanding how induction of oxidative stress by BPA may contribute to the pleiotropic effects observed after exposure.

Keywords

DNA damage; reactive oxygen species; antioxidant; prooxidant

1. Introduction

Bisphenol A (BPA) is a precursor industrial chemical that is widely used in the production of consumer products, including polycarbonate plastics, epoxy resins, and thermal paper [Vandenberg et al., 2007]. World-wide production of BPA has grown steadily over the past several decades, with greater than 10 billion pounds produced each year [Vandenberg et al., 2010, Vom Saal et al., 2012]. This growth in production has contributed to the ubiquity of BPA in consumer products and in the air, soil and water [Vandenberg et al., 2010]. As a

Conflicts of Interest

The author declares that there is no conflict of interests regarding the publication of this paper.

Contributions

N.R.G. conceived and wrote the manuscript.

result, human exposure through inhalation, ingestion, and/or absorption has resulted in circulating levels of BPA in the 10–100 nM range [LaKind and Naiman, 2015, Welshons et al., 2006].

The presence of free circulating BPA in biological samples is concerning, since BPA is structurally similar to diethylstilbestrol (DES) and has estrogenic character, though substantially weaker than DES or estradiol [Fang et al., 2000, Rochester, 2013]. This weak estrogenic character, coupled with initial predictions that BPA would be quickly metabolized into bisphenol A glucuronide (BPAG) or bisphenol A sulfate, (BPAS) and eliminated through urination, led to the belief that harmful endocrine disrupting effects from exposure would be minimized [Domoradzki et al., 2004, Volkel et al., 2002]. However, growing evidence demonstrates that free BPA circulates throughout the body [Vandenberg et al., 2012a, Vandenberg et al., 2013], and even at low doses can act as a potent endocrine disrupting chemical (EDC) (for reviews see [Kitraki, 2014, Mathieu-Denoncourt et al., 2015, Mileva et al., 2014, Rezg et al., 2014, Rochester, 2013]).

BPA binding and activation of estrogen receptors α and β (ERα and ERβ) is one of its most frequently implicated molecular mechanisms [Marino et al., 2012, Welshons et al., 2006, Wetherill et al., 2007]. BPA has been demonstrated to bind these receptors altering their localization and modulating transcription activities, despite have a lower affinity for these receptors than estradiol or other environmental xenoestrogens [Acconcia et al., 2015, Ascenzi et al., 2006, Bolli et al., 2010, Bolli et al., 2008, Marino et al., 2012, Singleton et al., 2006, Wetherill et al., 2007]. This lower potency makes it unlikely that BPA exerts is effects solely through interactions with these receptors. BPA has been also been demonstrated to be a potent activator of non-classical estrogen receptors, like G-coupled protein receptors (GPER) and estrogen-related receptor γ (ERRγ), as well as an activator for thyroid hormone receptor and androgen receptor [Alonso-Magdalena et al., 2012, Rochester, 2013]. The estrogenic character of BPA has been the focus of numerous studies, and there is growing evidence that BPA alters reproduction, development, metabolism, immune response, and neurobehaviors (reviewed in [Kitraki, 2014, Mathieu-Denoncourt et al., 2015, Mileva et al., 2014, Rezg et al., 2014, Rochester, 2013]). Further, population studies have associated BPA exposure with the development and progression of diseases, including asthma, diabetes, cardiovascular disease, obesity, and more recently cancer [Rochester, 2013, Seachrist et al., 2016].

Despite this weight of evidence, the health risks associated with the chronic, low dose BPA exposure the population experiences are still controversial [Kovacic, 2010, Trasande et al., 2016, Vandenberg and Prins, 2016]. One of the contributing issues to the controversy is the lack of molecular mechanisms describing the diverse and pleiotropic effects observed after BPA exposure. However, the increased demand for and focus on BPA research over the past ten years has resulted in more studies examining the underlying mechanisms of BPA exposure [Schug et al., 2013], and there is growing evidence that the induction of reactive oxygen species (ROS) by BPA may contribute significantly to its toxicity and carcinogenic potential [Gassman and Wilson, 2016, Rochester, 2013, Seachrist et al., 2016].

A cell's ability to maintain a balance in reduction and oxidation (redox) of chemicals plays an essential role in all aspects of cellular development, growth, and survival. Normal cellular metabolism generates ROS, such as superoxide anions, peroxides, and hydroxyl radicals, and cells have developed highly tuned pathways to utilize low levels of these species for gene regulation and to scavenge any excess ROS to prevent deleterious effects. This important redox balance is maintained by numerous components in the cell and is highly regulated and coordinated. However, when this balance is disrupted by environmental toxicants, like heavy metals, polycyclic aromatic hydrocarbons, or EDCs, the same ROS that were once beneficial to the cell can now cause mutations, unchecked cell growth, and insensitivity to cell death signals, including those induced by therapeutic agents [Liou and Storz, 2010]. Additionally, increased levels of oxidative stress have been implicated in aging, cardiovascular disease, neuronal degeneration, and the development and progression of cancer [Liou and Storz, 2010, Rahal et al., 2014].

