Cigarette Smoke Exposure Leads to Follicle Loss via an Alternative Ovarian Cell Death Pathway in a Mouse Model

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Cigarette smoking among reproductive-aged women is increasing worldwide. Cigarette smoking is a lifestyle behavior associated with important adverse health effects including subfertility and premature ovarian failure. We previously demonstrated that cigarette smoke (CS) exposure in mice decreases the primordial follicle pool; however, the mechanism of action is largely unknown. Therefore, the present study was designed to elucidate the mechanisms underlying CS exposure–induced ovarian follicle loss. CS exposure induced a significant decrease in the relative ovarian weight and the number of primordial and growing follicles. Oxidative stress, as shown by increased Hsp25 and decreased superoxide dismutase 2 protein expression, was found in mice exposed to CS for 8 weeks. Exposure decreased Bcl-2 but failed to induce apoptosis. An increased number of autophagosomes in granulosa cells of ovarian follicles together with increased expression of Beclin-1 and microtubule-associated protein light chain 3, key regulatory proteins in the autophagy (Atg) pathway, was found in CS-exposed mice compared with the control group. Taken together, our results suggest that CS exposure does not induce apoptosis but rather activates the Atg pathway ultimately leading to ovarian follicle loss. We further postulate that Atg is a novel mechanism of toxicant-induced ovarian follicle loss.

Key Words: autophagy; apoptosis; ovarian follicle; cigarette smoking.

Of the many environmental toxicants and lifestyle factors known to affect fertility and ovarian function, cigarette smoking is potentially the most clinically relevant and preventable toxic exposure in women (Dechanet et al., 2010; Sadeu et al., 2010). Although cigarette smoking is declining in men, the number of women and teenage girls who smoke is increasing, becoming a global health issue (World Health Organization, 2007, 2008). Approximately 250 million women worldwide are daily smokers (World Health Organization, 2008) and 30% of reproductive age women in the United States are smokers (Woodruff et al., 2011), whereas in Canada, approximately 17% of women are current smokers, smoking an average of 14.0 cigarettes/day (Health Canada, 2003, 2009). Moreover, a survey of high school students in Southwestern Ontario revealed that 36.2% of teenage girls smoke (Cohen et al., 2003). Regrettably, most women remain unaware of the adverse effects of cigarette smoking on fertility (Roth and Taylor, 2001). Our prior studies revealed that women exposed to cigarette smoke (CS) had decreased implantation (12–12.6% for those exposed to CS vs. 25% for nonsmokers) and pregnancy rates (19.4–20% for those exposed to CS vs. 48.3% for nonsmokers) (Neal et al., 2005). Long-term to-pregnancy, reduced in vitro fertilization success rates, altered ovarian steroidogenesis, depleted ovarian reserves, impaired oocyte function and viability, and earlier mean age of menopause have all been documented in women who smoke versus nonsmokers (Baird et al., 2005; Crha et al., 2001; Curtis et al., 1997; El-Nemr et al., 1998; Freour et al., 2008; Fuentes et al., 2010; Hughes and Brennan, 1996; Hughes et al., 1994; Jick and Potter, 1977; Neal et al., 2005; Rosevear et al., 1992; Rowlands et al., 1992; Sharara et al., 1994; Sterzik et al., 1996; Van Voorhis et al., 1996; Waylen et al., 2009; Weigert et al., 1999; Zenzes et al., 1995; Zenzes et al., 1997; Zenzes, 2000), each of which has enormous health, emotional, and financial consequences. Although it is well documented that cigarette smoking depletes the ovarian follicle reserve in women (Soares et al., 2007) and mice (Tuttle et al., 2009), the mechanism underlying ovarian follicle loss in women who smoke remains unknown.

The ovary is a dynamic organ whose main features include steroidogenesis and cyclical recruitment of a cohort of follicles from the primordial pool of follicles (Knight and Gister, 2006; Themmen, 2005). During fetal development (in humans), or shortly after birth (in mice), the total lifetime supply of primordial follicles is established. The primordial follicle pool is gradually depleted through a repetitive process of recruitment, selection of a dominant follicle, and ovulation (Hillier, 2001; Knight and Gister, 2006). Nondominant follicles are lost through atresia, which is thought to be driven...
by apoptosis (Flaws et al., 2001; Tilly, 1998). This process is tightly regulated and can easily be disrupted by lifestyle and environmental toxicants.

