SPECIAL
TARGETING AND REGULATION OF HORMONE SIGNALING
SECTION

# Carbon Black Nanoparticles Inhibit Aromatase Expression and Estradiol Secretion in Human Granulosa Cells Through the ERK1/2 Pathway

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Secretion of 17-$\beta$-estradiol (E2) by human granulosa cells can be disrupted by various environmental toxicants. In the current study, we investigated whether carbon black nanoparticles (CB NPs) affect the steroidogenic activity of cultured human granulosa cells. The human granulosa cell line KGN and granulosa cells from patients undergoing *in vitro* fertilization were treated with increasing concentrations of CB NPs (1 to 100 $\mu$g/mL) together or not with follicle-stimulating hormone (FSH). We observed that CB NPs are internalized in KGN cells without affecting cell viability. CB NPs could be localized in the cytoplasm, within mitochondria and in association with the outer face of the endoplasmic reticulum membrane. In both cell types, CB NPs reduced in a dose-dependent manner the activity of aromatase enzyme, as reflected by a decrease in E2 secretion. A significant decrease was observed in response to CB NPs concentrations from 25 and 50 $\mu$g/mL in KGN cell line and primary cultures, respectively. Furthermore, CB NPs decreased aromatase protein levels in both cells and reduced aromatase transcript levels in KGN cells. CB NPs rapidly activated extracellular signal-regulated kinase 1 and 2 in KGN cells and pharmacological inhibition of this signaling pathway using PD 98059 significantly attenuated the inhibitory effects of CB NPs on *CYP19A1* gene expression and aromatase activity. CB NPs also inhibited the stimulatory effect of FSH on aromatase expression and activity. Altogether, our study on cultured ovarian granulosa cells reveals that CB NPs decrease estrogens production and highlights possible detrimental effect of these common NPs on female reproductive health. (***Endocrinology* 158: 3200–3211, 2017)**

Ovarian estrogens play a key role in female fertility. Estrogens locally regulate ovarian follicular development, ovulation, steroidogenesis, and luteal function (1–3). They also provide feedback on the hypothalamus–pituitary complex and control growth and differentiation of reproductive organs such as the uterus and mammary gland. Estrogens are the main steroids produced by granulosa cells of mature ovarian follicles. Their synthesis requires two different cell types: thecal cells converting progesterone in androgens and granulosa cells in which thecal-derived androgens are converted in estrogens and notably in 17-$\beta$-estradiol (E2). The final step in the biosynthesis of E2 from androgens is catalyzed by cytochrome P450 aromatase (hereafter referred to as

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Abbreviations: CB NP, carbon black nanoparticle; DMEM/F12, Dulbecco's modified Eagle medium nutrient mixture F12; E2, 17-$\beta$-estradiol; ERK1/2, extracellular signal-regulated kinase 1 and 2; FBS, fetal bovine serum; FSH, follicle-stimulating hormone; MEK, mitogen-activated protein kinase kinase; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide; NP, nanoparticle; PS, penicillin/streptomycin; StAR, steroidogenic acute regulatory protein.



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aromatase). Aromatase is the product of the *CYP19A1* gene and, like most enzymes of the sex steroid biosynthesis pathway, is localized into the endoplasmic reticulum membrane. Ovarian E2 secretion and *CYP19A1* expression are controlled by several factors among which the pituitary gonadotropin follicle-stimulating hormone (FSH) plays a central role. FSH induces the expression of *CYP19A1* in granulosa cells and enhances aromatase activity in several mammalian species, including rodents and humans (4–6). The expression of *CYP19A1* is regulated by the cAMP/protein kinase A pathway, activated by the FSH receptor and by other kinases such as the mitogen-activated protein kinase kinases (MEKs) extracellular signal-regulated kinase 1 and 2 (ERK1/2) and P38, as well as phosphatidylinositol-3-kinase (7–11).

A growing number of studies indicate that granulosa cell steroidogenesis is affected by environmental contaminants. Bisphenol A, a compound widely used as a plasticizer in the industry, was shown to reduce aromatase expression and E2 secretion in primary cultures of human granulosa cells, as well as in the human granulosa cell line KGN (12). The active metabolite mono(2-ethylhexyl) phthalate of the plasticizer di-(2-ethylhexyl) phthalate significantly decreased aromatase transcript levels and E2 production in primary cultures of granulosa cells in both rats and humans (13, 14). An inhibitory effect of metabolite mono(2-ethylhexyl) phthalate was not only reported on basal E2 secretion but also on FSH and 8-Br-cAMP stimulated production (14). Exposure of granulosa cells to the toxicant dioxin (2,3,7,8- tetrachlorodibenzo-*p*-dioxin) also decreased E2 secretion in humans and rats (15–17).

Nanoparticles (NPs) are materials of at least one dimension less than 100 nM. A dramatic increase in their industrial production as well as in their industrial and medical use occurred over the past decades. There is emerging evidence that NPs may act as novel endocrine disrupters (18, 19). Among the few studies undertaken in ovarian steroidogenic cells, a recent work indicated that gold NPs enter rat granulosa cells and decrease E2 secretion (20). No such effect could, however, be detected with calcium phosphate NPs in human luteinized granulosa cells (21), highlighting the potential diversity of action of NPs associated with their structural diversity. Carbon black nanoparticles (CB NPs) are mostly produced by controlled incomplete combustion or thermal decomposition of hydrocarbons. They are intensively used in industry worldwide as reinforcing fillers in automobile tires and other rubber products. They are also used as a color pigment in plastics, paints, and printing inks. CB NPs can principally be taken up through inhalation, and experimental studies on animals have shown that CB NPs inhalation induce pulmonary inflammation and cardiovascular diseases (22–24). CB NPs and their respirable aggregates/agglomerates have been classified as possibly carcinogenic to humans [group 2B (25)]. The current limit for CB NPs exposure defined by the National Institute of Occupational Safety and Health is of 3.5 mg/m$^3$. However, under occupational settings, workers may be exposed to much higher concentrations of CB NPs, and amounts of 79 mg/m$^3$ or even 675 mg/m$^3$ were reported (26). Translocation of intratracheally instilled CB NPs into blood circulation has been described in mice (27). In an *in vitro* study (28), CB NPs were shown to enter mouse Leydig cells, the testis steroidogenic cells, and to decrease steroidogenic acute regulatory protein (StAR) expression, which is the rate-limiting factor in steroid biosynthesis. Consistently, intratracheal administration of CB NPs to adult mice altered testosterone production and reduced daily sperm production (29). The potential adverse effect of CB NPs on steroidogenic capacity of ovarian granulosa cells has never been investigated.