As with other aspects of BPA research, investigations into the induction of oxidative stress by BPA have generated numerous conflicting reports about prooxidant/antioxidant behavior [Babu et al., 2013, Chepelev et al., 2013], antioxidant depletion [Ge et al., 2014a, Huc et al., 2012], mitochondrial dysfunction [Kalb et al., 2016, Moon et al., 2012], alteration in cell signaling pathways [Chevalier et al., 2015, Ge et al., 2014b, Ge and Wang, 2016, Watson et al., 2005], and induction of cell death [Gassman et al., 2015, Huc et al., 2012, Leem et al., 2016, Ooe et al., 2005]. Here, a review of the current literature examining BPA exposure and the induction of ROS or oxidative stress will be provided to assess the landscape of the current BPA literature and potentially provide a framework for evaluating how induction of oxidative stress by BPA may influence the pleotropic and tissue-specific effects of BPA exposure.

2. Dosing

Contradictory *in vivo* and *in vitro* data has made evaluating the adverse health effects of BPA difficult for regulatory agencies and has fueled scientific and public controversy about the chemical. While there are a variety of factors that contribute to the generation of conflicting or contradictory data in the BPA field, including study designs, endpoints, and model systems, a significant contributor is the wide range of BPA doses utilized in the literature [Chapin et al., 2008]. BPA, like other estrogenic compounds and hormones, shows a non-monotonic dose-response [Vandenberg et al., 2012b], and the incomplete data on the dose-response effects of BPA in various model systems, make evaluating the health consequences of persistent BPA exposure more difficult [Chapin et al., 2008, NTP, 2008]. While a number of research efforts are focusing on developing an improved understanding of the toxicokinetics of BPA exposure [Schug et al., 2013, Vandenberg et al., 2013], studies into the adverse health effects of BPA continue to emerge, and the effects noted in these studies may provide important insight into the molecular mechanisms of BPA. So an important caveat to any review of the current literature is dosing.

The lowest observable adverse effect level (LOAEL) for *in vivo* studies has been defined as 50 μg/kg per day [EPA, 1993], while the *in vitro* LOAEL has been defined as 50 ng/ml or 2.19×10^{-7} M BPA [Welshons et al., 2006, Wetherill et al., 2007]. Examination of published

in vivo and *vitro* studies show that experimental BPA doses range from 0.2 to 50 μg/kg/bw in animal studies and from 10^{-15} to 10^{-4} M in cell studies (Tables I and II). Some researchers have attempted to address the non-monotonic dose-response by evaluating a number of BPA doses from low to high, while others have focused on a single dose, most often a low dose.

Human exposures to BPA have been estimated from 10–100 nM [LaKind and Naiman, 2015, Welshons et al., 2006], and studies frequently dose in this range in order to examine physiologically relevant effects. However, BPA contamination is ubiquitous, with the air, soil, and water revealing higher than expected levels of free BPA [Corrales et al., 2015, Fu and Kawamura, 2010]. This contamination can also extend to laboratory materials, and a report by Cao et. al in 2010 noted that laboratory plastics, while made of polystyrene or polypropylene rather polycarbonate, can be contaminated with low nanomolar concentrations of BPA (0.25–1.1 nM) [Cao et al., 2010]. They also reported this contamination extends to cell culture medium and fetal bovine serum, including charcoal stripped serum [Cao et al., 2010].

It is unclear how wide-spread this contamination may be since extremely low levels of detection are required by GC-MS instruments, and these contamination levels have not been confirmed by others in the field. However, these findings do suggests that low nanomolar dosing may be problematic due to contamination from laboratory materials and the inherent inaccuracies of achieving extremely low doses experimentally. These unknowns may be contributing to conflicting results and irreproducible data at low, but physiologically relevant doses. Higher doses might overcome background contamination, but it is unclear how these doses translate to human exposures.

Overall BPA dosing is still highly controversial. Here, dose and duration are noted in the text and in Tables I and II to assist in the evaluation of findings. Differences in dosing may significantly contribute to inconsistencies in the BPA literature, yet as highlighted here, a wide variety of BPA doses induce oxidative stress and adverse effects. Further research efforts in these areas should explore broad dose ranges and avoid extremely low doses to better evaluate the human health consequences of these effects.

3. Induction of free radicals and oxidative stress by BPA exposure

While a majority of BPA is converted into less toxic BPAG and BPAS, the remaining free BPA induces ROS through the enzymatic (H_2O_2/peroxidase and NADPH/CYP450) and non-enzymatic (peroxynitrite/CO_2 and $^{-}$OCl/HOCl) formation of phenoxyl radicals (described in [Atkinson and Roy, 1995a, Babu et al., 2013, Sakuma et al., 2010, Yoshida et al., 2001]). Subsequent reactions of these radicals with NADPH or intracellular glutathione (GSH) along with further enzymatic processing produce a variety of radical species, including superoxides, peroxides, and hydroxyl radicals [Babu et al., 2013, Sakuma et al., 2010]. Fluorescent reporters, such as dichlorodihydrofluorescein diacetate (DCFDA) and dihydroethidium (DHE) are frequently utilized to indirectly measure the generation of intracellular peroxides or superoxide after exposure to BPA in cells. These reporters have significant shortcomings in measuring reactive oxygen species and cannot be used to

identify specific radical species; however, they are frequently used to measure relative changes in oxidative stress [Kalyanaraman et al., 2012]. Most of the *in vitro* BPA studies utilize these probes, so general oxidative stress changes will be summarized and discussed here, and further work to identify radical species generated is needed in the field.

