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Cigarette Smoke Exposure Elicits Increased Autophagy and Dysregulation of Mitochondrial Dynamics in Murine Granulosa Cells¹

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ABSTRACT

Cigarette smoking is a lifestyle behavior associated with significant adverse health effects, including subfertility and premature ovarian failure. Cigarette smoke contains a number of chemicals, many of which are involved in the generation of reactive oxygen species, which can lead to apoptosis and autophagy. Autophagy is a fundamental process that removes damaged organelles and proteins through lysosomal degradation. The relevance of autophagy to toxicant-induced changes in ovarian function is largely unexplored. Previously, we reported that exposure to cigarette smoke causes follicle loss, oxidative stress, activation of the autophagy pathway, and decreased expression of manganese superoxide dismutase, which points to altered mitochondrial function. Therefore, our objective here was to test whether exposure to cigarette smoke results in the dysregulation of mitochondrial repair mechanisms leading to loss of follicles via autophagy-mediated granulosa cell death. In this study, mice were exposed to cigarette smoke or room air for 8 wk. The expression of genes and proteins of autophagy and mitochondrial repair factors was measured using quantitative real-time PCR and Western blot analysis, immunohistochemistry, and enzyme-linked immunosorbent assay. Increased expression of parkin and decreased expression of the mitofusins suggest that exposure to cigarette smoke triggers mitochondrial damage. Moreover, the autophagy cascade proteins, BECN1 and LC3, were upregulated, whereas the antagonist BCL2 was downregulated, following treatment. Taken together, our results suggest exposure to cigarette smoke induces dysfunction of mitochondrial repair mechanisms, leading to autophagy-mediated follicle death.

autophagy, cigarette smoke, fertility, follicle, granulosa cells, ovary, smoking

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INTRODUCTION

Although numerous sources of infertility have been identified, many otherwise healthy couples of childbearing age experience infertility for unknown reasons. Premature exhaustion of the ovarian follicle reserve has been identified as a possible causative factor for infertility. Several studies have shown that exposure to environmental toxicants results in the destruction of the follicle population, often in a stage-specific manner [1–9]. Of the numerous environmental toxicants and lifestyle factors known to affect fertility and ovarian function studied to date, cigarette smoking may be the single most clinically relevant and preventable toxic exposure in women, making it an ideal target for infertility prevention [10].

Cigarette smoke and female fertility have been strongly associated. That smoking depletes ovarian follicle reserve and impairs uterine receptivity is well documented [11]. Studies conducted in our laboratory have revealed that women exposed to cigarette smoke had greatly decreased implantation and pregnancy rates [12]. We also found that benzo[*a*]pyrene (BaP), a polycyclic aromatic hydrocarbon (PAH) present in cigarette smoke and a known agonist of the aryl hydrocarbon receptor (AhR), is detectable in the serum and follicular fluid of women who smoke or are exposed to cigarette smoke and that treatment with BaP impairs cumulus expansion in isolated rat follicle culture experiments [5, 13]. More recently, we reported that mice exposed to cigarette smoke have smaller ovaries and significantly fewer primordial follicles compared to sham controls [14].

Cigarette smoke contains more than 4000 chemical compounds, many of which are oxidants or free radicals that are inducers of oxidative stress. Previous studies have revealed that of the chemicals present in cigarette smoke, PAHs, most notably BaP, are present at 10-fold higher levels in sidestream than in mainstream smoke [15] and have been shown to lead to the production of free radicals, reactive oxygen species (ROS), and oxidative stress. Cigarette smoke contains a number of AhR agonists, the activation of which leads to induction of the cytochrome P450 enzymes CYP1A1, CYP1A2, and CYP1B1, which are involved in the generation of ROS [16]. Oxidative stress and the production of ROS can lead to both apoptosis and autophagy. Results of several studies have pointed to B-cell leukemia/lymphoma 2 (BCL2)-associated X protein (BAX) activation as the central pathway regulating follicle numbers. However, emerging evidence challenges this belief, and our data support an alternative cell death pathway as being important in regulating follicle demise. Destruction of developing fetal ovarian germ cells and induction of premature ovarian failure has been achieved in mice treated with AhR ligands that activated the intrinsic pathway leading to enhanced Bax expression [17–19]. However, although in vivo exposure to cigarette smoke decreased BCL2 protein expression, BAX protein expression remained unchanged, and enhanced apo-

ptosis was not evident in treated ovaries, suggesting a concentration-dependent effect of exposure to cigarette smoke on ovarian follicle loss and apoptosis [14]. Exposure to cigarette smoke instead resulted in activation of the autophagy cascade, as evidenced by an increase in the number of autophagosomes and expression of key regulatory genes in the ovaries of exposed mice [20].

Autophagy is a fundamental cellular process that was first described nearly six decades ago by Clark [21], using electron microscopy, in mammalian cells and later systemically characterized by de Duve and colleagues [22–24]. Derived from the Greek for “self-eating,” autophagy is evolutionarily conserved from yeast to mammals. To date, approximately 35 autophagy genes have been identified in yeast, a number of which have had mammalian homologues identified. These genes have been established as important regulators of both bulk and selective autophagy [25–27], including Beclin 1 (*Becn1*), microtubule-associated protein 1 light chain 3 (*Lc3*), and *Bcl2*. Traditionally considered to be a stress adaptation to avoid cell death (as in starvation conditions), autophagy has also been implicated as an alternative pathway to cell demise in recent years [28–31]. Programmed cell death (PCD) type I demonstrates the hallmarks of apoptosis, whereas PCD type II has been coined to describe cell death that demonstrates hallmarks of autophagy—namely, the accumulation of autophagosomes. This classification has generated considerable controversy, due primarily to debate over whether autophagy actually promotes cell death or is simply a reactive process upstream of PCD type I, but numerous studies have shown that autophagy acts independently of apoptosis to elicit cell death during development [32–34] as well as in response to cytotoxic [35–37] and metabolic stressors [38].