Animal studies have shown that exposure to relatively high concentrations of environmental toxicants, compared with human exposure, results in the destruction of the follicle population, often in a stage-specific manner (Detmar et al., 2008; Devine et al., 2002; Devine et al., 2004; Jurisicova et al., 2007; Neal et al., 2008; van Beek et al., 2007). Although some environmental toxicants disrupt intra-ovarian signaling, others increase follicle atresia, thereby depleting the follicle pool prematurely. Animal models have confirmed that environmental toxicants destroy follicles in a stage-specific manner. Exposure to polychlorinated biphenyls results in the destruction of growing follicles (Muller et al., 1978; Pocar et al., 2006), whereas 4-vinylcyclohexene diepoxide, a metabolite of 4-vinylcyclohexene and a solvent used in industry, induces apoptosis in primordial and primary follicles (Devine et al., 2002; Devine et al., 2004). Benzo[a]pyrene (BaP), a polycyclic aromatic hydrocarbon (PAH) and constituent of CS, also depletes the primordial follicle pool (Mattison et al., 1980; Mattison and Nightingale, 1982). Primordial follicle destruction is considered to be the most disastrous, as the effects of it are not detected until years after the exposure, often after ovarian failure is well established (Cortvrindt and Smits, 2002). However, the follicle destruction seen in these animal studies is invariably due to toxicological concentrations (Desmeules and Devine, 2006; Devine et al., 2001, 2002, 2004; Jurisicova et al., 2007; Mayer et al., 2002; Takai et al., 2003) that are not representative of human exposure. In earlier animal studies, BaP exposure was found to selectively destroy the follicles in the resting pool (Mattison and Thorgeirsson, 1978) leading to speculation that a similar exhaustion of the resting pool was occurring in women who smoke, culminating in premature menopause (Mattison and Thorgeirsson, 1978; Westhoff et al., 2000). Moreover, a prior study also demonstrated that a relatively high concentration of BaP (1 mg/Kg/week for 6 weeks) increased the expression of the harakiri, a Bcl-2 interacting protein (Hrk) (Jurisicova et al., 2007). However, we note that aryl hydrocarbon receptor (AhR) ligand–induced apoptosis may be both tissue dependent and concentration dependent. Unlike the results above, treatment of pregnant mice with high concentrations of B[a]P and 7,12-dimethylbenz[a]anthracene (DMBA) (1 mg/Kg each/week for 6 weeks) inhibited apoptosis in the murine placenta (Detmar et al., 2008). In this study, treatments increased FasL and Xiap expression but decreased Bax and apoptosis in the placenta, suggesting tissue specificity. Moreover, developmental exposure to the AhR ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is associated with vaginal dysmorphogenesis in mice through attenuated apoptosis (Gray and Osby, 1995). Therefore, we postulate that AhR-mediated effects on apoptosis are also target tissue dependent. In our studies, we found a significant loss of follicles at all stages of development in mice exposed to CS but exposure did not activate either the intrinsic or the extrinsic apoptosis pathways as shown by absence of an effect on terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL), DNA laddering, and activated caspase-3 or caspase-8 and caspase-9 expression versus controls (Tuttle et al., 2009). Our results diverge from those of prior studies (Jurisicova et al., 2007; Matikainen et al., 2001) using profoundly higher concentrations of B[a]P than are found in CS. In addition, treatment of 4-day-old mouse ovaries in vitro with B[a]P decreased Bcl-2 protein expression while Bax remained unchanged, suggesting a concentration-dependent effect of CS and B[a]P exposure on ovarian follicle loss and apoptosis (Tuttle et al., 2009). We suggest that prior studies showing evidence of PAH-induced follicle destruction via Bax activation are phenomenological and not relevant to human health because of the high concentrations of B[a]P and DMBA used. Finally, mice lacking both the proapoptosis members Bax and the Bak are completely resistant to apoptosis; yet cell death proceeded normally with the appearance of autophagosomes and autolysosomes (Shimizu et al., 2004). Nonapoptotic cell death in these mice was dependent on the autophagic proteins Atg5 and Beclin-1. Disassociation of Beclin-1 from Bcl-2/Bcl-XL in the endoplasmic reticulum (ER) but not the mitochondria is a key driver of autophagy (Atg) (Maiuri et al., 2010).