The generation of ROS by BPA has been examined using these fluorescent reporters in a large number of cell types and with doses ranging from 10^{-12} to 10^{-4} M (Table I) [Babu et al., 2013, Gassman et al., 2015, Ge et al., 2014a, Huc et al., 2012, Koong and Watson, 2015, Leem et al., 2016, Moon et al., 2012, Ooe et al., 2005, Pfeifer et al., 2015, Porreca et al., 2016, Xin et al., 2014]. Nanomolar BPA exposures can display short, transient bursts of ROS [Koong and Watson, 2015, Pfeifer et al., 2015], though longer sustained levels of oxidative stress were also reported [Huc et al., 2012, Porreca et al., 2016]. Differences in ROS generation and lifetime at these doses may be related to difference in cellular backgrounds, fluorescent reagents, or could result from concentration differences induced by background contamination [Cao et al., 2010].

Micromolar doses of BPA have been shown to increase oxidative stress levels 1–4 h after exposure [Babu et al., 2013, Gassman et al., 2015, Ge et al., 2014a, Huc et al., 2012, Leem et al., 2016, Ooe et al., 2005], and these levels may be maintained for up to 72 h [Huc et al., 2012, Xin et al., 2014]. Depending on cell type, some of the reported high micromolar doses, typically 10^{-4} M range, do result in cytotoxicity, likely mediated through ROS-induced DNA damage [Huc et al., 2012, Leem et al., 2016, Ooe et al., 2005]. However, a majority of BPA studies use doses that are not cytotoxic and significant generation of ROS has still been observed [Babu et al., 2013, Gassman et al., 2015, Ge et al., 2014a, Huc et al., 2012, Koong and Watson, 2015, Pfeifer et al., 2015, Porreca et al., 2016].

Given the wide range of cell types, doses, and endpoints measured, it is unclear if there are dose-dependent increases in ROS, despite suggestions of this effect by some authors. It is clear that levels of ROS vary significantly depending on the cell type and the hormone receptor status of the cells. A clear example of these effects were demonstrated by Koong and Watson using androgen-dependent and -independent prostate cell lines (LAPC-4 and PC3, respectively), where the same dose of BPA generated less ROS in the androgen-independent cells [Koong and Watson, 2015]. ERα status is also frequently examined in literature, with most studies reporting the induction of ROS, independent of ERα after exposure to BPA [Pfeifer et al., 2015]. Whether non-classical estrogen receptors significantly influence the production of ROS in cells has not been significantly addressed in the current literature, though some downstream effects (discussed in later sections) suggest they may play a role.

In human and animal studies, reactive species are also measured indirectly by examining damage induced to cellular macromolecules and DNA bases. Strong correlations between high urinary concentrations of BPA and increases in the levels of biomarkers for lipid peroxidation, malondialdehyde (MDA), and oxidatively-induced DNA damage, 8-hydroxydeoxyguanosine (8-OHdG), have been reported in a number of population studies [Asimakopoulos et al., 2015, Watkins et al., 2015, Yang et al., 2009, Yi et al., 2011, Zhang et al., 2016]. Increased levels of 8-OHdG and MDA are also observed in a number of animal

models with doses > 5 mg/kg/bw per day showing the most consistent and sustained generation of oxidative stress, though some low dose studies utilizing μg/kg/bw per day have reported increased levels of MDA and 8-OHdG as well (Table II) [Bindhumol et al., 2003, Chitra et al., 2003, Jain et al., 2011, Kabuto et al., 2004, Tiwari et al., 2012, Wu et al., 2013].

Taken together these data strongly support the prooxidant role of BPA. However, examination of the structure of BPA also illustrates its potential to act as a weak antioxidant via electron loss through the O-H bond [Chepelev et al., 2013, Kabuto et al., 2003, Kadoma and Fujisawa, 2000]. Several reports have noted a reduction in ROS after BPA exposure and indicated the potential for ROS scavenging by BPA [Chepelev et al., 2013, Ge et al., 2014a, Kabuto et al., 2003, Ponniah et al., 2015]. While the mechanisms underlying this effect have not been determined, contributing factors such as cellular microenvironment changes, cell type, and cell signaling responses could significantly contribute to the observed reduction in oxidative stress, as could differences in ROS measurement methods and time points [Chepelev et al., 2013, Ge et al., 2014a, Kabuto et al., 2003, Ponniah et al., 2015]. However, it is interesting to note that like other polyphenolic compounds, BPA may maintain weak antioxidant activities. While the overall energetics and competition of stronger antioxidants in the cellular milieu may not normally favor these activities, cell which experience high level of oxidative stress from their environments or functions, like trophoblasts, may have microenvironments that promote BPA's antioxidant activities [Chepelev et al., 2013, Ponniah et al., 2015].

