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In vitro exposure to environmental tobacco smoke induces CYP1B1 expression in human luteinized granulosa cells☆

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Abstract

Women smokers and women exposed to environmental tobacco smoke (ETS) have reduced ovarian function as evidenced by an earlier menopause, reduced follicular numbers, decreased levels of circulating estradiol, and decreased conception rates; however, the mechanism of action of altered ovarian function by ETS is poorly understood. The direct effects of ETS were evaluated using human luteinized granulosa cells (HLGCs) exposed to ETS in primary cell culture. Exposure to ETS caused a decrease in both estradiol and progesterone production. There was a concentration dependent increase in CYP1B1 gene and protein expression without a change in catechol-*O*-methyltransferase (COMT) expression. This is the first report of CYP1B1 induction secondary to ETS exposure in cells from the human ovary. CYP1B1 metabolizes both endogenous estrogens and polyaromatic hydrocarbons in ETS to a variety of reactive species and may contribute to the complex effects of ETS on ovarian function.

Keywords: Environmental tobacco smoke; CYP1B1; Catechol-*O*-methyltransferase; Ovary; Estradiol; Progesterone; Corpus luteum

1. Introduction

It is estimated that each year more than 400,000 deaths in the United States can be attributed to the adverse health effects from cigarette smoking [1,2]. In addition to the host of detrimental systemic effects, cigarette smoking has a number of adverse effects on the female reproductive system. Smoking is associated with reduced ovarian function as evidenced by an earlier menopause, decreased levels of circulating estradiol, and decreased conception rates [3–6]. Constituents and metabolites of tobacco smoke such as cadmium and cotinine as well as an increase in oxidative stress have been demonstrated in the follicular fluid of smokers [7–10]. This coupled with the demonstration of benzo(a)pyrene–DNA adducts in the corpus luteum and ovarian vasculature of smokers suggests a direct effect of smoking on the ovary [11].

Cigarette smoking has long been associated with a decrease in circulating estradiol levels during both the follicular and midluteal phases of the menstrual cycle [5]. This has functional significance as smokers have a secondary increase in osteoporosis and a decrease in estrogen responsive cancers such as endometrial cancer [12–14]. The mechanism of action is likely due to both a decrease in production and an increase in metabolism and elimination of estrogens. Constituents of tobacco smoke including anabasine and nicotine have been shown to decrease the production of estradiol in an *in vitro* system by directly competing with aromatase, the enzyme responsible for the conversion of androgens to estrogens [15,16]. In addition, livers from smokers have increased 7-ethoxyresorufin *O*-deethylase activity, [17,18] which correlates with increased hepatic P450 activity and may contribute to an increased rate of estrogen metabolism [19,20].

The effects of cigarette smoking on the incidence of ovarian cancer (tumors arising from the ovarian surface epithelium) are not well understood. A number of epidemiological studies have demonstrated an increased risk of ovarian cancer in

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smokers [21–26]. Other investigators failed to find an increase in the incidence of ovarian cancer among smokers [27,28]. However, differences in populations and tumor classification varies across studies and the most consistent reported effect of smoking on ovarian cancer risk is an increase in the incidence of the mucinous histologic subtype, but not other histologic subtypes [22–26].

The increased incidence of ovarian cancer in smokers is complicated and may relate to differences in the local environment within the ovary. Goodman et al. have demonstrated an increased risk of ovarian cancer in cigarette smokers with either a high activity polymorphism of CYP1B1 or a low activity polymorphism in COMT [29]. These polymorphisms may lead to altered local metabolism of estradiol causing an increase in 4-OH-E_2 formation or a decrease in metabolism, respectively. 4-OH-E_2 can be further oxidized to the quinone, which, in addition to generating oxygen free radicals can directly adduct DNA [30]. In addition, increased expression or activity of CYP1B1 may increase local metabolism of various pro-carcinogens present in tobacco smoke such as benzo[*a*]pyrene. In mice lacking CYP1B1, there is a decreased incidence of ovarian tumors (granulosa cell tumors) secondary to exposure to the polyaromatic hydrocarbon 7,12-dimethylbenz[*a*]anthracene (DMBA) [31]. This is coupled with a decreased formation of adducts suggesting that the lack of CYP1B1 prevents local metabolism of DMBA to the carcinogenic metabolite 1,2-epoxide-3,4-diol-DMBA. Previous work from our laboratory has demonstrated a concentration dependent increase in the expression of CYP1B1 in human luteinized granulosa cells (HLGC) exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) *in vitro*. The direct effects of ETS on the expression and regulation of CYP1B1 and COMT in the human ovary remains unexplored and potential increases in CYP1B1 may contribute to the altered ovarian function noted in women smokers.