Mitophagy is the selective degradation of mitochondria via autophagy. Mitochondria are essential energy-producing organelles that exist in a dynamic, interconnected network that is constantly reshaped by a strictly regulated balance between fission and fusion to maintain proper mitochondrial content in daughter cells and allow repair of damaged mitochondria [39–43]. During fission, mitochondria become fragmented and are targeted for autophagosomal degradation by parkin protein (PARK2), while at the same time, the ubiquitination and degradation of central fusion proteins, mitofusin 1 (MFN1) and mitofusin 2 (MFN2), is occurring [44–46]. During apoptosis, mitochondrial cristae are remodeled, opening their tubular junctions, which leads to release of proapoptotic factors, such as cytochrome *c*, and activation of the apoptosis cascade [47, 48], a process that we have consistently shown is not upregulated by exposure to cigarette smoke [14, 20]. In contrast to fission, key regulatory proteins in mitochondrial fusion drive mitochondrial elongation, increased cristae density, and maintenance of ATP output [49] to sustain cell viability. Thus, unopposed mitophagy results in an energy-deficient state in the affected cells that culminates in the death of those cells.

Although exposure to cigarette smoke increases the number of autophagosomes [20], it is not known if the adverse effects are primarily mitochondrial-specific (mitophagy) or if multiple organelles undergo autophagy (bulk autophagy). Therefore, the objective of the present study was to explore the mechanistic pathway linking exposure to cigarette smoke to mitochondrial dysfunction and autophagy, a novel alternative cell death pathway important in follicle development and demise. Decreased expression of superoxide dismutase 2 (SOD2) suggests mitochondrial damage in ovaries exposed to cigarette smoke [20]. Fusion and elongation provide a mechanism for mitochondrial repair, a process that opposes autophagy [49,

50]. Taken together, we postulate that cigarette smoke exposure-induced mitochondrial dysfunction in granulosa cells leads to autophagy-mediated cell death, a novel alternative ovarian cell death pathway.

MATERIALS AND METHODS

Ethics Statement

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

Animals

The ovarian effects of exposure to cigarette smoke were studied in female C57BL/6 mice (8 wk of age at the start of exposure) obtained from Charles River Laboratories. Mice were maintained in polycarbonate cages at 22°C ± 2°C and 50% ± 10% relative humidity on a 12L:12D photoperiod and were provided with food (LabDiet; PMI Nutrition International) and tap water ad libitum throughout the experiment.

Exposure to Cigarette Smoke

Mice were exposed to cigarette smoke twice daily, 5 days a week, for 8 wk using a whole-body smoke exposure system (SIU48; Promech Lab AB). Details of the exposure protocol have been described previously [51]. Briefly, cigarette smoke from twelve 3R4F reference cigarettes (Tobacco and Health Research Institute, University of Kentucky) with filters removed was mixed with room air and delivered into the exposure chamber over a 50-min period twice daily. Animals were placed in the restrainer, which was then placed in the smoke-exposure box. No lead-up period was required for smoke exposure; however, mice were acclimatized to the restrainer over a 3-day period before commencement of the experiment. This acclimatization was accomplished by placing mice in the restrainer for 20 min on Day 1, 30 min on Day 2, and 50 min on Day 3. Following the acclimatization period, mice were exposed to cigarette smoke for 50 min twice daily, 5 days a week, for 8 wk before euthanization. Control animals were placed in the restrainer for 50 min twice daily, 5 days a week, and exposed to room air only. Mice were euthanized at the end of the exposure by exsanguination, and ovaries were collected and weighed before processing.

Electron Microscopy

Ovaries were collected and processed for electron microscopy as described previously [20]. Briefly, ovaries were excised and fixed with 2% glutaraldehyde buffered in 0.1 M sodium cacodylate buffer containing 0.05% calcium chloride (pH 7.4) at 4°C. The tissue was washed in 0.1 M sodium cacodylate buffer with 4% sucrose and stored at 4°C. Tissue blocks from six mice per treatment group were sectioned (section thickness, 75 µm) with a Sorvall TC-2 tissue sectioner and postfixed in 1.5% ferrocyanide reduced osmium tetroxide, followed by dehydration in ethanol and infiltration in propylene oxide, and then embedded in Epon (Miller-Stephenson Chemical Co. Inc.). Sections were analyzed for the presence of autophagosomes in the granulosa cells.

Immunohistochemistry

Ovaries were fixed in 10% (v/v) formaldehyde, washed in cold water, and transferred to 70% ethanol 24 h later. Following fixation, ovaries were dehydrated in graded ethanol solutions, cleared in xylene, and embedded in paraffin. Serial sections (section thickness, 4 µm) were prepared and mounted on glass slides, deparaffinized in xylene, and rehydrated in graded ethanol solutions. Following rehydration, endogenous peroxidase activity was quenched and antigen retrieval carried out using citrate buffer (pH 3.0) at 37°C for 30 min. Sections were blocked with horse serum. Avidin/biotin blocking was carried out before incubation with primary antibody (1:100; BECN1; Cell Signaling) was performed on 4-µm-thick ovarian slices for 16 h at 4°C. Immunohistochemical targets were localized using diaminobenzidine (DAB; 0.25 mg/ml [w/v]; Sigma Aldrich) in PBS (8 g/L [w/v] NaCl, 0.2 g/L [w/v] KCl, 1.44 g/L [w/v] Na₂PO₄, and 0.24 g/L [w/v] KH₂PO₄; pH 7.4) and counterstained using Harris hematoxylin (Sigma Aldrich). Sections were dehydrated and cover slips mounted using Permount (Fisher Scientific). Slides were examined by a reader blinded to treatment group using an Olympus IX81

microscope, and images were captured using Image Pro-AMS (Media Cybernetics). Positive-staining cells were counted using Image Pro (Media Cybernetics) to identify cells within each follicle stained with DAB and expressed as a percentage of granulosa cells per follicle that were positive for BECN1.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from ovaries using an RNeasy Mini Kit (Qiagen) with On-Column DNase Digestion (Qiagen) as per manufacturer's instructions. Potential genomic DNA contamination was removed from the samples by treatment with RNase-free DNase (Invitrogen, Life Technologies) for 15 min at 37°C. Following confirmation of RNA integrity by gel electrophoresis and spectrophotometric quantification, cDNA was reverse transcribed using an iScript kit (Bio-Rad).