In this study, we analyzed the consequences of CB NPs exposure on granulosa cell steroidogenesis using both the KGN human granulosa cell line and granulosa cells from subjects undergoing *in vitro* fertilization. We present evidence that CB NPs affect basal and FSH-induced aromatase expression and E2 secretion in human granulosa cells. In addition, we show that recruitment of ERK1/2 signaling pathway in granulosa cells contributes to CB NP-induced downregulation of aromatase and E2 secretion. Altogether, our study suggests that CB NPs may act as an endocrine disruptor, reducing E2 synthesis by the human ovary and hence affecting reproductive capacity.

## Materials and Methods

### Cell culture

The immortalized human granulosa cell line KGN was from the RIKEN BioResource Center (30). KGN cells display aromatase activity and express functional FSH receptors, as observed in normal granulosa cells (31). Cells were cultured at 37°C with 5% $CO_2$ in Dulbecco's modified Eagle medium nutrient mixture F12 (DMEM/F12) without red phenol containing 10% fetal bovine serum (FBS) and 0.5% penicillin/streptomycin (PS).

Human luteinized granulosa cells were collected from women undergoing *in vitro* fertilization at Antoine Beclère Hospital in Clamart, France. Nine patients aged 31 to 39 years were included in this study according to the following inclusion criteria: (1) both ovaries present, with no morphological abnormalities; (2) menstrual cycle length range between 25 and 35 days; (3) no current or past diseases affecting ovaries or gonadotropin or sex steroid secretion, clearance, or excretion; (4) no clinical signs of hyperandrogenism; and (5) inclusion in a standard long gonadotropin-releasing hormone agonist suppression protocol. An informed consent was obtained from all

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women, and this investigation received the approval of Beclère Hospital internal institutional review board. Follicular fluids from each patient were pooled and centrifuged through a one-step density Percoll gradient (vol/vol, phosphate-buffered saline/Percoll) at 4000 × g for 15 minutes to remove red blood cells. Luteinized granulosa cells were collected at the interface, washed with PBS and seeded at $3 \times 10^5$ cells per well in six-well plates in 2 mL of DMEM/F12 without red phenol containing 10% fetal calf serum and 0.5% PS. Primary cell cultures were used to measure E2 secretion (n = 6) or aromatase protein expression (n = 3).

## Nanoparticles preparation and treatment of cells

FW2 (13 nm) CB NPs were obtained from Evonik Industries/Degussa (Frankfurt, Germany). Their physicochemical characteristics have been determined previously (32). Stock solutions of CB NPs were diluted in pure water at a concentration of 20 mg/mL and sonicated 10 minutes to improve dispersal of particles as previously described (33). CB NPs were then diluted in a solution of bovine serum albumin (1.5 mg/mL) at a final concentration of 1 mg/mL, sonicated 3 minutes at 30 to 60 W using an induced ultrasonic probe (Ultrasonic Processor, Fisher Bioblock Scientific, Illkirch, France), and stored at –20°C. CB NPs were sonicated again just before use.

Cells were incubated with CB NPs concentrations ranging from 1 to 100 μg/mL, corresponding to 0.2 to 20 $\mu g/cm^2$. Based on morphometric and particle deposition data in the human lung (34, 35), it has been calculated that exposure to permissible limit concentrations over a year would lead to deposition of at least 2.5 $\mu g/cm^2$. Moreover, as indicated earlier, workers can be submitted to a much higher exposition in occupational settings, suggesting that the range of CB NPs concentrations used in the current study may be environmentally relevant. These concentrations are in the range or even lower than those classically used in *in vitro* studies [macrophages (36), human umbilical vein endothelial cells (37), human epithelial (32, 33), and fibroblast (38) cell lines] including the study performed on testis Leydig cells (28), the male counterpart of ovarian granulosa cells.

## Cell viability

For the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, KGN cells were seeded at $0.75 \times 10^5$ cells per well in 24-well plates in 0.5 mL of DMEM/F12 without red phenol containing 10% FBS and 0.5% PS. Twenty-four hours later, cells were serum-starved overnight and incubated the next day with increasing concentrations of CB NPs (25 to 100 μg/mL, corresponding to 5 to 20 $\mu g/cm^2$) for 24 hours. At the end of the incubation, media were aspirated and replaced by 0.5 mL medium containing MTT (1 mg/ mL). After 2 hours of incubation, cells were lysed and purple crystal granules dissolved in 200 μL of dimethyl sulfoxide. The absorbance of the solubilized formazan crystals was read at 575 nm on a Flexstation3 (Molecular Devices, Sunnyvale, CA).

## Transmission electron microscopy

KGN cells were seeded at $3.5 \times 10^5$ cells per well in six-well plates in 2 mL of DMEM/F12 without red phenol containing 10% FBS and 0.5% PS. Forty-eight hours later, cells were serum starved overnight and incubated or not the next day with CB NPs (10 or 25 μg/mL) for 24 hours. At the end of the incubation, cells were extensively washed with culture medium. Then, cells were washed in 0.05 M sodium cacodylate buffer (pH 7.4) and fixed *in situ* with 2.5% glutaraldehyde diluted in the same buffer at 4°C for 1 hour, followed by five washes in sodium cacodylate buffer. The monolayers were postfixed with 2% osmium tetroxide in sodium cacodylate buffer for 1 hour at room temperature in the dark. The postfixed monolayers were dehydrated *in situ* through an ethanol ascending series and embedded in epoxy resin (Epoxy-Embedding Kit; catalog no. 45359; Sigma-Aldrich, Saint-Quentin Fallavier, France). Gelatin capsules filled with resin were returned to the cell layers and the polymerization was performed in a 60°C oven for 48 hours. At the end, capsules were detached from the wells and ultrathin sections (70 nm) of the cell monolayer were cut and collected on copper grids. Sections were contrasted using 2% uranyl acetate for 5 minutes and Reynold's lead citrate for 2 minutes, and they were examined under a transmission electron microscope (80-120 kV 912 Omega ZEISS) equipped with a digital camera (Veleta Olympus, Hamburg, Germany).

## Estradiol secretion

E2 secretion by KGN cells was determined by measuring E2 accumulation in the conditioned medium by radioimmunoassay. As observed in normal granulosa cells, KGN cells secrete negligible amounts of E2 unless they are provided with an androgen precursor (31). The accumulation of E2 was thus measured during a 48-hour period in the presence of a high concentration of androstenedione (100 nM) and was taken to reflect the level of aromatase activity. KGN cells were seeded at $2 \times 10^5$ cells per well in six-well plates in 2 mL of DMEM/F12 without red phenol containing 10% FBS and 0.5% PS. Seventy-two hours later, medium was aspirated and replaced by the same medium containing only 2% FBS. The next day, cells were incubated with increasing concentrations of CB NPs (10 to 100 μg/mL) for 24 hours. At the end of the incubation, cells were extensively washed and incubated for the next 48 hours with serum-depleted medium supplemented with 100 nM of androstenedione in the presence or not of 5.5 UI/mL of FSH (Gonal F-150; Merck Serono, Lyon, France). Some cells were cotreated with the MEK inhibitor PD 98059 (25 μM; Calbiochem, Sigma-Aldrich), the inhibitor being added 30 minutes before the introduction of CB NPs. At the end of the incubation, media were collected and centrifuged to eliminate CB NPs. We verified using E2 radioimmunoassay that no substantial amount of E2 was trapped in CB NP pellets (not shown). Supernatants were then stored at –80°C before measurement of synthetized E2 levels.