Atg is an evolutionarily conserved process in eukaryotes, which translates to “self-eating,” and involves the cytoplasmic sequestering of cellular debris and organelles inside a double-membrane vesicle, termed an autophagosome, which is delivered to the lysosome for degradation (Mizushima and Levine, 2010; Szegedi et al., 2009; Yang and Klionsky, 2010). Atg is a fundamental cellular process that removes long-lived proteins and damaged organelles (mitochondria and ER) through lysosomal degradation. Key regulatory steps involve induction of Atg via nutrient starvation, genotoxic agents, or oxidative stress (Bursch, 2001; Vilser et al., 2010), which leads to activation of Beclin-1 and membrane nucleation. Beclin-1 is part of the class III phosphoinositide 3-kinase complex and is crucial for Atg but is inhibited by Bcl-2 (Li et al., 1999, 2001; Pattingre et al., 2005). During the autophagic process, microtubule-associated protein light chain 3 (LC3), the mammalian homologue of yeast Atg8, is processed from LC3-I to LC3-II in the endoplasmic reticulum and can be found in the membrane of the autophagosome. Mechanisms regulating cross talk between the apoptosis and Atg pathways are unclear; however, we note that Bcl-2 is at the interface between both pathways and emerging studies have identified Atg as an important alternative pathway of cell death in mammalian cells including human and rodent granulosa cells (Choi et al., 2010; Gawriluk et al., 2011; Vilser et al., 2010). However, the relevance of Atg to granulosa cell death and toxicant-induced changes in ovarian function are completely unexplored. Hence, we hypothesized that CS exposure would induce ovarian
follicle loss via Atg rather than apoptosis. Therefore, the present study was designed to elucidate the mechanism(s) underlying follicle loss following CS exposure. The exposure protocol used in our prior study (Tuttle et al., 2009) delivers a dose of CS that results in a serum cotinine (the metabolite of nicotine) concentration that is representative of that seen in women who smoke a pack of cigarettes per day.

MATERIALS AND METHODS

Ethics statement. All animal work described in this study was conducted using protocols approved by the McMaster University Animal Research Ethics Board and is in accordance with the Canadian Council for Animal Care guidelines for the use of animals in research.

Animals. The ovarian effects of CS exposure were studied in female C57BL/6 mice (8 weeks old at the start of exposure) obtained from Charles River Laboratories (Montreal, PQ, Canada). Mice were maintained in groups of three to five mice per cage in polycarbonate cages at 22 ± 2°C and 50 ± 10% relative humidity on a 12-h light-dark cycle and were provided with food (LabDiet; PMI Nutrition International, Saint Louis, MO) and tap water ad libitum throughout the experiment.

CS exposure. Mice were exposed to CS twice daily, 5 days/week for 4, 8, 9, or 17 weeks using a whole-body smoke exposure system (SIU-48; Promech Lab AB, Vintrie, Sweden). Details of the exposure protocol have been described previously (Botelho et al., 2010) and in Supplementary materials and methods.

Histology and follicle counts. One ovary from each mouse in each treatment group was collected for histology. Ovaries were processed as described in Supplementary materials and methods. Follicles were identified and classified under light microscopy using a modification of Pedersen and Peters’ classification system (Pedersen and Peters, 1968) and as described in Supplementary materials and methods.

Immunohistochemistry. Immunohistochemical staining was conducted using sections from the same ovaries as above but not needed for follicle counts as described previously (Tuttle et al., 2009) and in Supplementary materials and methods.