Quantitative Real-Time PCR

Gene- and species-specific primers for prosurvival factor *Bcl2* and mitochondrial repair mechanism markers *Parkin*, *Mfn1*, and *Mfn2* were obtained from SA Biosciences. Control reactions without cDNA and a no-RT control were run to verify the absence of primer dimerization and genomic DNA contamination, respectively. PCR amplification was carried out in a 20- μ l reaction volume containing 1–5 ng of cDNA, 0.5 μ M each of forward and reverse primers, and 10 μ l of Fast SYBR Green Master Mix (SABiosciences). The PCR reactions were initiated with denaturation at 95°C for 10 min, followed by 40 amplification cycles of 95°C for 15 sec and 60°C for 1 min. Samples were run in triplicate, and results were averaged. Crossing threshold (CT) was calculated using the analysis software SDS 2.2.1 (Applied Biosystems, Life Technologies). Changes in gene expression were calculated according to the method described by Livak and Schmittgen [52].

Beclin 1 Enzyme-Linked Immunosorbent Assay

Ovaries from mice exposed for 8 wk to either sham or cigarette smoke were homogenized in 0.02 mol/L of PBS (pH 7–7.2) and quantified using the Bradford method of protein quantification. BECN1 concentrations in ovarian homogenates were assayed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Beclin 1 ELISA; Shanghai BlueGene Biotech Co. Ltd.). Sample BECN1 concentration was calculated using a four-parameter logistic curve-fit with the formula $y = \min + (\max - \min) / (1 + \text{abs}(x / \text{EC50})^{\text{Hillslope}})$. The sensitivity in this assay is 0.1 ng/ml. Values were calculated and expressed as the mean \pm SEM.

Western Blot Analysis

Protein expression was measured in whole-ovarian homogenates from either sham or smoke-exposed mice as described previously [20]. Following SDS-PAGE and transfer to polyvinylidene difluoride blotting membrane (Bio-Rad), membranes were blocked overnight with 5% (w/v) skim milk in Tris-buffered saline (TBS; 8 g/L [w/v] NaCl, 0.2 g/L [w/v] KCl, and 3 g/L [w/v] Tris base; pH 7.4) with Tween-20 (TBS-T; 1 \times TBS and 0.5% [v/v] Tween-20) and incubated with primary antibody. The following antibodies were used for the present study: beta actin (ACTB; 1:5000; Abcam), BCL2 (1:2000; Abcam), BECN1 (1:1000; Cell Signaling), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:5000; Abcam), LC3 (1:2000; Novus Biologicals), MFN1 (1:2000; Sigma Aldrich), MFN2 (1:2000; Sigma Aldrich), and PARK2 (1:1000; Cell Signaling). Following washing with TBS-T, blots were incubated with horseradish peroxidase-conjugated secondary anti-rabbit immunoglobulin (Ig) G (1:4000; Amersham Biosciences) or anti-mouse IgG (1:4000; Amersham Biosciences) antibodies for 1 h at room temperature. Blots were thoroughly washed in TBS-T followed by TBS, whereupon reactive protein was detected using ECL Plus chemiluminescence substrate (Amersham Biosciences) and Bioflex x-ray film (Clonex Corporation). Densitometric analysis of immunoblots was performed using ImageJ 1.37v software (National Institutes of Health); all proteins were quantified relative to the loading control.

Statistical Analysis

All statistical analyses were performed using SigmaStat (v.3.1; SPSS). Results are expressed as the mean \pm SEM unless otherwise stated. Data were checked for normality and equal variance, and treatment effects were tested using *t*-test. A value of $P \leq 0.05$ was considered to be significant.

RESULTS

General Health of Animals Exposed to Cigarette Smoke

Treatment with cigarette smoke had no effect on the general health of the mice, as shown by the absence of stereotypical behaviors, hunched back, and signs of lacrimation, porphyria, or ruffled coat. Our previous work has shown changes in whole-body and relative ovarian weights following 8 wk of exposure to cigarette smoke [20] in the absence of any signs of adverse effects on the general health of the animals.

Autophagosome Formation Is Evident Following Exposure to Cigarette Smoke

Ovaries from sham and smoke-exposed ovaries were collected and processed for transmission-electron microscopy (TEM) to confirm our previous findings of autophagosomes in the granulosa cells of ovarian follicles treated with cigarette smoke. Nuclei were normal in appearance in granulosa cells from both sham and smoke-exposed mice (Fig. 1), although nuclei were displaced by autophagosomes in smoke-exposed mice (Fig. 1B). Autophagosomes were more abundant in granulosa cells of smoke-exposed mice compared with controls, in keeping with our previous findings [20]. Additionally, though mitochondria were visible in the cytoplasm of granulosa cells from both treatment groups (Fig. 1, arrowheads), autophagosomes located in smoke-exposed ovaries contained large, swollen organelles resembling mitochondria (Fig. 1B, arrows).

Exposure to Cigarette Smoke Alters Immunolocalization of BECN1 Protein

Immunohistochemical analysis of ovaries sectioned and stained using the rabbit anti-BECN1 antibody revealed changes in the intensity, amount, and location of staining in treated versus control ovaries. BECN1 staining was seen in all stages of follicle development, including primordial follicles. Low-power magnification of sham and smoke-exposed ovaries (Fig. 2, A and B, respectively) showed BECN1 staining in both treatment groups. Primordial follicles showed evidence of staining in both sham (Fig. 2C) and smoke-exposed (Fig. 2D) follicles; however, localization in the smoke-exposed ovaries differed from that in the sham ovaries. In smoke-exposed primordial follicles, the granulosa cells stained positive for BECN1, whereas in sham follicles, only the oocyte stained positively. This pattern did not hold true for larger follicles, however. In primary (Fig. 2, E and F), preantral (Fig. 2, G and H), and antral (Fig. 2, I and J) follicles, staining was evident in the theca, granulosa cells, and oocyte in both treatment and sham follicles. However, follicles in the smoke-exposed ovaries had a higher percentage of positive-stained cells (only significant in preantral follicles, $P = 0.008$) (Fig. 2K), and the intensity of staining was greater. Interestingly, the pattern of staining was also different. In the sham ovaries, BECN1 staining was more punctate, with less cytoplasmic staining, whereas in the smoke-exposed mice, staining was evident in the cytoplasm and more diffuse than punctate in nature.

