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DBP-induced endoplasmic reticulum stress in male germ cells causes autophagy, which has a cytoprotective role against apoptosis in vitro and in vivo

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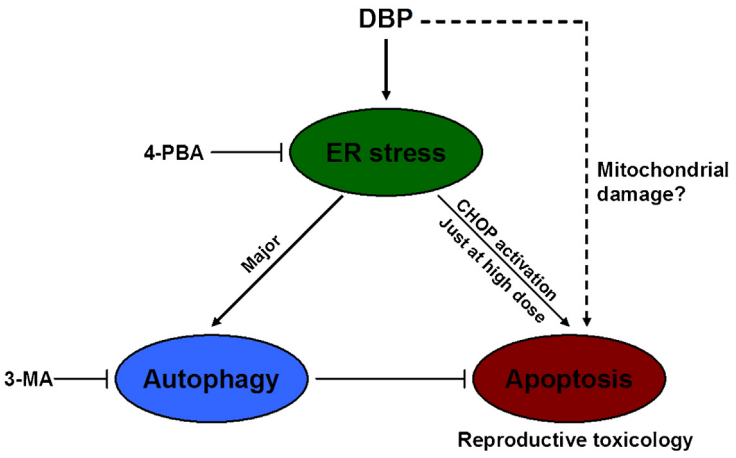
HIGHLIGHTS

- DBP exposure induced apoptosis both in vitro, in GC-2 cells, and in vivo, in prepubertal rat testis germ cells.
- DBP induced ER stress in germ cells both in vitro and in vivo.
- ER stress-mediated CHOP activation contributed to DBP-induced germ cell apoptosis.
- DBP-induced ER stress also triggered autophagy, which has a protective effect against germ cell death.

GRAPHICAL ABSTRACT



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ABSTRACT

Recently, spermatogenic cell apoptosis was shown to play a key role in the induction of testicular atrophy by dibutyl phthalate (DBP), thus causing reproductive toxicology. However, the molecular events induced by DBP in apoptotic germ cells remain unclear. In the present study, the mouse spermatocyte-derived GC-2 cell line was exposed to different doses of DBP. We found that DBP induced marked apoptosis in GC-2 cells. The levels of the major endoplasmic reticulum (ER) stress markers GRP-78, ATF-6, and p-EIF2α were elevated when GC-2 cells were exposed to 25 μM DBP and increased in a dose-dependent manner at higher concentrations. Furthermore, at a concentration that resulted in significant apoptosis (100 μM), CHOP, which plays a convergent role in ER stress-mediated apoptosis and is regulated by various upstream ER stress signals, was activated and partially contributed to the DBP-induced apoptosis.

Abbreviations: DBP, dibutyl phthalate; ER, endoplasmic reticulum; UPR, unfolded protein response; IRE1α, inositol-requiring protein-1α; PERK, protein kinase RNA (PKR)-like ER kinase; ATF-4, activating transcription factor 4; ATF-6, activating transcription factor 6; CHOP, transcription factor C/EBP homologous protein; LC3, microtubule-associated protein 1 light chain 3; 4-PBA, 4-phenylbutyrate; 3-MA, 3-methyladenine; TEM, transmission electron microscopy.

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However, inhibition of ER stress by 4-PBA, a chemical with chaperone-like activities, augmented the GC-2 cell apoptosis induced by DBP. Further experiments demonstrated that DBP-induced ER stress additionally had a protective role, mediated through the activation of autophagy. These results were confirmed in prepubertal rat testis germ cells; DBP treatment significantly induced testicular atrophy, accompanied by apoptosis, ER stress, and autophagy. Inhibition of ER stress and autophagy significantly aggravated the DBP-induced damage to the germ cells and testes. Taken together, our data suggest that DBP-induced ER stress in germ cells has a cytoprotective effect that is mediated through autophagy activation. These findings provide novel clues regarding the molecular events involved in DBP-induced germ cell apoptosis.

1. Introduction

Dibutyl phthalate (DBP) is one of the most widely studied phthalate derivatives and is often found in personal care products in addition to polyvinyl chloride (PVC) plastics. Because the phthalate is not covalently incorporated into the plastic matrix and is released into the environment under certain conditions, inevitable human exposure through ingestion, inhalation, and dermal contamination has caused public health concerns (Bosnir et al., 2003; Wittassek et al., 2011). In recent years, concerns have specifically been raised regarding toxicity to the male reproductive system (Hauser and Calafat, 2005; Heudorf et al., 2007). In particular, epidemiological research has indicated a close connection between DBP exposure and poor sperm quality (Jurewicz et al., 2013; Pant et al., 2008), and scientists have also reported an association between a decreased anogenital distance in male infants and their mothers' urinary levels of DBP (Swan et al., 2005). Because of its high production volume, the extent of human exposure, and published evidence of reproductive and developmental toxicity, among seven phthalates, DBP was prioritized for evaluation of potential human reproductive and developmental effects by the National Toxicology Program Center for the Evaluation of Risks to Human Reproduction (CERHR) (National Toxicology Program, 2003).