TUNEL labeling. TUNEL was conducted using sections from the same ovaries as above but not needed for either follicle counts or immunohistochemistry. Apoptotic cells in the ovary were labeled using the ApopTag Fluorescein In Situ Apoptosis Detection Kit (Chemicon International S7110, Temecula, CA) as per manufacturer’s instructions and outlined briefly in Supplementary materials and methods.

DNA damage. DNA was extracted from smoke-exposed and control samples using a QiAmp DNA Mini Kit (Qiagen, Mississauga, ON) and quantified using a spectrophotometer. For procedure on conversion of DNA to single-stranded DNA, see Supplementary materials and methods.

Protein carbonyl ELISA. Samples (10 μg/ml) in 1× PBS were assayed in triplicate using the OxiSelect Protein Carbonyl ELISA Kit (Cell Biolabs) as per manufacturer’s instructions and outlined briefly in Supplementary materials and methods.

Glutathione assay. Ovaries from smoke-exposed and control mice were prepared for use with the Glutathione Assay Kit (Cayman Chemical Company, Ann Arbor, MI) as described in Supplementary materials and methods.

Western blot. Protein expression was measured in whole-ovarian homogenates of smoke-exposed and control animals. Details of the protocol have been previously described (Tuttle et al., 2009) and are summarized in Supplementary materials and methods.

Electron microscopy. Ovaries were fixed for transmission electron microscopy (TEM) and summarized in Supplementary materials and methods. Autophagosomes in granulosa cells were counted in seven fields of view per ovary at ×7500 magnification and the average number of autophagosomes per female per treatment group was calculated. Only granulosa cells with a visible nucleus were counted. Autophagosomes were counted independently by two observers blinded to treatment.

Quantitative real-time PCR. Total RNA was isolated from ovaries using a Qiagen RNeasy mini kit with on-column DNase digestion (Qiagen) as per manufacturer’s instructions. Specifics of the procedure are summarized in Supplementary materials and methods and primer sequences can be found in Supplementary table 1. Analysis of gene expression changes were calculated according to the method of Livak and Schmittgen (2001) using the 2−ΔΔCt method.

Statistical analysis. All statistical analyses were performed using SigmaStat (v.3.1, SPSS, Chicago, IL), see Supplementary materials and methods.

RESULTS

General Health of Animals Exposed to CS

Treatment with CS had no effect on the general health of the mice, as shown by absence of stereotypical behaviors, hunched back and signs of lacrimation, porphyria, or ruffled coat. CS exposure for 8 weeks resulted in whole body and relative ovarian weights that were significantly lower compared with sham controls (Supplementary fig. 1 A–D). CS exposure also resulted in significant reductions in the number of follicles in different stages of development in ovaries of mice exposed to CS for 4, 8, 9, and 17 weeks compared with the sham exposed mice (Supplementary fig. 1 E–H and fig. 2).

Based on the above data, it was decided that an exposure of 8 weeks was sufficient to induce significant total and primordial follicle loss. Subsequently, all other experiments were performed on ovaries from mice exposed for this time period.

CS Exposure Results in a Stress Response and in Reactive Oxygen Species Damage

Whole ovary homogenates from mice exposed to CS for 8 weeks were examined to determine if there was a stress response in these animals compared with sham controls. Expression of the small heat shock protein Hsp25 was significantly induced (p < 0.001) in the ovaries of mice exposed to CS (Fig. 1A).

Protein expression of intracellular copper, zinc superoxide dismutase (SOD) 1, and mitochondrial manganese (Mn) SOD2 were measured. Although there was no significant change in SOD1 expression (Fig. 1B), expression of SOD2 was significantly lower (p < 0.001) in the ovaries of smoke-exposed mice (Fig. 1C), indicating a decreased ability to deal with reactive oxygen species (ROS). Treatment had no effect on 8-hydroxydeoxyguanosine (8-OHdG) (Fig. 1D), protein carbonyl formation (Fig. 1E), or total glutathione levels (Fig. 1F).
There was no difference in the number of TUNEL-positive cells between the two groups (Figs. 2A and B). Immunohistochemical staining of sham and smoke-exposed ovaries using antibodies directed against Bcl-2 and Bax showed a marked decrease in the expression of the antiapoptotic Bcl-2 in exposed ovaries with no change in the expression of the proapoptotic Bax (Fig. 2C), which was quantified and confirmed by Western blot (Fig. 2D). Despite a significant decrease in the expression of Bcl-2, there was no increase in the expression of Bax (Fig. 2E), a proapoptotic protein, which is directly regulated by Bcl-2. Although the Bax:Bcl-2 ratio was shifted in favor of apoptosis (Fig. 2F), there was no other evidence of increased apoptosis.