Exposure to Cigarette Smoke Results in Upregulation of Autophagy Machinery

Homogenates from whole ovaries were examined to determine if the genes of the autophagy cascade and the proteins for which they code were altered following treatment. Changes in gene expression, as measured by changes in mRNA

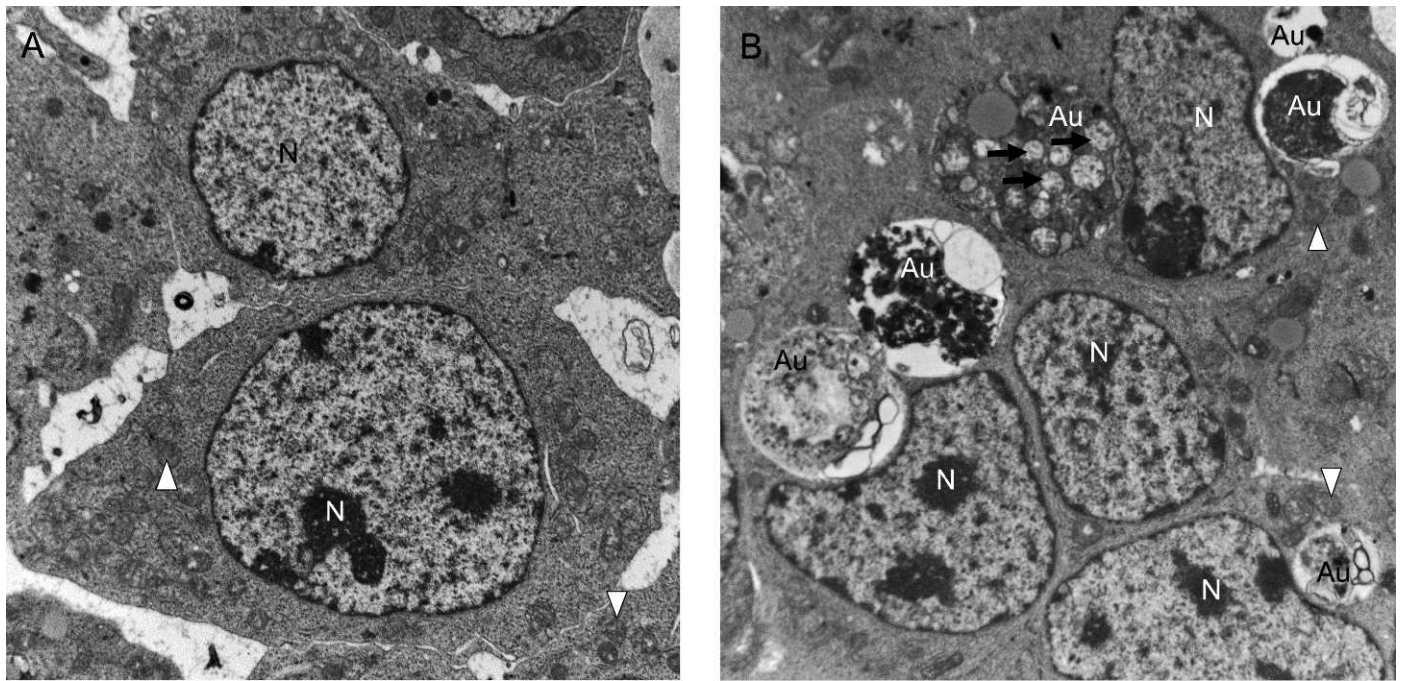


FIG. 1. Autophagosomes are present in the granulosa cells of ovarian follicles exposed to cigarette smoke. Representative transmission-electron micrographs of granulosa cells from growing follicles in sham (A) and cigarette smoke-exposed (B) ovaries are shown. Nuclei (N) appear normal but are displaced by autophagosomes (Au) in smoke-exposed ovaries. Mitochondria are visible in both sham and smoke-exposed ovaries (arrowheads) and can be visualized within an autophagosome (arrows) in a smoke-exposed ovarian follicle. Original magnification $\times 10\,000$.

expression using real time RT-PCR, were assessed for *Bcl2*. Whereas gene expression of *Bcl2* was not changed, a trend toward downregulation was seen (Fig. 3). Protein expression of BCL2, however, was significantly lower in the ovaries of mice exposed to cigarette smoke ($P = 0.003$) (Fig. 3). In keeping with gene expression data from our previous experiment [20], both BECN1 and LC3 proteins were significantly upregulated in smoke-exposed mice ($P = 0.002$ and 0.037 , respectively) (Fig. 4, A and B, respectively). An ELISA also revealed that BECN1 was more abundant in smoke-exposed mice compared to sham controls ($P = 0.003$) (Fig. 5).

Mitochondrial Repair Mechanisms Are Disrupted by Exposure to Cigarette Smoke

Gene and protein expression of three important regulators of mitochondrial repair was examined in the homogenates of ovaries from smoke-exposed and control mice. Significant increases in the expression of both the gene ($P = 0.001$) and protein ($P = 0.006$) of the proflission marker parkin were seen following exposure to cigarette smoke (Fig. 6, A and B, respectively), whereas expression of the genes *Mfn1* and *Mfn2*, encoding the profusion proteins MFN1 and MFN2, was decreased ($P = 0.003$ and 0.052 , respectively) (Fig. 6, C and E, respectively). In line with the changes in gene expression, expression of both MFN1 and MFN2 was significantly downregulated following treatment ($P < 0.001$ and $P = 0.02$, respectively) (Fig. 6, D and F, respectively).

DISCUSSION

Our results show that exposure to cigarette smoke causes overexpression of the autophagy proteins BECN1 and LC3 and underexpression of the autophagy inhibitor BCL2. Coupled with induction of autophagy, mitochondrial dysfunction appears to occur following exposure to cigarette smoke, as

evidenced by increased expression of the profission protein PARK2 and decreased expression of the profusion proteins MFN1 and MFN2. These findings, together with our previous results that revealed a profound increase in autophagosomes in granulosa cells and overexpression of the proautophagy genes *Becn1* and *Lc3* [14, 20], suggest that exposure to cigarette smoke induces mitochondrial dysfunction, culminating in mitochondrial-specific autophagy.