These conflicting findings for prooxidant and antioxidant roles highlight the difficulty in assessing BPA effects, and the controversy that can result. Fluorescent reporters may cloud this issue further, and there are key differences in the doses, durations, and cellular microenvironments used in these reports that may be influencing the prooxidant/antioxidant role of BPA. The prooxidant actions of BPA are supported by numerous *in vivo* and *in vitro* studies, while the antioxidant role has only been reported *in vitro*. Together, this evidence suggests that BPA induces oxidative stress over a range of doses, and further work is needed to identify radical species generated.

4. Depletion of cellular enzymatic and non-enzymatic antioxidants

The ROS generated by the metabolic processing of BPA may exceed the capacity of the intracellular antioxidant system. These enzymatic and non-enzymatic antioxidant systems play a critical role in maintaining redox homeostasis, addressing the reactive species generated by the mitochondrial electron transport, NADPH oxidases, xanthine oxidases, or cytochrome P450s [Valko et al., 2007]. Free radical scavengers, such as reduced glutathione, ascorbic acid, thioredoxins, and α-tocopherol, and enzymes, like superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), play critical roles in redox reactions in the cell. Concentrations of these small molecules and expression levels of these enzymes determine the antioxidant capacity of cells, and several endpoints have been assayed after BPA exposure to determine its effect on antioxidant capacity.

The most common assays determine gene expression levels and activities of key enzymes (SOD, CAT, and GPx), determine the ratios of reduced and oxidized glutathione

(GSH:GSSG), or examine both enzymatic and non-enzymatic contributions at once by measuring the total antioxidant capacity (TAC) of a cell or tissue lysates. Exposure to high micromolar doses of BPA showed slight depletion of GSH (~10–30%) in INS-1, Sertoli, and mouse embryonic fibroblast cells, which correlated with observed increases in ROS [Gassman et al., 2015, Ge et al., 2014a, Xin et al., 2014]. While several studies have confirmed the induction of ROS with nanomolar doses of BPA, measurements of antioxidant capacities were not included in a majority of these studies [Huc et al., 2012, Koong and Watson, 2015, Moon et al., 2012, Pfeifer et al., 2015, Porreca et al., 2016]. GSH levels were examined after nanomolar dosing of Sertoli cells, and a 35% increase in GSH was observed, which was consistent with the lack of ROS detected at that dose [Ge et al., 2014a].

In animal studies, a range of BPA doses from μg/kg/bw to mg/kg/bw per day were shown to significantly reduce the TAC of a number of tissues and organs, including liver, pancreas, and testes [Hassan et al., 2012, Kalb et al., 2016, Moghaddam et al., 2015], and decreased activities of SOD, CAT, and/or GPx were also reported in brain, epididymal sperm, liver, kidney, pancreas, testes, and germ cells [Aydogan et al., 2008, Bindhumol et al., 2003, Chitra et al., 2003, Hassan et al., 2012, Jain et al., 2011, Kabuto et al., 2004, Kabuto et al., 2003, Kalb et al., 2016, Moghaddam et al., 2015, Moon et al., 2012, Tiwari et al., 2012, Wu et al., 2013]. Levels of thiobarbituric acid reactive substances (TBARS) were also increased in the liver and testes after BPA exposure [Bindhumol et al., 2003, Kabuto et al., 2003, Kalb et al., 2016, Wu et al., 2013]. However, studies of ovarian tissue from newborn mice dosed with 0.1, 1, 5, and 10 μg/ml or mice dosed *in utero* with 0.5, 20, or 50 μg/kg/bw per day showed no significant changes in antioxidant enzymes, though transgenerational increases in SOD, CAT, and GPx were observed from the *in utero* BPA exposure [Berger et al., 2016, Zhou et al., 2015].

While it appears that the observed reduction in antioxidant activities correlates well with the induction of ROS by BPA over a variety of doses, as noted for ROS induction, alterations in the enzymatic and non-enzymatic antioxidant schemes appear to be highly cell, tissue, and organ specific. To further evaluate this effect and its dose-dependence, there is a significant need for more systematic inclusion of these endpoints in cell and animal studies.

5. Induction of DNA damage and cytotoxicity

ROS-induced cytotoxicity is a well-studied mechanism of action for drugs, toxins, and toxicants and is often mediated by the induction of reactive species and the depletion of antioxidant activities in cells [Deavall et al., 2012, Orrenius et al., 2011]. Generated ROS can damage cellular macromolecules, induce DNA strand breaks, base lesions, and DNA proteins cross-links, and these effects are further enhanced through the depletion of antioxidant pathways. In addition to producing ROS, the enzymatic processing of BPA by cytochrome P450 also generates the DNA-reactive quinone form of BPA, by a mechanism similar to how natural estrogens are metabolized into to catechol estrogen-3,4-quinones [Cavalieri and Rogan, 2010]. BPA-DNA adducts have been observed *in vivo* and *in vitro* after high dose exposure of BPA [Atkinson and Roy, 1995a, Atkinson and Roy, 1995b, Izzotti et al., 2009].