2. Materials and methods

2.1. Media and chemicals

Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY) was modified with the following additions and used in all aspects of the study: sodium bicarbonate, 3.7 mg/ml; antibiotic/antimycotic containing amphotericin B, penicillin G, and streptomycin sulfate at final concentrations of 0.25 μg/ml, 100 U/ml, and 10 μg/ml, respectively (Gibco); 10% fetal calf serum (Gibco); and 2 U/ml of the synthetic human chorionic gonadotropin Pregnyl (Organon, West Orange, NJ). Media was filtered through a 0.22-μm sterile syringe filter (Fisher, Santa Clara, CA) and equilibrated at 37 °C and 5% CO_2 in air before use.

2.2. Cell collection

Human luteinized granulosa cells (HLGC) were collected by ultrasound-guided follicular aspiration from women undergoing fertility treatment at the Northern California Fertility Medical Center (Roseville, CA). These cells were a by-product of treatment and are normally discarded. All samples were provided coded without knowledge of the individual's identity and were exempt from review by the University Human Subjects Review Committee (HSRC) under Federal exemption category number 4. Exemption from full HSRC review was approved by the HSRC coordinator and was renewed annually. However, samples were only collected with prior written approval from the individual. Patients received varying amounts of Metrodin (Serono Laboratories, Randolph, MA) and Pergonal (Serono) and received 10,000 IU Pregnyl 36 h before follicular aspiration. HLGC from multiple follicles from an individual patient were pooled into a single sample.

2.3. Cell culture

HLGC, prepared by initial centrifugation at 300 × *g* for 5 min and increased to 500 × *g* for an additional 5 min, layered onto 40% Percoll (Sigma) and centrifuged at 500 × *g* for 30 min. The cells were transferred to 15 ml of DMEM and centrifuged again at 500 × *g* for 10 min. The supernatant was discarded, and the pellet was resuspended in 2 ml DMEM. HLGC were filtered through an 89-μm polyester filter (Spectra/Mesh, Spectrum Medical, Laguna Hills, CA), counted and plated. HLGC were plated at either 1×10^6 in 100 mm plates with 10 ml of DMEM or 5×10^5 in 60 mm plates with 5 ml of DMEM. HLGC were cultured at 37 °C in an atmosphere of 5% CO_2 in air. HLGC were given 24 h to equilibrate and attach then media was changed to remove debris This first media change was considered day 0 of the culture period and media was changed every 48 h thereafter for a maximum of 10 days.

2.4. Exposure system and generation of ETS

The exposure system and monitoring methods have been previously described in detail [34]. Briefly, ETS is generated by a smoke exposure system (Teague Enterprises, Davis, CA) using IR4F conditioned cigarettes from the Tobacco and Health Research Institute of the University of Kentucky. Two to three cigarettes at a time were smoked under Federal Trade Commission conditions in a staggered fashion at a rate of one puff (35 ml, 2 s duration) per minute. The side-stream smoke was drawn into a conditioning chamber where it was aged and diluted. The side-stream smoke was then further diluted as it passed into the exposure chambers in such a way as to produce total suspended particulates (TSP) concentrations of 1.0 mg/m^3. The exposure chambers were stainless steel and glass Hinners-type and 4.2 m^3 in size.

2.5. Smoke exposed media

In order to perform in vitro smoke exposure using the HLGC, the culture media was exposed to ETS in the laboratory of Dr. Kent Pinkerton (Center for Health and the Environment, University of California, Davis). The modified DMEM as described above was placed in a small side-stream smoke chamber connected to the smoking machine for approximately 6 h. The relative humidity within the chamber was 43% and the temperature was 22 °C. Carbon monoxide (CO) levels were measured every 30 min, nicotine was measured once, and the TSP were measured every 2 h. The levels in the chamber for the batch of smoke exposed media used in these studies were 287 ± 19 ppm CO, 12 mg/m^3 nicotine and 110 ± 5 mg/m^3 TSP. This method for exposure of culture media has been used successfully to produce effects of smoke exposure in vitro [32,33].

2.6. ETS exposure

The ETS exposed DMEM was diluted to 10% and 25% with DMEM to give low and high concentrations, respectively. Concentrations of ETS exposed media were based on results of exposure of recombinant hepa1 cells, stably transfected with a reporter plasmid containing the luciferase gene under control of several dioxin responsive enhancers (DREs), which was generously performed in the laboratory of Dr. Michael Denison (University of California, Davis) as previously described [35]. Increasing concentrations of ETS-exposed media resulted in a concentration dependent induction of luciferase activity (data not shown). The high concentration of ETS used in all HLGC experiments was the lowest maximally active concentration in the luciferase assay. Concentrations of ETS greater than 25% markedly decreased HLGC survival (data not shown). Cells from a given patient were separated into an untreated control, low concentration of ETS, and a high concentration of ETS. In the first experiment, cells from each of seven patients ($n = 7$) were cultured in six 60 mm plates with 5 ml of media and given a day to adhere. The following day, two plates received the low concentration of ETS, two plates received the high concentration, and two were left untreated. After 24 h, one plate of each condition was rinsed in ice cold PBS and