In the present study, exposure to cigarette smoke results in changes in the localization and expression of the BECN1 protein. BECN1 was present but restricted to the oocyte in resting primordial follicles of sham mice. In addition to the oocyte, BECN1 was present in both granulosa and theca cells of larger follicles. These findings are inconsistent with those of Gaytan et al. [53], who found that staining was restricted to the theca cells of secondary, antral, and preovulatory follicles in the human ovary; however, this disparity could be explained by interspecies differences as well as variation in the sensitivity of the antibody used. To our knowledge, no published studies have identified the immunolocalization pattern of BECN1 expression in murine ovaries. Expression of BECN1 in untreated ovaries was expected, however, because numerous studies have identified a basal level of expression in a variety of cells, including rat pheochromocytoma cells [54], human umbilical vein endothelial cells [55], ovarian follicles [53], melanoma cells [56], mouse ovarian tissue [20], and mouse cortical and hippocampal tissues [57]. BECN1 is a key protein initiator of autophagy, a fundamental cellular process that constitutively eliminates damaged organelles (mitochondria and endoplasmic reticulum [ER] and long-lived, insoluble proteins via lysosomal degradation and that is essential for survival, differentiation, development, and homeostasis. BECN1, a BCL2-regulated protein, is required for normal mammalian development [53] and is at the intersection between apoptosis and autophagy, suggesting a role in the interrelationship between the two processes. In the present

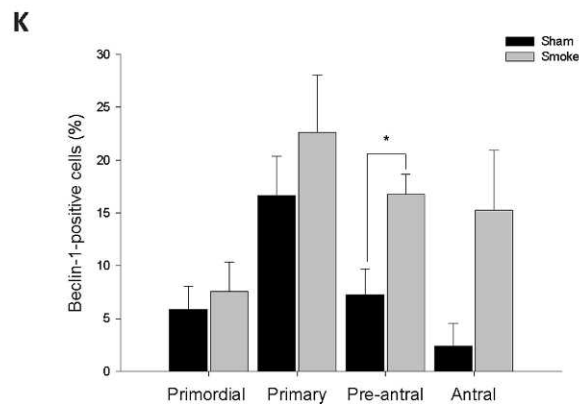
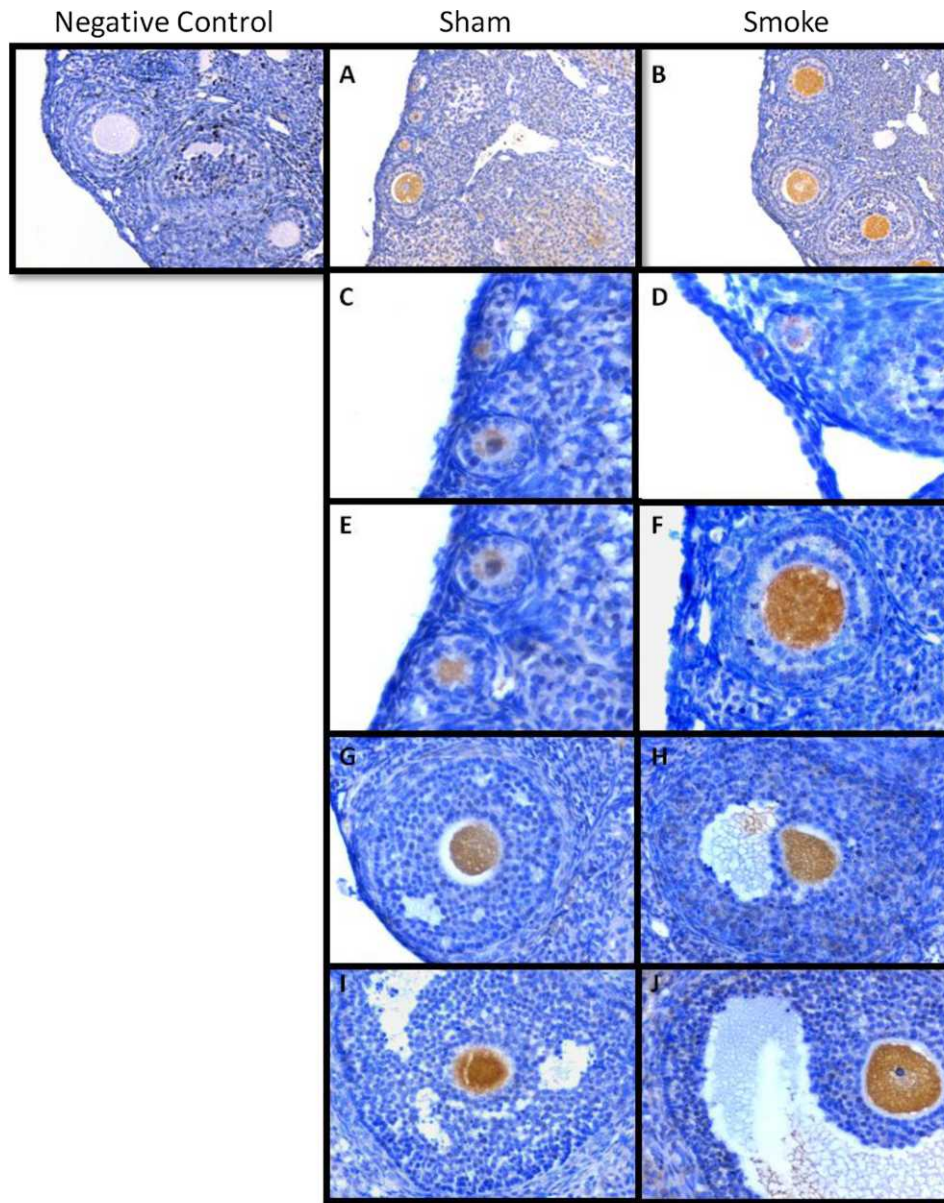


FIG. 2. BECN1 protein localization. Immunohistochemical staining for BECN1 in ovaries from negative control, sham (A, C, E, G, and I), and cigarette smoke-exposed (B, D, F, H, and J) mice is shown. Follicles at all stages of maturity (C and D, primordial; E and F, primary; G and H, preantral/secondary; I and J, antral) expressed BECN1 protein in both sham and smoke-exposed mice. The intensity of the staining as well as the localization differed between treatment groups and follicle stages. The percentage of BECN1-positive cells present in follicles at each stage of development is also shown (K). Data were checked for normality and equal variance, and treatment effects were tested using *t*-test. Values are expressed as the mean \pm SEM. * $P \leq 0.05$. Original magnifications $\times 10$ (negative control), $\times 4$ (A and B), and $\times 20$ (C–J).