CS Exposure Induces Atg

Ovaries from sham and smoke-exposed mice were processed for TEM to determine whether treatment with CS-induced Atg-mediated cell death. TEM micrographs of granulosa cells from the ovaries of sham and smoke-exposed mice showed normal mitochondria and ER (Figs. 3A and 3B). In contrast, granulosa cells from CS-exposed animals contained large lysosomes and pleomorphic nuclei and an abundance of autophagosomes (Fig. 3B). Events of the Atg cascade beginning at phagophore formation through to autophagolysosome development were evident in the granulosa cells of smoke-exposed ovaries (Figs. 3C–F). Although the same number of granulosa cells were examined in each treatment group (Fig. 3G), the mean number (±SEM) of autophagosomes per ovary was significantly greater ($p < 0.001$; Fig. 3H) in smoke-exposed mice compared with controls. Real-time PCR of Beclin-1 and LC3 gene expression was upregulated 1.46-fold ($p < 0.018$; Fig. 3I) and 1.49-fold ($p < 0.001$; Fig. 3J), respectively, confirming activation of the Atg pathway.

DISCUSSION

Cigarette smoking is a well-documented health hazard and is potentially the most toxic and preventable hazard for reproductive function in women. Although CS exposure has been linked with earlier menopause and loss of ovarian follicles, the mechanisms underlying this phenomenon are unknown. Our results show that exposure to CS causes primordial follicle loss...
and decreased numbers of all follicle populations together with markers of oxidative stress in the ovary but failed to induce apoptosis as shown by absence of an effect on TUNEL staining. Our previous study also showed that expression of activated Caspase-3, the common executioner in apoptosis, was not changed (Tuttle et al., 2009). However, a profound increase in the number of autophagosomes in granulosa cells was found in ovarian sections from mice exposed to CS together with increased expression of Beclin-1 and LC3, key regulatory proteins in the Atg cascade. Taken together, our results suggest that CS exposure, at exposures representative of average smokers, induces granulosa cell Atg and ultimately depletion of the ovarian reserve of primordial follicles. Our results expand the literature by demonstrating that Atg is a novel alternative mechanism of follicle loss in the ovary that can potentially be activated by toxicant exposure.