Twenty-four hours after seeding, primary cultures of human luteinized granulosa cells were serum starved for 3 hours and then cultured for 24 hours in a serum-depleted medium containing increasing concentrations of CB NPs (10 to 100 μg/mL). At the end of the incubation, CB NPs were removed, cells were washed extensively and then incubated for the next 48 hours with 2 mL of serum-depleted medium containing 100 nM of androstenedione. At the end of the incubation, media were collected as described earlier for E2 levels measurement.

Radioimmunoassay of E2 concentrations was performed following the manufacturer's instructions (Beckman Coulter, Villepinte, France). The intra- and interassay coefficients of variation were inferior or equal to 8.9% and 12.2%, respectively. Final data are plotted as nanograms of synthetized E2 per mL in the conditioned medium.

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### *Aromatase expression*

KGN cells were seeded at $2 \times 10^5$ cells per well in six-well plates in 2 mL of DMEM/F12 without red phenol containing 10% FBS and 0.5% PS. Seventy-two hours later, the medium was aspirated and replaced by serum-depleted medium containing 0.5% PS. The next day, cells were pretreated or not with PD 98059 (30 minutes, 25 μM) and then were incubated with increasing concentrations of CB NPs (from 0.1 to 100 μg/mL) for 24 hours. At the end of the incubation, cells were extensively washed, incubated or not for the next 48 hours with serum-depleted medium supplemented with 5.5 UI/mL of FSH. Total RNAs were extracted using the RNA Plus extraction kit (Qiagen, Valencia, CA). Reverse transcription was performed using 1 μg RNA in a total volume of 10 μL with the Superscript II reverse transcription (Invitrogen, Thermo Fisher, Villebon sur Yvette, France) using random primers according to the manufacturer's instructions. For messenger RNA quantification of *CYP19A1* and *G6PDH*, encoding glucose-6-phosphate deshydrogenase, real-time polymerase chain reaction was carried out in duplicates with one-fourth dilution of the complementary DNAs using the Lightcycler 480 Probes Master kit (Roche Diagnostics, Meylan, France). Cycling conditions included an initial heat-denaturing step at 95°C for 3 minutes, followed by 45 cycles at 95°C for 10 seconds, 60°C for 10 seconds and 72°C for 10 seconds. Primers used for complementary DNA amplification were Fwd 5′ CTTTGCCACTGAGTTGATTTTAGC 3′ and Rev 5′ ATTAGGGTGCTTTGCAATGAGAAA 3′ for *CYP19A1* and Fwd 5′ ATCGACCACTACCTGGGCAA 3′ and Rev 5′ TTCTGCATCACGTCCCGGA 3′ for *G6PDH*. Data were analyzed using the advanced E method with standard curve-derived efficiencies obtained from the LightCycler 480 software. Expression levels of *CYP19A1* were normalized to the *G6PDH* messenger RNA level. The specificity of amplification was checked by gel electrophoresis and melting curve analysis.

For protein expression, primary cultures or KGN cells were lysed in lysis buffer [10 mM Tris-HCl (pH 7.4), 30 mM sodium pyrophosphate, 50 mM sodium chloride, 1% TritonX-100, and 1 mM dithiothreitol supplemented with protease and phosphatase inhibitor cocktails (Roche Diagnostics)]. Lysates were passed through a $G26^{1/2}$ syringe and then centrifuged at 10,000 × g for 30 minutes at 4°C. Supernatants were discarded and pellets containing cell membranes were resuspended in 25 μL of lysis buffer. Membrane proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Expression of aromatase was assessed by immunoblotting with a polyclonal antiaromatase antibody (catalog no. A7981; 1:1000 dilution; Sigma-Aldrich; Table 1). Equal protein loading was verified by reprobing blots with an antivinculin antibody (catalog no. hVIN-I; 1:20,000 dilution; Sigma-Aldrich).

### ERK1/2 signaling

KGN cells were seeded at $2 \times 10^5$ cells per well in six-well plates in 2 mL of DMEM/F12 without red phenol containing 10% FBS and 0.5% PS. Seventy-two hours later, medium was aspirated and replaced by serum-depleted medium containing 0.5% PS. The next day, cells were incubated with CB NPs (50 μg/ mL) for various periods of time (0, 5 minutes, 15 minutes, 30 minutes, 1 hour, or 6 hours). At the end of the incubation, cells were extensively washed and lysed in lysis buffer. Lysates were passed through a $G26^{1/2}$ syringe and then centrifuged at 10,000 × g for 30 minutes at 4°C; 20 μg of the supernatants were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Phosphorylation of ERK1/2 proteins was assessed by immunoblotting with the polyclonal antipERK1/2 antibody (catalog no. 9101; 1:1000 dilution; Cell Signaling, Promega, Charbonnières les Bains, France; Table 1). Equal protein loading was verified by reprobing blots with an anti-ERK1/2 antibody (catalog no. 9102; 1:1000 dilution; Cell Signaling). Western blots were visualized using ECL+ (Amersham, GE Healthcare Life Sciences) and analysis was performed using a Fuji LAS-4000 imager.

### Statistical analyses

Experiments were performed in duplicate and all given values are the mean ± standard error of the mean of at least three independent experiments (see details in the legend of each figure). Statistical analyses were performed using Prism software (GraphPad). Differences were determined by one-way analysis of variance followed by Dunnett *post hoc* test or two-way analysis of variance followed by Tukey *post hoc* test for multiple comparisons.