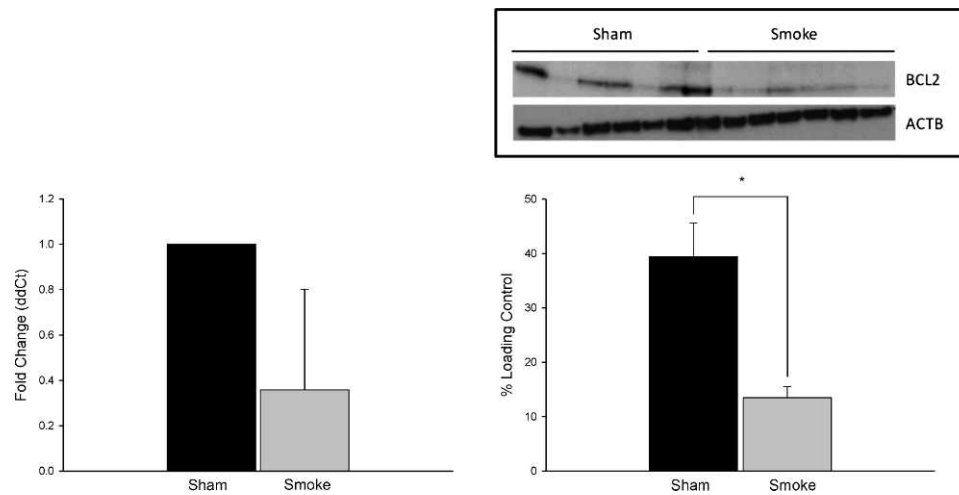


FIG. 3. Changes in *Bcl2* gene and BCL2 protein expression following 8 wk of exposure to cigarette smoke. Analysis of *Bcl2* gene and BCL2 protein expression was performed on whole-ovarian homogenates from sham and smoke-exposed mice. The graph in the left column depicts gene expression changes relative to *Actb* control ($n = 6$ per group). The graph and representative blot in the right column depict protein expression changes relative to ACTB loading control ($n = 7$ sham and 6 smoke). Data were checked for normality and equal variance, and treatment effects were tested using *t*-test. Values are expressed as mean \pm SEM. * $P \leq 0.05$.

study, BECN1 expression is increased following exposure to cigarette smoke, and immunohistochemical staining reveals a shift from focal to diffuse staining throughout the cytoplasm. Activation of the autophagy cascade begins with induction of BECN1 expression and membrane nucleation. Taken together, we interpret these data as evidence that exposure to cigarette smoke increases BECN1 expression, leading to wider distribution in the cytoplasm to sites of membrane nucleation and induction of autophagolysosome development.

In the present study, we examined the effect of exposure to cigarette smoke on the expression of genes and their respective proteins involved in the autophagy cascade. Of these, we found a significant increase in both gene and protein expression of two proautophagy cascade members, BECN1 and LC3. Moreover, whereas we saw no change in *Bcl2* gene expression, an autophagy antagonist, a significant decrease was observed in BCL2 protein expression following cigarette smoke

exposure. Our findings are consistent with those of others who have demonstrated that drugs such as etoposide and staurosporine (known apoptosis inducers) as well as cigarette smoke and its extract induce autophagy in a variety of cell types and species [35, 38, 58–62], suggesting that although tissue and/or species differences exist, autophagy-mediated cell death can act as a substitute for apoptosis under certain conditions. Interestingly, not all treatments that elicited an autophagic response resulted in the same type of autophagy; to wit, whereas etoposide treatment caused death-associated autophagy in Hep3B hepatoma cells, staurosporine treatment in the same cells was cytoprotective [62]. Our results are in keeping with the above-mentioned studies, but Shimizu et al. [35] have demonstrated that overexpression, rather than reduction in the expression of either BCL2 or BCL2-like 1 (BCL2L1; a BECN1-interacting protein), resulted in autophagy-mediated cell death similar to that seen in their double *Bax*/

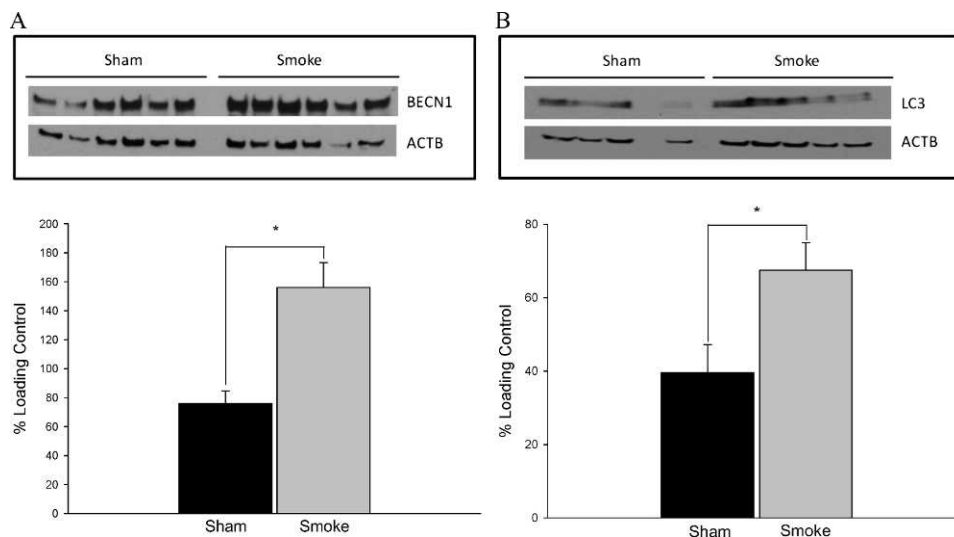


FIG. 4. Autophagy-related proteins are altered following 8 wk of exposure to cigarette smoke. Analysis of BECN1 (A) and LC3 (B) protein expression was performed on whole-ovarian homogenates from sham and smoke-exposed mice. Protein expression was measured relative to ACTB loading control (A, $n = 6$ per group; B, $n = 5$ per group). A representative blot is shown for each graph. Data were checked for normality and equal variance, and treatment effects were tested using *t*-test. Values are expressed as mean \pm SEM. * $P \leq 0.05$.

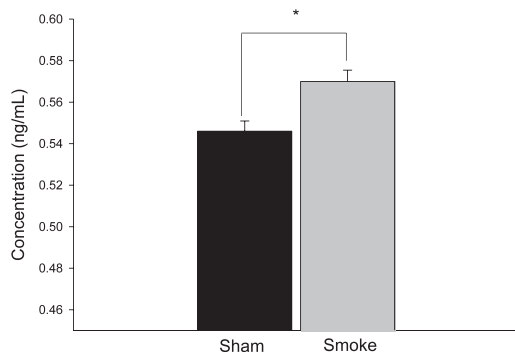


FIG. 5. BECN1 protein changes following exposure to cigarette smoke. Protein expression changes measured by Western blot analysis were confirmed using a Beclin 1 ELISA ($n = 15$ per group). Data were checked for normality and equal variance, and treatment effects were tested using t -test. Values are expressed as mean \pm SEM. $*P \leq 0.05$.