High micromolar and millimolar doses of BPA have been reported to induce ROS, DNA damage and cytotoxicity in bone mesenchymal stem cells (hBMSC), hepatocytes, hepatocellular carcinoma (HepG2), neuronal cells (Neuro2a), and spermatogonia (GC-1) [Audebert et al., 2011, Leem et al., 2016, Nakagawa and Tayama, 2000, Ooe et al., 2005]. Induction of DNA strand breaks have also been observed in some studies at low nanomolar and micromolar doses of BPA, which are not cytotoxic [Fic et al., 2013, Gassman et al., 2015, Pfeifer et al., 2015, Xin et al., 2014]. The induction of DNA strand breaks by BPA has been linked to the induction of ROS in several studies with the addition of antioxidants or specific protein inhibitors that reduce the generation of ROS, reducing the strand break signaling observed [Pfeifer et al., 2015, Xin et al., 2014]. However, induction of strand breaks by non-toxic doses of BPA is not observed in all cell lines, with Audebert et al. report that BPA exposure did not induce γH2AX signaling in HepG2 cells being frequently cited [Audebert et al., 2011]. While there are several possible explanations, including biotransformation difference, for the observed differences in strand break generation or signaling after BPA exposure, another possible explanation may be that BPA induces alterations in chromatin structure and/or DNA damage response and repair that effect the generation and signaling of breaks [Allard and Colaiácovo, 2010, Fernandez et al., 2012, Gassman et al., 2015, Porreca et al., 2016].

We recently reported that high micromolar doses of BPA promoted the transient compaction of chromatin and the down-regulation of key DNA repair proteins involved in the recognition and excision of oxidatively-induced DNA damage [Gassman et al., 2016]. Compaction was observed within 4 h of exposure and was resolved within 24 h [Gassman et al., 2016]. Other studies have also noted changes in chromatin methylation after BPA exposure [Doherty et al., 2010, Warita et al., 2013], and a number of studies have determined that BPA exposure can alter the expression levels of DNA damage response and repair proteins [Acharya et al., 1996, Allard and Colaiácovo, 2010, Betancourt et al., 2010, Fernandez et al., 2012, Naciff et al., 2002, Naciff et al., 2010, Porreca et al., 2016, Yin et al., 2014]. Given that increases in ROS have been observed within 15 min of BPA exposure [Koong and Watson, 2015], rapid changes in chromatin compaction and methylation state may be occurring but have not been observed by the current literature. Examining changes in the chromatin state within 1–2 h of exposure and after longer exposure may help to reveal how epigenetic changes are induce by BPA.

Together these data suggest that the cytotoxicity and genotoxicity observed after BPA exposure may be linked to the generation of phenoxyl radicals and ROS [Babu et al., 2013, Fic et al., 2013, Gassman et al., 2015, Pfeifer et al., 2015, Sakuma et al., 2010]. Further, there is compelling evidence that BPA exposure may alter the recognition and repair of DNA damage, particularly oxidatively-induced DNA damage, through chromatin structure or modulation of DNA damage response and repair proteins [Gassman et al., 2016]. More studies are required to determine doses and durations that induce these effects and the consequences of these effects, since they could significantly contribute to the mutagenicity and genotoxicity of BPA.

6. Mitochondrial dysfunction

Early work on BPA indicated that its cytotoxic mechanism may be mediated through inhibition of mitochondrial energy production [Nakagawa and Tayama, 2000]. Millimolar doses of BPA were shown to deplete intracellular ATP by inhibiting both NAD- and FAD-linked respiration and uncoupling oxidative phosphorylation [Nakagawa and Tayama, 2000]. There is also growing evidence that nanomolar and micromolar doses of BPA accumulate in the mitochondria of cells and induces mitochondrial dysfunction [Chepelev et al., 2013, Ooe et al., 2005, Pfeifer et al., 2015]. The lipophilic nature of BPA may drive its accumulation into the mitochondrial membrane, though further work is required to validate the localization and potential interactions of BPA accumulated in mitochondria.

Several groups have specifically examined mitochondrial ROS and membrane potential after exposure. High micromolar doses were shown to increase mitochondrial ROS, particularly superoxide, in hBMSC, HepG2, Neuro2a, and GC-1 [Huc et al., 2012, Leem et al., 2016, Ooe et al., 2005]. Nanomolar doses of BPA also showed increased generation of ROS in mitochondria in HepG2, breast epithelial cells (184A1, ERα negative), and breast cancer cells (MCF7, ERα positive) [Huc et al., 2012, Pfeifer et al., 2015]. The increased ROS was typically observed for 24 h, though in the HepG2 study elevated mitochondria ROS was observed 48 and 72 h after exposure to extremely low nanomolar and picomolar doses of BPA [Huc et al., 2012], though these doses many be significantly higher than reported due to background contamination by BPA [Cao et al., 2010]. Further, superoxide production by the mitochondria was found to increase, contributing to cytosolic levels of ROS and driving lipid peroxidation [Huc et al., 2012].