**Table 1. Antibody Table**

| Peptide/ Protein Target | Antigen Sequence (if Known) | Name of Antibody | Manufacturer, Catalog No. | Species Raised in; Monoclonal or Polyclonal | Dilution Used | RRID |
|---|---|---|---|---|---|---|
| Aromatase | Amino acids 472-486 of human aromatase (GeneID: 1588), | Aromatase | Sigma-Aldrich, A7981 | Rabbit; polyclonal | 1:1000 | AB_1845043 |
| Vinculin | Vinculin, purified from human uterus | Vinculin | Sigma-Aldrich, V9131 | Mouse; monoclonal | 1:20,000 | AB_477629 |
| P-ERK 1/2 | Synthetic phosphopeptide corresponding to residues surrounding Thr202/Tyr204 of human p44 MAP kinase | P-ERK 1/2 | Cell Signaling, 9101 | Rabbit; polyclonal | 1:1000 | AB_331646 |
| Total ERK 1/2 | Synthetic peptide corresponding to a sequence in the C-terminus of rat p44 MAP Kinase | Total ERK 1/2 | Cell Signaling, 9102 | Rabbit; polyclonal | 1:1000 | AB_330744 |

Abbreviation: RRID, Research Resource Identifier.

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## Results

### Cell viability

Cells were incubated during 24 hours with increasing concentrations of CB NPs (25 to 100 μg/mL). To assess the potential cytotoxic effect of CB NPs, we determined cell viability with the commonly used assay MTT. Before assessing cell viability, we verified that CB NPs did not significantly interfere with MTT assay. As indicated in Table 2, incubation of cells with CB NPs did not have any incidence on cell viability.

### Uptake and intracellular localization of CB NPs in KGN cells

Analysis by transmission electron microscopy in KGN cells was used to determine the uptake and intracellular localization of CB NPs at different concentrations: 10, 25 (Fig. 1), and 50 μg/mL (not illustrated). CB NPs were taken up by KGN cells at every concentration tested and were found as aggregates/agglomerates either freely dispersed within the cytoplasm or associated with different subcellular compartments as illustrated in transmission electron microscopy images (Fig. 1). Interestingly, CB NPs were found associated with organelles containing enzymes of the steroidogenic pathway. Many CB NPs were indeed observed closely associated with the outer face of endoplasmic reticulum membrane (Fig. 1a), which contains most steroidogenic enzymes including the aromatase. Some CB NPs were also detected within mitochondria and notably associated with the inner membrane (Fig. 1b) where cytochrome P450scc, the first enzyme of sex steroids biosynthesis pathway, is localized. CB NPs were also found along the outer nuclear membrane and Golgi apparatus membrane (Fig. 1c). Macropinocytosis images were observed, suggesting that this could be one of the mechanism by which CB NPs gain access into the cell (Fig. 1d). No aggregates/agglomerates were detected in cells that were not incubated with CB NPs (not shown).

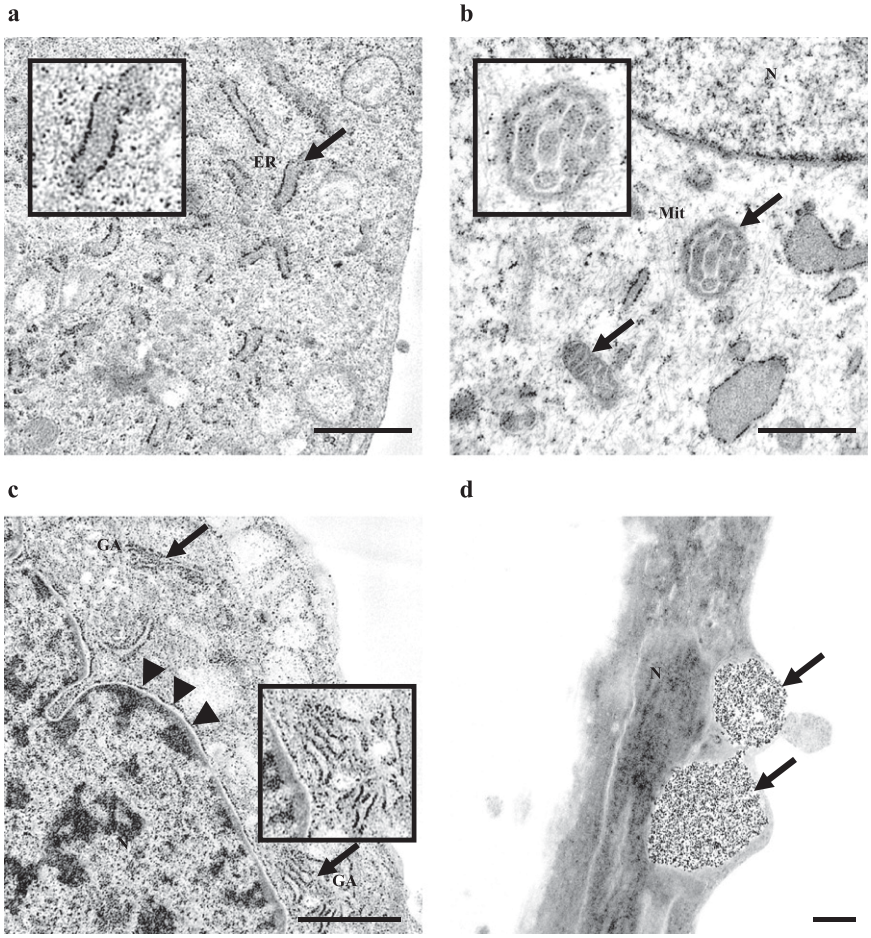


**Figure 1.** Transmission electron microscopy study of uptake and intracellular localization of CB NPs in KGN cells. KGN cells were incubated for 24 hours with CB NPs at a concentration of (a, c) 10 μg/mL or (b, d) 25 μg/mL. Cells were then extensively washed and processed for transmission electron microscopy as described in Materials and Methods. Arrows indicate (a) endoplasmic reticulum (ER), (b) mitochondria (Mit), (c) Golgi apparatus (GA), and (d) large aggregates of CB NPs entering the cell by macropinocytosis. Magnifications illustrate the close association of CB NPs with membranes of (a) endoplasmic reticulum, (b) mitochondria, and (c) Golgi apparatus. Arrowheads indicate (c) the outer nuclear membrane. Bar = 1 μm. N, nucleus.

**Table 2. Effect of a 24-Hour Treatment With CB NPs (13 nm) on the Viability of KGN Cells**

| CB NP (μg /mL) | 0 | 25 | 50 | 100 |
|---|---|---|---|---|
| Cell viability, % of no CB NP | 100 | 98 ± 2 | 97 ± 2 | 94 ± 3 |

KGN cells were incubated for 24 hours with increasing concentrations (25 to 100 μg/mL corresponding to 5 to 20 μg/cm$^2$) of CB NP and cell viability was determined by MTT assay as described in Materials and Methods. Results are mean ± standard error of the mean of five independent experiments and are expressed as percentage of untreated cells.