Bak mutants [35]. Once again, death-associated autophagy appeared to be regulated differently than starvation-induced autophagy (survival). For instance, BCL2L1 is required for regulation of BECN1 in death-associated, but not in starvation-induced, autophagy, and upregulation of BECN1 expression is only seen in death-associated autophagy [63].

Emerging evidence challenges the long-held belief that BAX activation is the central pathway regulating follicle density, and our data support the view that an alternative cell death pathway is important in regulating follicle demise. Mechanisms regulating cross-talk between the apoptosis and autophagy pathways are unclear. However, we note that BCL2 is at the interface between both pathways, and several studies have identified autophagy as an important alternative pathway of cell death in mammalian cells, including human and rodent granulosa cells. We and others have shown stress-induced cell death often proceeds in an apoptosis-independent manner. Specifically, mice lacking both *Bax* and BCL2-antagonist/killer 1 (*Bak*) are completely resistant to apoptosis, yet cell death, the execution of which is dependent upon the induction of autophagy, progresses normally [35]. Similarly, human granulosa cells with unopposed oxidative stress demonstrated increased autophagy following increased expression of lectin-like oxidized low-density receptor (LOX-I), a scavenger receptor and membrane glycoprotein that is activated by oxidized low-density lipoprotein [61, 64]. During yolk removal in some fish species, autophagosomes containing degenerating mitochondria and other material were evident in follicular cells [29]. Finally, our lab reported a significant loss of follicles at all stages of development in mice exposed to cigarette smoke without activating either the intrinsic or extrinsic apoptosis pathways despite a significant reduction in the expression of BCL2 [14, 20].

We have previously shown that both cigarette smoke condensate and BaP delayed follicle development and decreased estradiol-17 and antimullerian hormone output of follicles in isolated follicle cultures [13, 65, 66], whereas exposure to cigarette smoke induced oxidative stress [20], as shown by increased heat shock protein 25 (HSP25) and decreased SOD2 expression, but did not induce apoptosis in mice [14, 20], as shown by no changes in active caspase 3 (CASP3) expression, TUNEL staining, or DNA laddering. The observed absence of changes in active CASP3, the common executioner in the apoptosis cascade, coupled with a lack of increased TUNEL staining in treated ovaries led us to conclude that apoptosis was not upregulated following exposure to cigarette smoke. This conclusion was further supported by our

TEM experiments, which showed a distinct lack of hallmarks of apoptosis and an abundance of autophagosomes in various stages of maturity [20]. These findings, coupled with our present findings, suggest that cigarette smoke induces oxidative stress via an AhR-dependent process, leading to dysregulation of mitochondrial dynamics, as evidenced by the observed increase in PARK2 expression and the decrease in MFN1 and MFN2 expression, leading to mitophagy and ultimately follicle demise. Overall, our findings suggest that exposure to cigarette smoke, representative of exposure in women who smoke a pack a day, does not activate the apoptosis machinery of ovarian follicles [14, 20] but instead induces autophagy (present study and [20]), which leads to death of the granulosa cells and ultimately death of the follicle. Numerous studies have shown that treatment with ovarian toxicants results in apoptosis in the ovary [2, 7, 67–77]. We propose that the concentrations of chemicals used in those studies were significantly higher than the concentrations achieved in women who smoke and thus resulted in activation of the canonical apoptosis cell death pathway, whereas our treatment initiated autophagy that in all likelihood was initially an adaptive response to protect follicle development and mediate oxidative stress caused by the cigarette smoke but that ultimately was overcome due to the chronic activation of the cascade, which led to autophagy-mediated cell death. This hypothesis is not without precedent. Previous studies have shown that prolonged activation of the autophagy cascade, particularly nonphysiological assaults (i.e., chemotherapy), lead to autophagy-mediated cell death without the activation of apoptosis [78, 79].

Mitochondrial homeostasis is vital to the survival of cells. Mitochondria are responsible for the execution of a number of processes upon which cells depend to maintain appropriate energy levels, reduce ROS accumulation, and carry out PCD [39]. In mouse pancreatic beta cells, metabolically stressed mitochondria, in the form of free fatty acids and high glucose levels, displayed elevated levels of membrane potential heterogeneity and increased autophagy [43, 80, 81]. Before mitophagy, mitochondria have been shown to depolarize and are sequestered in a preautophagy pool [42]. These mitochondria no longer contribute to the pool of mitochondria capable of fusing. Mitofusins play an important role in maintaining mitochondrial integrity. In mouse embryonic fibroblasts, the absence of either MFN1 or MFN2 resulted in reduced fusion and increased numbers of fragmented mitochondria [82] as well as increased sensitivity of the cells to various apoptotic stimuli [83]. Interestingly, inhibition of autophagy (by deletion of autophagy related gene 5 (*Atg5*) or silencing of *Becn1*) or induction of mitophagy (starvation-induced) both resulted in mitochondrial membrane depolarization [42], suggesting that changes in membrane potential are indicative of perturbations in the delicate balance between mitochondrial survival and elimination.