Hyperpolarization of mitochondria was also observed over a range of BPA doses in HepG2 cells [Huc et al., 2012] and at 10 nM dosing of Sertoli cells [Ge et al., 2014a]. Micromolar dosing of Sertoli cells with BPA resulted in less dense mitochondria with significant loss in membrane potential [Ge et al., 2014a]. Proteomic profiling, along with increased levels of ATP, in the Sertoli cells dosed with 10 nM BPA indicate that an increase in energy metabolism is promoted by BPA exposure [Ge et al., 2014a], which was hypothesized by Huc et al. to explain their observations in the HepG2 cells [Huc et al., 2012]. Proteomic profiling of colorectal cancer cells dosed with 10 nM BPA also support large scale changes in energy metabolism, though mitochondrial function was not specifically examined [Chen et al., 2015].

Mitochondrial outcomes have not been well investigated in animal models, though two reports indicate BPA exposure may alter mitochondrial function. Spermatozoa from male mice exposed to BPA via breast milk from dams receiving 3000 μg/kg bw per day BPA were found to have lower mitochondrial functionality [Kalb et al., 2016]. While mice exposed to 0.05 and 1.2 mg/kg bw per day were shown to have reduced mitochondrial function, alter energy metabolism and impair autophagy in the liver [Moon et al., 2012].

BPA exposure has been implicated in cardiovascular disease, obesity, metabolic disorders, and diabetes [Gore et al., 2015, Kirkley and Sargis, 2014]. Given the evidence that low doses of BPA can alter superoxide production and mitochondrial integrity, further investigation

into the impact BPA exposure has on mitochondria is warranted. Particularly since mitochondria do not have the full suite of DNA repair pathways contained in the nucleus, mitochondria DNA damage, induced by ROS or BPA-DNA adducts, may induce mutations impacting mitochondria functions in exposed individuals or their offspring [Cline, 2012].

7. Cell signaling effects on ROS induction

While a comprehensive review of all the cell signaling changes that have been attributed to BPA exposure is beyond the scope of this review, it should be briefly noted that BPA exposure has been shown to alter cell signaling pathways that can be induced by ROS, contribute to the generation of ROS, or promote cell proliferation and pro-survival [Rochester, 2013, Watson et al., 2005]. In particular, activation of the mitogen-activated protein kinase (MAPKs), PI3K/AKT, and NF-κB pathways have been implicated in BPA-induction of oxidative stress and inflammation response and are proposed to be mediated through the nuclear or membrane ER signaling mechanisms [Ge et al., 2014b, Vinas and Watson, 2013, Zhu et al., 2015]. Overactivation of these pathways has been linked to increased cell proliferation, up-regulation of pro-survival proteins, and increased migration and invasion in a number of cells, including breast, colorectal, ovarian, and prostate [Chen et al., 2015, Koong and Watson, 2015, Ptak et al., 2014, Rubin, 2011, Song et al., 2015, Tohme et al., 2014]. Additionally, gene expression changes in several important oncogenes, such as Myc and Stat3, have also been reported [Dairkee et al., 2013, Goodson et al., 2011, Pfeifer et al., 2015, Ptak et al., 2014, Weinhouse et al., 2015, Zhu et al., 2015], and knockdown of c-Myc during BPA exposure was shown to reverse the induction of ROS and DNA damage [Pfeifer et al., 2015]. Conversely, studies that noted a reduction of ROS after BPA exposure observed an increase in Nrf proteins [Chepelev et al., 2013, Ponniah et al., 2015], and mRNA levels of heme oxygenase-1 (*ho-1*) and NAD(P)H Quinone Dehydrogenase 1 (*nqo1*) [Chepelev et al., 2013]. These changes indicated that BPA, when acting as an antioxidant, stimulated antioxidant response elements in the genome to protect cells from oxidative stress [Chepelev et al., 2013].

Further investigation of these cell signaling responses are required to better understand the balance between prooxidant and antioxidant roles of BPA, and the cell signaling changes that they produce. However, the strong pro-survival effects observed when BPA is co-dosed with other cytotoxic agents, like cisplatin, doxorubicin, X-rays, and KBrO3, may indicate that BPA can promote adaptive responses, using both its prooxidant and antioxidant properties, to ensure cell survival [Dobrzyńska and Radzikowska, 2013, Gassman et al., 2016, Gassman et al., 2015, Lapensee et al., 2009].

8. Conclusions

A significant impediment for regulatory agency in evaluating BPA induced exposure effects is inconsistencies or contradictory findings. These may arise from issues with dosing, background contamination, the wide variety of cell culture or animal models used, or the various endpoints used. However, these inconsistencies or contradictory findings might also arise from the complexity and pleiotropic actions of BPA.