### CB NPs reduce estradiol synthesis in KGN and in human luteinized granulosa cells

To determine the effects of CB NPs on aromatase activity, KGN cells or primary cultures of human luteinized granulosa cells were cultured for 24 hours with increasing concentrations (10 to 100 μg/mL) of CB NPs, extensively washed and reincubated for 48 hours in a medium supplemented with a high concentration of androstenedione. E2 secretion was measured by radioimmunoassay and was taken to reflect aromatase activity. Without CB NPs, basal E2 production was 0.25 ± 0.03 ng/mL in KGN cells. CB

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NPs dose-dependently inhibited aromatase activity as observed by a dose-dependent decrease in E2 secretion (Fig. 2a). Inhibition was detected at 25 μg/mL and maximum inhibition was of 88 ± 1% at 100 μg/mL of CB NPs. In primary cultures of human luteinized granulosa cells, basal E2 production was of 1.86 ± 0.2 ng/mL (Fig. 2b). Treatment of cells with CB NPs decreased aromatase activity as reflected by a significant decrease of 48.4 ± 5% with 50 μg/mL and of 81.8 ± 9% with 100 μg/mL of CB NPs (Fig. 2b).

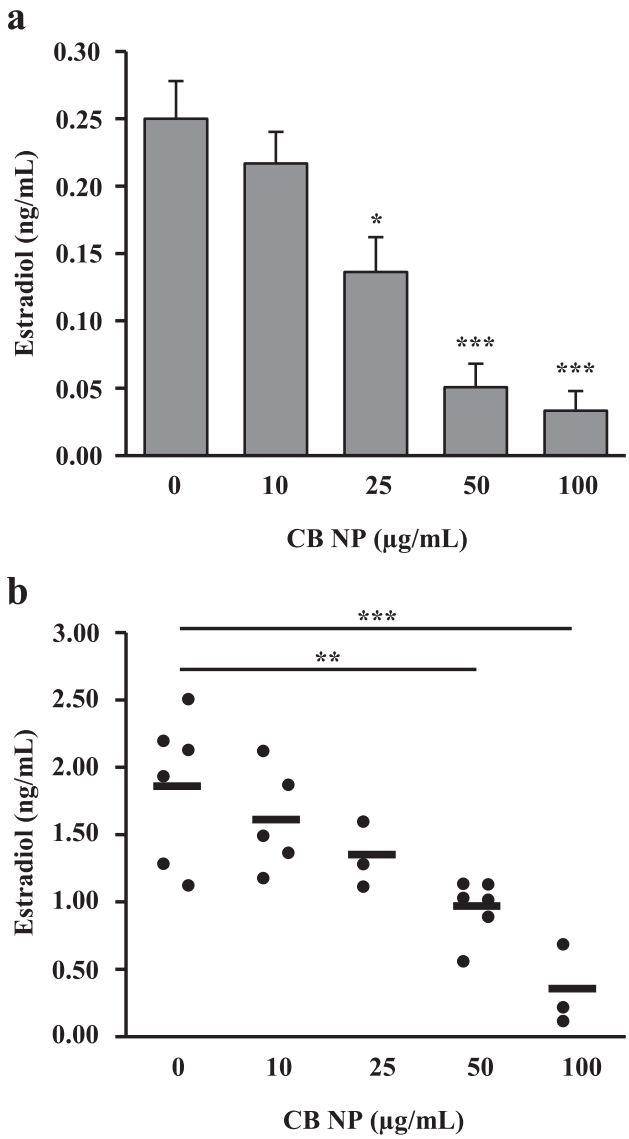


**Figure 2.** Effects of CB NPs on E2 secretion in KGN cell line and in primary cultures of human luteinized granulosa cells. Cells were incubated for 24 hours with increasing concentrations of CB NPs, extensively washed, and E2 secretion during the next 48 hours was measured by assaying E2 concentration in the conditioned media by radioimmunoassay. (a) Dose-response inhibitory effects of CB NPs on E2 secretion (ng/mL) by KGN cells. Data are means ± standard error of the mean from six to seven independent experiments. (b) Dose-response inhibitory effects of CB NPs in primary cultures of human luteinized granulosa cells. Data are from six women for 0 and 50 μg/mL CB NPs, five women for 10 μg/mL, and three women for 25 and 100 μg/mL. Concentrations are represented as dot plots. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with untreated cells.

### CB NPs downregulate aromatase expression and CYP19A1 transcript levels

We further investigated the effects of CB NPs on aromatase expression. Immunoblotting studies revealed a band of approximately 55 kDa in agreement with the known molecular weight of aromatase in both primary culture and KGN cells. Exposure of KGN cells to CB NPs (50 μg/mL) induced a downregulation of aromatase protein expression in a time-dependent manner, with a reduction reaching 55 ± 4% at 24 hours (Fig. 3a). Consistent with such inhibition, we observed that *CYP19A1* transcript levels were significantly reduced by a 24-hour treatment with CB NPs with an inhibition of 39 ± 5% at 25 μg/mL that was maintained at higher (up to 100 μg/mL) concentrations (Fig. 3b). CB NPs also decreased aromatase protein levels in human luteinized granulosa cells (Fig. 3c). Contrasting with KGN cells, no effect was observed at 50 μg/mL. At 100 μg/mL, CB NPs significantly decreased (37 ± 8%) aromatase expression.

### Role of ERK1/2 in mediating inhibitory effects of CB NPs on estradiol synthesis and aromatase expression

Given that ERK1/2 inhibit *CYP19A1* expression and E2 secretion in granulosa cells from several mammals including humans and that NPs activate various cellular signaling pathways (39), we tested the hypothesis that ERK1/2 could mediate the inhibitory effects of CB NPs on *CYP19A1* expression. To test this hypothesis, we first examined the ability of CB NPs to activate ERK1/2 pathway in KGN cells using immunoblotting with antibodies recognizing the phosphorylated forms of ERK1/2. Time-course activation studies (Fig. 4a) showed that CB NPs rapidly increase the amount of phosphorylated ERK1/2 without affecting the total amount of ERK1/2 in KGN cells. Maximal activation (× 4.5) was observed after 15 minutes, and significant activation could still be detected after 1 hour of incubation with CB NPs. Phosphorylation levels then returned to basal levels at 6 hours. To determine whether activation of the ERK1/2 pathway contributes to CB NPs-induced inhibition of *CYP19A1* expression, the MEK inhibitor PD 98059 (25 μM) was used to block ERK1/2 activation. Treatment with CB NPs decreased *CYP19A1* transcript levels (Fig. 4b), as observed previously, and interestingly, PD 98059 prevented the inhibitory effect of both concentrations of CB NPs. To further assess the role of ERK1/2 pathway in mediating the action of CB NPs, we next determined the consequences of PD 98059 treatment on aromatase activity (Fig. 4c). CB NPs alone decreased aromatase activity in a dose-dependent manner as previously