The primordial follicle pool in mice was significantly lower as early as 4 weeks of exposure to CS. By 8 weeks, CS exposure was sufficient to induce a significant reduction in ovarian weight and primordial follicle numbers, which was extended to other follicle populations with continued exposure. In women, premature onset of menopause is characterized by the exhaustion of the resting pool of follicles, known as primordial follicles, resulting in anovulation, changes in circulating hormone levels, and cessation of menses. In the mouse, premature ovarian failure and primordial follicle loss can be seen in animals exposed to environmental toxicants (Li et al., 1995; Miller et al., 1992), including CS (Tuttle et al., 2009); however, as in humans, the molecular mechanisms are unknown. In the present study, CS exposure had no effect on the number of apoptotic granulosa cells, a finding that is consistent with our previous study (Tuttle et al., 2009). Mice exposed to CS for 8 weeks had significantly fewer follicles, notably in the primordial follicle pool, in the absence of differences in the expression of active caspase-3, the terminal effector enzyme of the apoptosis cascade (Tuttle et al., 2009). Our findings are contrary to previous studies, which show that exposure to a number of different ovarian toxicants result in follicle loss via apoptosis (Stacchiotti et al., 2009; Tabuchi et al., 2003). In one such study, BaP upregulated the expression of Hrk, a cell death gene activated downstream of the AhR which is involved in the regulation of the follicle pool in mice.
FIG. 3. Autophagosomes are present in the granulosa cells of CS-exposed ovaries. TEM micrographs of granulosa cells of (A) sham and (B) CS-exposed ovaries. Nuclei (N), mitochondria (arrows), ER (*), and autophagosomes (P) are visible within the cells of both treatment groups. Autophagolysosome formation in the granulosa cells of CS-exposed ovaries can be visualized at all stages of development in the granulosa cells of CS-exposed ovaries. (C) Developing phagophores (open arrows). (D) Sequestering of organelles and cytoplasmic materials within the developing autophagosome (arrowhead) and autophagosomes (P). (E) An autophagosome (P). (F) An autophagolysosome (Au). Original magnification: ×7500. (G) The number of cells counted in each treatment group was not different.
HRK functions by facilitating apoptosis via sequestration of Bcl-2 thereby leaving Bax free to form pores in the outer mitochondrial membrane causing the release of additional apoptotic factors stored within (Jurisicova et al., 2007). We propose that the divergent results are potentially due to differences in the concentration of BaP used in the previous study and that present in CS, which is significantly lower than doses of toxicants administered in previous studies (Devine et al., 2002; Devine et al., 2004; Jurisicova et al., 2007). Furthermore, CS is a complex mixture of chemical toxicants including nicotine, carbon monoxide, polyhalogenated hydrocarbons, and metals such as cadmium (Hoffmann and Wynder, 1986). Hence, the potential interactions among these chemicals at low concentrations cannot be excluded by the current study. Experiments designed to characterize the toxic chemicals underlying activation of Atg are currently underway using chemical fractionation of CS. It has been previously shown that the mechanism, stage of follicle affected, and speed at which follicle loss occurs is both compound and dose specific (Hoyer et al., 2009; Pandini et al., 2009; Shirota et al., 2007). Exposure to CS condensate, the particulate phase of CS, resulted in delayed follicle development and premature luteinization of follicles (Sadeu and Foster, 2011), whereas BaP exposure, at concentrations representative of human exposure, decreased steroidogenesis and anti-Mullerian hormone output of follicles (Sadeu and Foster, 2010).

In the present study, CS exposure induced oxidative stress, as shown by a threefold increased expression of Hsp25, a small heat shock protein that is upregulated under oxidative stress conditions (Stacchiotti et al., 2009). Moreover, SOD2 protein expression was decreased in exposed mice, which is in line with others who found that a loss of antioxidant activity, specifically SOD2 and glutathione, leads to oxidative damage in neuronal cells (Tabuchi et al., 2003). Of note, HeLa cells transfected with SOD2 siRNA and subsequently treated with thienyl trifluoroacetone had elevated ROS production, Atg, and cell death, whereas SOD2 overexpressing cells had reduced levels of cell death by 45% compared with controls (Chen et al., 2007). Mice exposed to CS for 16 and 32 weeks showed a marked increase in ROS generation in leukocytes (Talukder et al., 2011). In human neutrophils, exposure to CS extract resulted in tissue damage mediated by oxidative stress (Matthews et al., 2011). Circulating progenitor cells taken from smokers had a higher incidence of ROS production, lower plasma antioxidant concentration, and higher MnSOD protein expression and enzyme activity than those isolated from nonsmoking control animals (Talukder et al., 2011). CS contains more than 4000 chemical compounds, many of which are oxidants or free radicals that are inducers of oxidative stress. Many of these are also AhR agonists, the activation of which leads to induction of cytochrome P450, which is involved in the generation of ROS (Tagawa et al., 2008), which can lead to apoptosis and Atg (Tagawa et al., 2008).