Finally, in the newly fertilized oocyte, selective mitophagy has been documented whereby sperm mitochondria are selectively targeted for mitophagy immediately following fertilization [84]. The oocyte requires a large amount of energy to produce a mature oocyte that contains the appropriate number of chromosomes and, as such, contains the most mitochondria of all the cells in the body, making the repair and maintenance of mtDNA integrity paramount. Hence, the proper balance between mitochondrial fission and fusion and its core proteins is essential to producing a viable oocyte. Numerous studies have also shown that to produce a viable oocyte, healthy granulosa cells are required [85–98]. Thus, our findings showing increased autophagy and mitochondrial dysfunction in granulosa cells suggest that cigarette smoke decreases

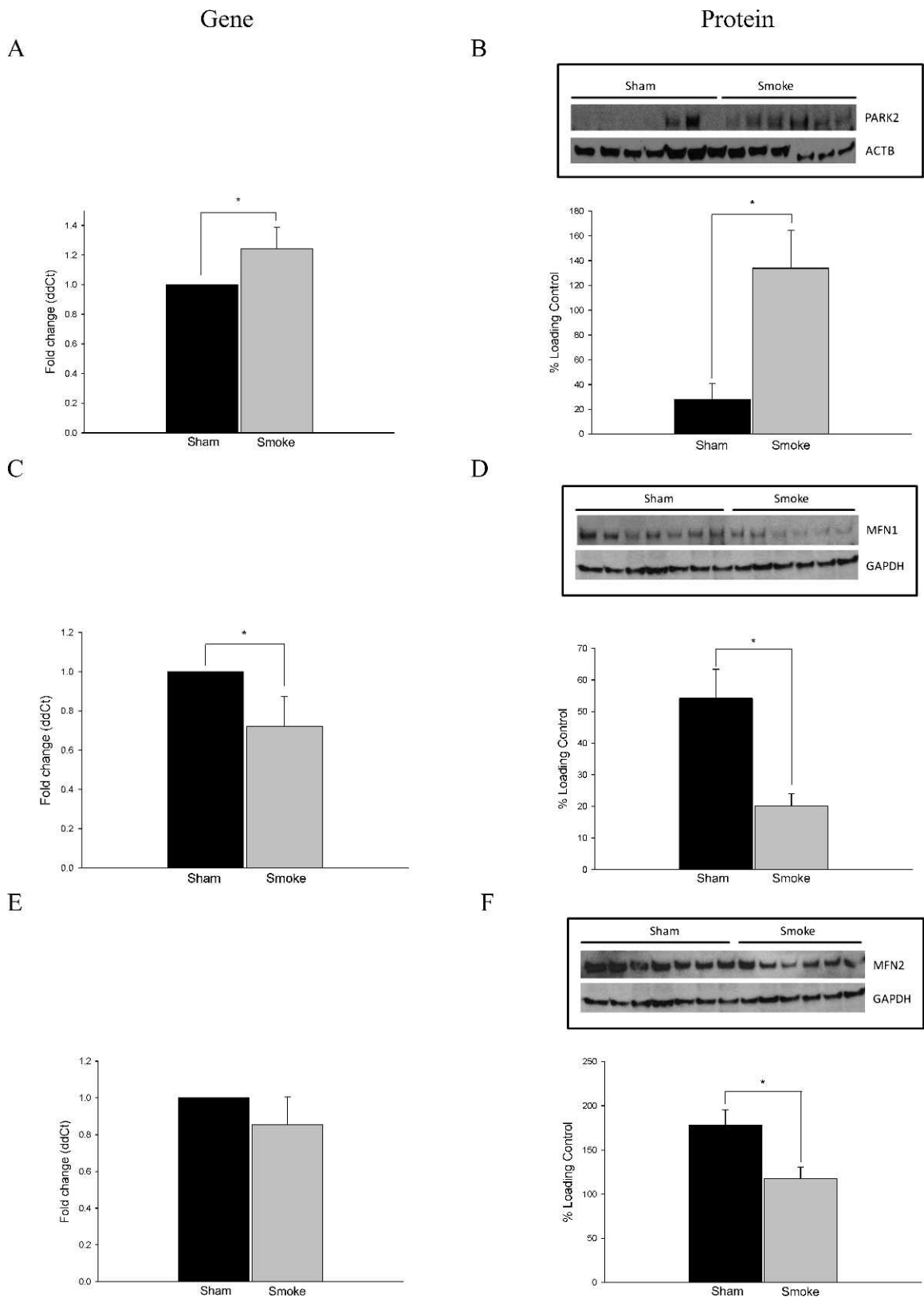


FIG. 6. Dysregulation of mitochondrial repair mechanisms is evident in ovaries exposed to cigarette smoke for 8 wk. Analysis of PARK2 (**A** and **B**), MFN1 (**C** and **D**), and MFN2 (**E** and **F**) gene and protein expression in whole-ovarian homogenates from sham and smoke-exposed mice is shown. Gene expression changes (**A**, **C**, and **E**; $n = 6$ per group) were measured relative to *Actb* control and protein expression changes (**B**, **D**, and **F**; $n = 7$ sham and 6 smoke) were measured relative to ACTB (PARK2) or GAPDH (MFN1 and MFN2) loading controls. Data were checked for normality and equal variance, and treatment effects were tested using *t*-test. Values are expressed as mean \pm SEM. * $P \leq 0.05$.

granulosa cell numbers via autophagy and, in so doing, decreases follicle survival.

In summary, we have shown in the present study that exposure to cigarette smoke results in mitochondrial dysfunction, as supported by increased expression of the profission PARK2 and the subsequent decreased expression of its targets, the profusion proteins MFN1 and MFN2. Enhanced autophagy activity was also apparent, as evidenced by the increased presence of autophagosomes in treated ovaries and via increases in gene and protein expression of BECN1, which is a key initiator of autophagy, and of LC3, which is responsible for sequestration of organelles within the autophagosome, as well as the decreased expression of the autophagy antagonist BCL2. Taken together, our results suggest that cigarette smoke, in doses relevant to human exposure, causes mitochondrial damage and dysfunction, leading to enhanced autophagy activity in the granulosa cells of ovarian follicles. Although our results show that exposure to cigarette smoke induces changes in the expression of several mitochondrial-specific proteins, we cannot rule out potential adverse effects on other organelles following cigarette smoke exposure. Given that all smokers do not suffer from infertility, we postulate that reparative autophagy is inadequate to manage cigarette smoke-induced oxidative stress; the threshold needed to support follicle growth and oocyte development is exceeded, resulting in follicle loss and subfertility. The number of young women who start smoking is growing [99], suggesting that prevention strategies are not effective deterrents in this population. Coupled with the addictive quality of nicotine, often requiring multiple attempts to quit smoking [100], this implies that counseling women to quit is not an effective strategy when managing smoking-related infertility. Moreover, it is unknown if cigarette smoke-induced ovarian damage is reversible by smoking cessation. Therefore, the mechanistic pathway elucidated in the present study is important in identifying the potential targets of antioxidant therapies for fertility preservation. With mitochondrial dysfunction being implicated in a broad spectrum of diseases covering every aspect of medicine, our results may have far-reaching implications both in reproductive medicine and throughout medicine in general.

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