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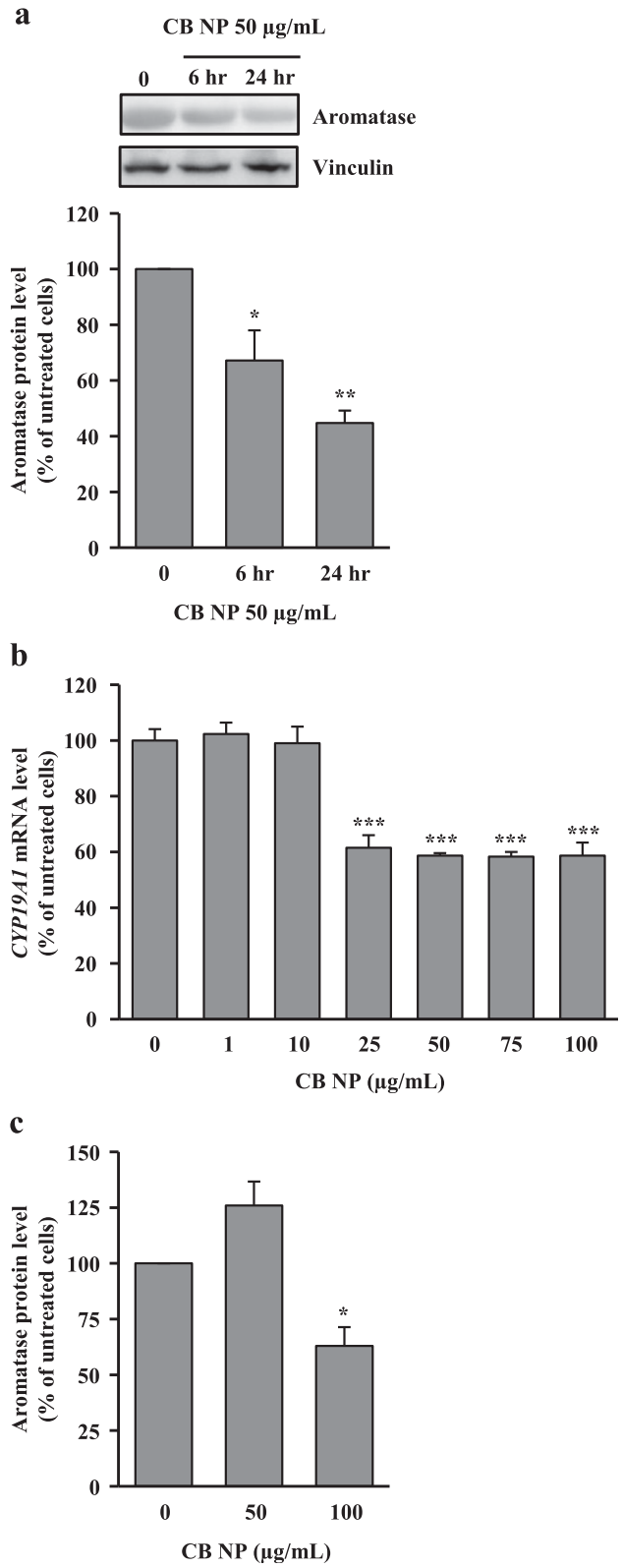


**Figure 3.** Effects of CB NPs on aromatase expression level and *CYP19A1* messenger RNA level. (a) Time-dependent inhibitory effects of CB NPs on aromatase expression levels in KGN cells. Cells were incubated for 6 or 24 hours with 50 µg/mL of CB NPs and, after extensive washings, aromatase expression level was determined by immunoblotting. Vinculin was used as an internal loading control. A representative immunoblot is shown in the inset. (b) Inhibitory effect of CB NPs on *CYP19A1* messenger RNA level.

observed (Fig. 4c). In the presence of PD 98059, incubation of cells with CB NPs still decreased aromatase activity but the extent of inhibition was significantly reduced (maximum inhibition of 49.3 ± 2% vs 74.4 ± 6%, in presence or absence of PD 98059; Fig. 4c). Noteworthy, in absence of CB NPs, PD 98059 treatment led to a significant increase of E2 secretion indicating that the ERK1/2 signaling pathway exerts a tonic inhibition on aromatase in KGN cells.

### CB NPs inhibit FSH stimulatory effects on CYP19A1 expression and aromatase activity in KGN cells

Because FSH is a crucial regulator of granulosa cell steroidogenesis and of *CYP19A1* expression, we examined the effects of CB NPs on FSH-stimulated KGN cells. Treatment with FSH markedly increased *CYP19A1* transcript levels, as expected (1162 ± 149% increase, data not shown). Treatment of cells with CB NPs reduced FSH-stimulated increase in *CYP19A1* transcripts (Fig. 5a). The effect was significant with only 10 µg/mL of CB NPs and a maximal 57 ± 6% inhibition was observed at 100 µg/mL. CB NPs inhibitory effect was also observed on FSH stimulation of aromatase activity. Indeed, CB NPs either at 50 µg/mL or 100 µg/mL completely blocked the enhancement of E2 secretion induced by FSH (Fig. 5b).

## Discussion

In the current study, we show that CB NPs enter human KGN granulosa cells and localize in the cytoplasm and within mitochondria, as observed for gold and calcium phosphate NPs in rat and human luteinized granulosa cells, respectively (20, 21). Interestingly, our study reveals for the first time that CB NPs can be closely associated with the outer face of the endoplasmic reticulum membrane in which aromatase is localized. Furthermore, we demonstrate here that CB NPs decreased E2 production in human granulosa cells and that this involves a decrease in steady-state levels of aromatase transcript and protein. Significant effects on E2 secretion and *CYP19A1*

**Figure 3. (Continued).** Cells were incubated for 24 hours with increasing concentrations of CB NPs and, after extensive washings, *CYP19A1* messenger RNA level was determined by quantitative polymerase chain reaction. (c) Inhibitory effect of CB NPs on aromatase expression levels in primary cultures of human luteinized granulosa cells. Primary cultures of luteinized granulosa cells collected from three women were incubated for 24 hours with 50 µg/mL or 100 µg/mL of CB NPs and, after extensive washings, aromatase expression level was determined by immunoblotting. Vinculin was used as an internal loading control. Results are means ± standard error of the mean from (a, c) three or (b) three to four independent experiments and are expressed as percentage of untreated cells. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with untreated cells.