Several distinct lines of evidence lead us to believe that CS exposure induce oxidative/ER stress leading to activation of the autophagic pathway to play an important role in ovarian follicle loss. Oxidative stress can trigger ER stress (Borradaile et al., 2006; Brookheart et al., 2009; Sorensen et al., 2006), which results in activation of the unfolded protein response (UPR). The UPR functions to maintain cellular homeostasis and several ER chaperone proteins including calreticulin, glucose regulated protein 78/immunoglobulin binding protein, those proteins containing the amino acid sequence KDEL (Lys-Asp-Glu-Leu), and protein disulfide isomerase mediate protein folding to stabilize nascent proteins and restore homeostasis (Sage et al., 2010). The inability of chaperone proteins to restore homeostasis leads to increased expression of CCAAT-enhancer binding protein homologous protein, an indicator of ER stress and a potent inhibitor of Bcl-2 expression (Sage et al., 2010; Tagawa et al., 2008). Furthermore, induction of ER stress leads to phosphorylation of Bcl-2 by c-Jun N-terminal kinase, which targets Bcl-2 for proteosomal degradation (Szegedzi et al., 2009). Specifically, we found that CS exposure results in a significant decrease in Bcl-2 expression together with a significant increase in the number of autophagosomes in the granulosa cells of ovarian follicles and an increase in the gene expression of two key Atg cascade mediators, Beclin-1 and LC3. Although Bcl-2 is a known inhibitor of apoptosis, it is also implicated in preventing the progression of Atg. In the Atg pathway, Bcl-2 interacts with Beclin-1, preventing it from facilitating membrane nucleation of the autophagosome (Glick et al., 2010; Kaushik et al., 2010). In addition to serving as a mechanism to rid the cell of misfolded, long-lived proteins, and superfluous or damaged organelles, Atg functions as an adaptive response to various stresses, including oxidative stress.

Mice lacking both Bax and Bak, proapoptosis members, are completely resistant to apoptosis; yet cell death proceeds in a normal manner with the appearance of autophagosomes and autolysosomes (Shimizu et al., 2004). Nonapoptotic cell death in these mice was dependent on the autophagic proteins Atg5 and Beclin-1. Disassociation of Beclin-1 from Bcl-2/Becl-XL in the ER but not mitochondria is a key driver of Atg (Fig. 4). Taken together, we postulate that CS exposure–induced decreased expression of Bcl-2 leads to follicle loss via Atg ultimately resulting in depletion of the primordial follicle pool. This finding is in keeping with and extends the literature by inculpating Atg as a novel alternative cell death pathway in granulosa cells that can be activated by CS exposure. Mechanisms regulating cross talk between apoptosis and Atg are unclear; however, emerging

(H) The incidence of autophagosomes in the granulosa cells of smoke-exposed ovaries compared with that in sham ovaries. Sham and smoke-exposed ovaries (n = 5). Real-time PCR of (I) Beclin-1 and (J) LC3 gene expression in smoke-exposed ovaries compared with sham controls. Fold change relative to actin (Beclin-1) and glyceraldehyde 3-phosphate dehydrogenase (LC3). Sham and smoke-exposed ovaries (n = 6). Values are expressed as the mean (± SEM).

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studies have identified Atg as an important alternative cell death pathway in mammalian cells, including human and rodent granulosa cells (Choi et al., 2010; Vilser et al., 2010). In human granulosa cells, unchecked oxidative stress led to increased expression of lectin-like oxidized low-density receptor1, a scavenger receptor and membrane glycoprotein that is activated by oxidized low-density lipoprotein (Vilser et al., 2010). Furthermore, CS extract increased conversion of LC3-I to LC3-II and increased Atg in cultures of human bronchial epithelial cells (Kim et al., 2008). Similarly, CS extract treatment induced Atg in lung epithelial cells, macrophages, and fibroblasts (Hwang et al., 2010). Therefore, we postulate that CS exposure can induce oxidative/ER stress leading to Atg in granulosa cells and that this nonapoptotic cell death pathway is central to follicle depletion as previously demonstrated.

In summary, CS exposure resulted in decreased ovarian weight and follicle numbers, increased oxidative stress as measured by Hsp25 and SOD2 expression, and profoundly increased the number of autophagosomes in granulosa cells of ovarian follicles but did not increase granulosa cell apoptosis. Moreover, CS-induced increased expression of Beclin-1 and LC3 further supports the hypothesis that Atg is the central underlying mechanism of CS-induced ovarian follicle loss. We further postulate that Atg is a novel pathway of follicle destruction activated by CS and potentially other environmental toxicants.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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