Among the effects of phthalates on the male reproductive system, the adverse effects following postnatal exposure are relatively well known. Animal experiments have shown that the most prominent effect of DBP in the testis is testicular atrophy (Bao et al., 2011). Tubular atrophy is accompanied by spermatogenic cell apoptosis (Alam et al., 2010b; D'Souza et al., 2005; Kondo et al., 2006). Several mechanisms have been proposed to explain the induction of apoptosis by DBP, including collapse of vimentin filaments in Sertoli cells (SCs), resulting in inadequate physical and metabolic support for germ cells (Alam et al., 2010b; Kavlock et al., 2002); dysfunction of Leydig cells (LCs), which may ultimately lead to interference with spermatogenesis (Chen et al., 2013); and activation of estrogen receptors in the testes of prepubertal rats as well as induction of spermatogenic cell apoptosis (Alam et al., 2010a). Moreover, a commonly used phthalate, di-(2-ethylhexyl) phthalate (DEHP), has been found to selectively induce the apoptosis of spermatocytes. Reactive oxygen species (ROS) are also increased selectively by DEHP in isolated germ cells, but not in SCs (Kasahara et al., 2002). Recent evidence and our previous study suggested that DBP induced oxidative stress and subsequent spermatogenesis defects in rats (Aly et al., 2015; Chen et al., 2011). Collectively, these findings indicate that the direct and indirect apoptotic effects of DBP on germ cells may coexist. However, the events in apoptotic germ cells remain unclear. Specifically, the target organelles and molecular events that are triggered by DBP to initiate apoptosis remain to be elucidated.

The endoplasmic reticulum (ER) is the central intracellular organelle required for protein translocation, folding, and post-translational modification. Various perturbations at the cellular level can disturb ER homeostasis, causing accumulation of unfolded proteins in the lumen of the ER. A process known as "ER stress" then triggers the unfolded protein response (UPR), which is mediated by three transmembrane ER proteins: inositol-requiring protein-1α (IRE1α), protein kinase RNA (PKR)-like ER kinase (PERK), and activating transcription factor 6 (ATF-6). The ER stress response was first proposed to be primarily an adaptive mechanism to alleviate the accumulation of misfolded proteins. Upon activation, the UPR proteins induce signal transduction reactions designed to increase protein-folding activity and to degrade misfolded proteins in the ER lumen (Hetz, 2012; Walter and Ron, 2011). Among those degradative pathways, autophagy has been extensively described as a pro-survival mechanism that eliminates the unfolded proteins and damaged organelles that accumulate during ER stress (Kroemer et al., 2010; Urra et al., 2013). Autophagy is specifically an intracellular self-defense process in which cytosolic contents are sequestered into autophagic vesicles that are subsequently degraded upon fusion with lysosomes (Amaravadi et al., 2011). More recently, ER stress was shown to increase cell viability via autophagy activation (Yan et al., 2014).

However, excessive ER stress can also trigger cell apoptosis by activating pro-apoptotic molecules, including transcriptional factor C/EBP homologous protein (CHOP), apoptosis signal-regulating kinase 1 (ASK1)/c-Jun amino terminal kinase (JNK), and caspase-12. Increasing evidence has also demonstrated that ER stress plays a key role in reproductive toxicant-induced spermatogenic cell apoptosis (Ji et al., 2011, 2013).

Although direct evidence for a relationship between DBP exposure and ER stress in testis germ cells has not been found, it has been demonstrated that phthalates (DBP and DEHP) increase the levels of intracellular Ca^{2+} and ROS, which are both closely related to ER stress (Nakamura et al., 2002; Rosado-Berrios et al., 2011; Wan et al., 2004). The ER is the major storage compartment for intracellular Ca^{2+} and the main source of free radicals, and disruption of calcium homeostasis or excessive calcium production contributes to the perturbation of ER function. These findings provide clues as to the potential effects of ER stress on the DBP-induced apoptosis of germ cells.

In the present study, we utilized chemical-exposed mouse spermatocyte-derived GC-2 cells as an in vitro exposure model and administration to rats as an in vivo exposure model to explore the molecular events and mechanisms influencing the apoptosis induced by DBP in spermatogenic cells, focusing on the roles of ER stress and autophagy.

2. Materials and methods

2.1. Chemicals and antibodies

DBP (99% pure), 4-phenylbutyrate (4-PBA), and 3-methyladenine (3-MA) were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA). The primary antibodies used in