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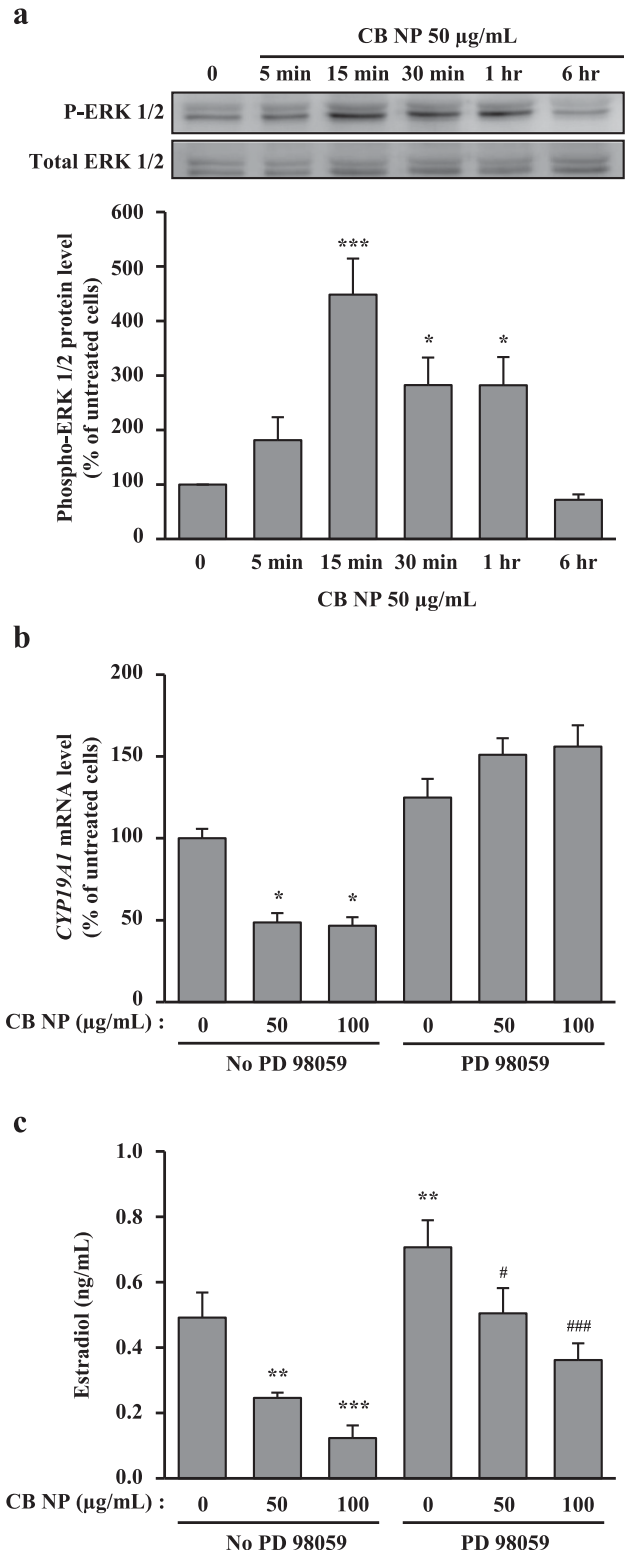


**Figure 4.** Activation of ERK1/2 signaling pathway by CB NPs in KGN cells and involvement in CB NPs-induced decrease of *CYP19A1* messenger RNA and E2 secretion. (a) Time-course activation of the ERK1/2 pathway by CB NPs in KGN cells. KGN cells were incubated with 50 µg/mL of CB NPs for increasing periods of time, and P-ERK1/2 proteins level was measured by immunoblotting and normalized with total ERK1/2. A representative immunoblot is shown in the inset. Results are means ± standard error of the mean of five independent experiments for 0, 5, and 30 minutes and 6 hours; four independent experiments for 1 hour; and three

expression in KGN cells were observed in response to CB NPs starting at a concentration of 25 µg/mL. We did not observe downregulation of transcripts encoding StAR or P450scc, two other rate-limiting proteins of steroids biosynthesis, that were instead up-regulated in a dose-dependent manner by CB NPs suggesting that CB NPs inhibitory effects are specific of *CYP19A1* (Simon *et al.*, unpublished data). The decrease in *CYP19A1* transcripts in KGN cells was paralleled by a reduction in its 55 kDa protein that has been characterized as the active form of the enzyme (40, 41) and a reduction was also observed in primary cultures of granulosa cells. Altogether, our data indicate that CB NPs may act at a transcriptional level to regulate *CYP19A1* expression in KGN cells. An increasing body of evidence gained from several cellular models indicates that gene expression can be modulated by different NPs (37, 42, 43). Our study provides the first demonstration to our knowledge that the gene encoding the key enzyme in estrogen biosynthesis is targeted by CB NPs. Effects of CB NPs on both E2 secretion and aromatase protein expression were observed at lower CB NPs concentrations in the granulosa cell line KGN than in primary cultures. This may be due to some features of each cellular model such as the tumoral origin of KGN cells or the initiation of luteinization in granulosa cells collected after induction of ovulation during *in vitro* fertilization.

A growing amount of data indicates that cell exposure to NPs leads to the activation of several signaling pathways. Among them, activation of ERK1/2 has been reported in response to a large variety of NPs such as copper oxide, silver, silica NPs (44–46), as well as carbon NPs (47, 48). Our study establishes that CB NPs recruit also this signaling pathway in human granulosa cells. Furthermore, we report that ERK1/2 significantly contribute to CB NPs inhibition of *CYP19A1* expression and E2

**Figure 4. (Continued).** independent experiments for 15 minutes. (b) Effect of the ERK signaling pathway inhibitor PD 98059 on basal and on CB NPs regulated *CYP19A1* messenger RNA level. KGN cells were preincubated for 30 minutes with the MEK inhibitor PD 98059 (25 µM) before treatment with CB NPs (50 or 100 µg/mL) for 24 hours. After extensive washings, *CYP19A1* messenger RNA level was determined by quantitative polymerase chain reaction. Results are means ± standard error of the mean of three independent experiments. (c) Effect of the ERK signaling pathway inhibitor PD 98059 on basal and on CB NPs regulated E2 secretion. KGN cells were preincubated for 30 minutes with the MEK inhibitor PD 98059 (25 µM) before treatment with CB NPs (50 or 100 µg/mL) for 24 hours. Cells were then extensively washed, and E2 secretion during the next 48 hours was measured by assaying E2 concentration in the conditioned media by radioimmunoassay. Results are means ± standard error of the mean of four independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with untreated cells. # $P < 0.05$; ### $P < 0.001$ compared with PD 98059 treated cells with no CB NPs.