As illustrated here, BPA can induce complex oxidative stress effects in cells related to the estrogen receptor status [LaPensee et al., 2010, Yin et al., 2014], the presence of non-classical estrogen receptors like ERRγ [Ge et al., 2014a, Ge et al., 2014b], and even in absence androgen receptors [Koong and Watson, 2015]. Additionally, cellular microenvironment appears to play a key role in BPA's actions as well, with cells already experiencing stress from serum starvation or co-exposed to other genotoxic agents showing pro-survival [Dobrzyńska and Radzikowska, 2013, Gassman et al., 2016, Gassman et al., 2015, Lapensee et al., 2009] and even antioxidant roles for BPA [Chepelev et al., 2013, Ponniah et al., 2015], which have not been observed in other cells lines or animal models.

While there still needs to be further and more rigorous study to establish the free radicals, key metabolites, and cellular conditions that induce oxidative stress from BPA exposure or even tip the balance toward a potential antioxidant role. Examination of the existing BPA literature, with a critical eye on dose, duration, and model system, reveals that there is growing and compelling evidence that a wide variety of BPA doses promote the generation of ROS, alter the antioxidant balance, induce mitochondrial dysfunction, and promote changes in a number of cell signaling pathways related to oxidative stress.

This clearly indicates that active, unconjugated BPA and its numerous metabolites have more significant exposure effects than originally considered. It further demonstrates that BPA's induction of oxidative stress, whether it acts in concert with or independent of its endocrine disrupting properties, may produce a number of pleiotropic effects that may profoundly influence disease development, reproductive toxicology, and cancer.

Acknowledgments

This work was supported by the National Institute of Environmental Health Sciences (NIEHS) grant number R00ES023813.

Abbreviations

8-OHdG	8-hydroxydeoxyguanosine
BPA	bisphenol A
BPAG	bisphenol A glucuronide
BPAS	bisphenol A sulfate
CAT	catalase
DCFDA	dichlorodihydrofluorescein diacetate
DES	diethylstilbestrol
DHE	dihydroethidium
EDC	endocrine disrupting chemical
ERα	estrogen receptor α

ERRγ	estrogen-related receptor γ
GPER	G-coupled protein receptors
GSH	glutathione
GPx	glutathione peroxidase
ho-1	heme oxygenase-1
LOAEL	lowest-observed-adverse-effect level
MDA	malondialdehyde
MAPK	mitogen-activated protein kinase
nqo1	NAD(P)H Quinone Dehydrogenase 1
ROS	reactive oxygen species
redox	reduction and oxidation
SOD	superoxide dismutase
TAC	total antioxidant capacity
TBARS	thiobarbituric acid reactive substances

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Table I

Induction of oxidative stress by BPA in cultured cells

Model system	Dose	Duration	ROS (Method)	Antioxidant	DNA strand breaks	Cell proliferation	Cytotoxicity	Reference
GT1-7 (hypothalamic neurons)	25 – 100 μM	up to 6 h	Increased at 50 and 100 μM, (DCFDA, MitoSox™)	n.d.	n.d.	n.d.	n.d.	[Babu et al., 2013]
Mouse embryonic fibroblasts	150 μM	up to 24 h	Increased (DCFDA, 1h); Increase in DNA lesions (4 h, GC-MS/MS)	Decrease in GSH (24 h)	Increased γH2AX at 4h	No increase	70% viable	[Gassman et al., 2015]
Sertoli TM4 (testis)	10 nM and 10 μM	24 h	No change at 10 nM; Increased at 10 μM (DCFDA)	Increased GSH at 10 nM; Decreased GSH at 10 μM	n.d.	Increased at 10 nM; Decreased at 10 μM	no	[Ge et al., 2014a]
HepG2 (hepatocellular carcinoma)	10^{-12}–10^{-4} M	24, 48, or 72 h	Increased (DCFDA, DHE and MitoSOX™)	n.d.	n.d.	n.d.	10^{-4}	[Huc et al., 2012]
LAPC-4 and PC-3 (prostate)	10^{-13}–10^{-7} M	15 min	Increased (DCFDA)	n.d.	n.d.	small effect on LAPC-4 >10^{-12}	no	[Koong and Watson, 2015]
MCF10A, 184A1, MDA-MB-231, MCF7 (breast epithelial and breast cancer)	10 nM	2, 3 or 24 h	Increased at 2 h (DCFDA, MitoTracker™)	n.d.	Increased γH2AX (24 h); Increased olive tail moment in neutral comet assay	Increased in MCF10A and 184A1; Decreased in MCF7; Slight increase in MDA-MB-231	no	[Pfeifer et al., 2015]
INS-1 pancreas	0, 25, 50, and 100 μM	24 h	Increased at 50 and 100 μM (DCFDA)	Decreased GSH all doses	Increased tail moment for 50 and 100 μM in comet assay	ND	ND	[Xin et al., 2014]
Neuro2a (neuronal) and GC-1 (spermatogonia)	50 and 100 μM	24 or 48 h	Increased at 50 and 100 μM, (DCFDA, MitoSox™, MitoTracker™)	n.d.	n.d.	n.d.	100 μM	[Ooe et al., 2005]
Bone Mesenchymal Stem Cells (hBMC)	200 μM to 500 μM	18 h	Increased MDA	n.d.	n.d.	no	>200 μM	[Leem et al., 2016]