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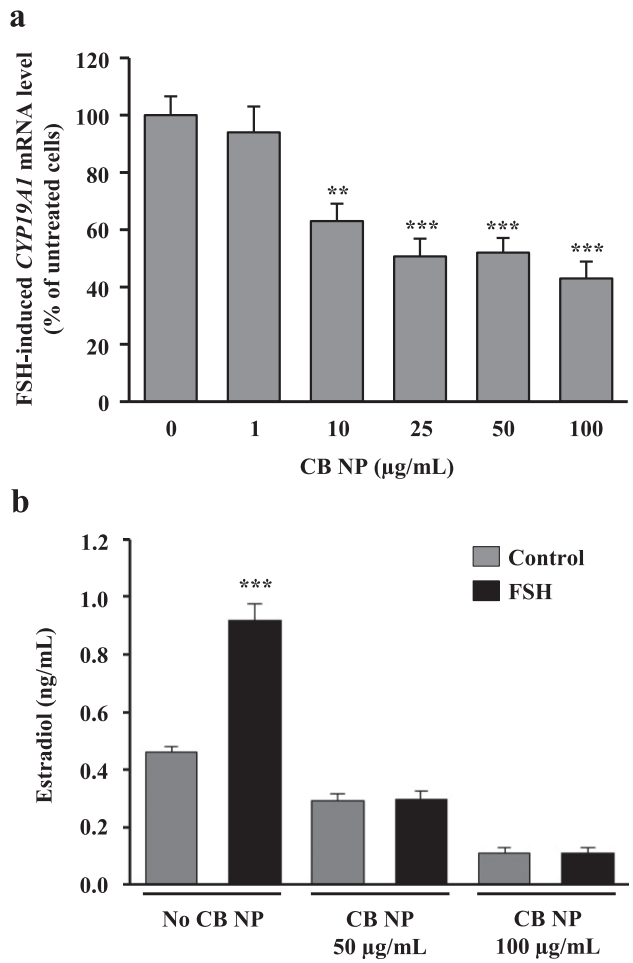


**Figure 5.** Effects of CB NPs on FSH-induced *CYP19A1* messenger RNA and E2 secretion in KGN cells. (a) Dose-response inhibitory effects of CB NPs on FSH-induced *CYP19A1* messenger RNA by KGN cells. Cells were incubated for 24 hours with increasing concentrations of CB NPs, extensively washed and E2 secretion in response to FSH (5.5 UI/mL) was measured during the next 48 hours by assaying E2 concentration in the conditioned media by radioimmunoassay. (b) Dose-response inhibitory effects of CB NPs on FSH-induced E2 secretion by KGN cells. Cells were incubated for 24 hours with increasing concentrations of CB NPs, extensively washed and stimulated with FSH for 48 hours. *CYP19A1* messenger RNA level was determined by quantitative polymerase chain reaction. Results are means ± standard error of the mean from (a) three or (b) four independent experiments and are expressed as percentage of untreated cells. ** $P < 0.01$; *** $P < 0.001$ compared with untreated cells.

secretion. Indeed, blocking ERK1/2 activation using the pharmacological inhibitor PD 98059 completely blocked CB NPs induced decrease in *CYP19A1* transcript levels. In agreement with such result, several studies have demonstrated that activation of the ERK1/2 signaling pathway inhibits FSH-induced aromatase in granulosa as well as in Sertoli cells (8, 10, 49, 50). PD 98059 treatment also reduced the extent of E2 secretion inhibition by CB NPs. However, a significant inhibition could still be detected indicating that CB NPs probably use additional mechanisms to disrupt granulosa cells steroidogenesis. The close association of CB NPs with endoplasmic reticulum membrane suggests that CB NPs may directly interact with aromatase. In line with this, we recently showed that CB NPs, exhibiting the same physicochemical characteristics as the ones used in the current study, bind to arylamine *N*-acetyltransferase, an aromatic amine-metabolizing enzyme, leading to protein conformational changes and loss of enzyme activity (33). If such a mechanism exists in KGN cells, CB NPs may reduce E2 secretion through direct interaction with aromatase. CB NPs would also possibly affect fluidity or integrity of the endoplasmic reticulum membrane, as already observed in plasma membrane exposed to gold or silicon NPs (51, 52). Given the structural complexity of the catalytically active aromatase, changes in organization of the reticulum membrane may reduce enzyme capacity. Interestingly, the ERK signaling cascade has been described to regulate in an opposite manner *CYP19A1* and other components of the steroidogenic pathway such as *StAR*. Evidence from *in vitro* and *in vivo* experiments indeed indicate that ERK1/2 increase *StAR* expression (8, 53, 54). Keeping this in mind, our demonstration that CB NPs downregulate *CYP19A1* but upregulate *StAR* expression is consistent with the role of ERK1/2 in mediating the effects of CB NPs in KGN cells.

The physiological regulation of E2 secretion by the ovary is complex. In addition to physiological regulators such as gonadotropins, steroids, and growth factors (2, 4, 50), several environmental contaminants including phthalates and bisphenol A can affect E2 biosynthesis (12, 13). These compounds are defined as endocrine disruptors and are considered as a serious threat to human health. Here, we demonstrate that CB NPs decrease E2 production by human granulosa cells and this was observed both in the KGN cell line and in primary cultures of human granulosa cells. This finding is in agreement with the study of Stelzer and Hutz (20) using another type of NPs and demonstrating that gold NPs decreased E2 secretion by rat granulosa cells. In contrast, no such effect could be detected with calcium phosphate NPs in human luteinized granulosa cells (21). This discrepancy may relate to the different types of NPs used but also to different experimental procedures such as the duration of exposition. In the current study, we provide evidence that, in addition to decrease basal E2 secretion, CB NPs significantly counteracted FSH regulation of E2 production and *CYP19A1* expression. This was observed with CB NPs concentrations beginning from 10 µg/mL. Given the main regulatory role of FSH, such effect may profoundly affect ovarian steroidogenesis.

Altogether, our data suggest that exposure to CB NPs leads to disruption of ovarian endocrine activity. Although the concentrations used in our study can be

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considered as environmentally relevant when considering lung exposure, as explained previously, the amount of inhalated CB NPs that would reach the ovaries remain unknown. Translocation of CB NPs and other NPs to blood and lymphatic circulations has been described (27, 55). It is likely that, as for other NPs, only limited amount translocate to peripheral organs (56). However, as NPs, including carbon nanomaterials, have been described to accumulate in organs without being effectively eliminated (57, 58), there is a need to assess their toxicity on peripheral organs. Supporting the potential endocrine toxicity of inhalated CB NPs *in vivo* is the demonstration that intratracheal administration of CB NPs to male mice exerts adverse effects on male testicular gametogenesis and steroidogenesis (29, 59, 60). In conclusion, our study in human ovarian granulosa cells has revealed that CB NPs disrupt E2 secretion notably through the recruitment of the ERK1/2 signaling pathway. Because exposure to CB NPs is currently rising worldwide, further investigation is required to better assess their potential detrimental effects on female reproductive health.

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***Disclosure Summary:*** The authors have nothing to disclose.

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