Model system	Dose	Duration	ROS (Method)	Antioxidant	DNA strand breaks	Cell proliferation	Cytotoxicity	Reference
FRTL-5 (thyroid)	1 nM	24 or 72 h	Increased after 24 and 72 h (DCFDA)	n.d.	n.d.	no	no	[Porreca et al., 2016]
HepG2	10 or 100 nM	2, 6, 12, or	Increased MDA; Increased ROS (DHE) at 100 nM	n.d.	n.d.	n.d.	n.d.	[Moon et al., 2012]
HEK293 (epithelial kidney)	0–200 μM	24 h	Reduced ROS (DCFDA)	Increased GSH at 100 μm		Increased at 50 and 100 μM	no	[Chepelev et al., 2013]
BeWo trophoblast (model of placental trophoblasts)	0, 0.09, 0.9, and 9 μM	72 h	No change (DCFDA)	no change	n.d.	n.d.	> 45 μm	[Ponniah et al., 2015]
Hepatocytes, rat	0.25–1 mM	3 h	n.d.	n.d.	n.d.	n.d.	> 0.25 mM	[Nakagawa and Tayama, 2000]
HepG2	0.1 μM to 10 μM	4 and 24 h	n.d.	n.d.	Increased tail moment at 24 h for both doses in comet assay	n.d.	No	[Fic et al., 2013]

n.d. - not determined

Table II

Induction of oxidative stress by BPA in animal models

Model system	Dose (per day)	Duration	ROS	Antioxidant	Reference
Male mice (C57BL/6), liver	0.05 and 1.2 mg/kg/bw	5 days	MDA increased at both doses	GPx decreased at both doses	[Moon et al., 2012]
Male rats (Sprague-Dawley), germ cells	200 mg/kg/bw	10 days	Increased TBARS	Decreased SOD activity	[Wu et al., 2013]
Male rats (Wistar), liver	0.2, 2.0 and 20 μg/kg/bw	30 days	Increased hydrogen peroxide and TBARS	Decreased SOD, CAT, and GPx in mitochondrial and microsome rich fractions of liver.	[Bindhumol et al., 2003]
Male rats (Wistar), epididymal sperm	0.2, 2.0 and 20 μg/kg/bw	45 days	Increased hydrogen peroxide and TBARS	Decreased SOD, CAT, and GPx	[Chitra et al., 2003]
Male rats (Wistar), brain	2 and 20 μg/kg/bw per day	28 days	Increased MDA at both doses	Decreased GSH at both doses	[Jain et al., 2011]
Male and female rats (Holtzman), plasma and liver	2.4 and 10 μg/kg/bw per day and 5 and 50 mg/kg/bw	6 days	Increased 8-OHdG at 5 mg/kg/bw in plasma and increased MDA in liver at doses above 2.4 μg/kg/bw	Decreased GSH in liver at doses above 2.4 μg/kg/bw	[Tiwari et al., 2012]
Male mice (ICR), brain, kidney, liver, testis	25 and 50 mg/kg/bw	5 days	No significant change	Increased SOD in liver (25 and 50 mg/kg/bw); Decreased CAT in liver at 50 mg/kg/bw; Decreased GPx in kidney at 50 mg/kg/bw.	[Kabuto et al., 2004]
Male mice (ICR), brain, liver, testis	5 and 10 μg/ml	*In utero* and 4 weeks postnatal exposure	Increased TBARS at 10 μg/ml	Increased CAT in liver at 5 μg/ml; CAT decreased in testis at 10 μg/ml; Increased GPx in kidney at 10 μg/ml.	[Kabuto et al., 2003]
Male mice, serum and pancreas	0.5 and 2 mg/kg	4 weeks	Increased MDA observed in serum; MDA increased in pancreas	Decreased GSH in serum; GSH, TAC, SOD and CAT decreased in pancreas	[Moghaddam et al., 2015]
Male mice (Swiss albino), testes	300, 900, and 3000 μg/kg/bw	21 days	n.d.	Decrease in TAC at 900 and 3000 μg/kg/bw	[Kalb et al., 2016]
Male rats (Wistar), liver	0.1, 1, 10, 50 mg/kg/bw	4 weeks	n.d.	Decreases in GSH, TAC, SOD, CAT, and GPx	[Hassan et al., 2012]
Male rats(Wistar), brain	25 mg/kg/bw	45 days	Increased MDA	Decreased GSH	[Aydogan et al., 2008]
Female mice, (Inbred FVB), ovary	0.5, 20, or 50 μg/kg/bw	*in utero*	No significant change	Increased SOD, CAT, and GPx in second and third generation mice.	[Berger et al., 2016]
Female mice (CD-1), ovary	0.1, 1, 5, and 10 μg/ml	On isolated ovaries for 1–8	Increased ROS observed with DCFDA for 5 μg/ml at	No significant change	[Zhou et al., 2015]

Model system	Dose (per day)	Duration	ROS	Antioxidant	Reference
		days	day 8		

n.d.- not determined