Characterization of the adverse effects of nicotine on placental development: in vivo and in vitro studies


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Cigarette smoke exposure is associated with many adverse pregnancy outcomes, including preterm labor, preterm premature rupture of membranes, placental abruption, and fetal growth restriction/low birth weight (4, 12). There is a direct relationship between the number of cigarettes smoked during pregnancy and the relative risk of low birth weight (12). In developed countries, it has been suggested that maternal cigarette smoking during pregnancy is a principal environmental cause of fetal growth restriction (60). Moreover, although cigarette smoking during pregnancy has been identified as a significant modifiable risk factor for low birth weight, many women continue to smoke during their pregnancies (3).

Cigarette smoke is estimated to contain as many as 4,000 chemicals (49, 53), including the addictive component nicotine. Data from animal studies suggest that nicotine exposure may be a critical component in the development of adverse reproductive effects in women who smoke (6, 42). In previous reports, we have shown that exposure of gravid rats to nicotine impaired fetal growth, resulting in reduced birth weight (27), an effect that is similar to the growth restriction seen in women who smoke (55). It is well established that normal fetal growth depends on the proper growth and development of the placenta; importantly, nicotine and its metabolites have been shown to accumulate in the placenta (35), where they may have direct effects on placental development and function. However, few reports have addressed the direct effect of nicotine insults on placental development and cell differentiation.

Normal placental development and vascularization depend on a tightly orchestrated coordination between the maternal decidua and trophoblast cells. They depend on adequate transformation of the uteroplacental vasculature by trophoblast cells following their proliferation, migration, and invasion into the maternal decidua (16, 52). During pregnancy, the depth of invasion by placental trophoblast cells into the uterine wall is critical and finely controlled. Poor invasion of maternal vessels has been correlated with placental pathologies such as fetal growth restriction, whereas an excessive trophoblast invasion is associated with choriocarcinoma (59).

Trophoblast cells are the earliest cell lineage to differentiate during mammalian development, arising from trophectoderm of the blastocyst (48). These stem cells can continue to proliferate or go on to differentiate along a multilineage pathway (25), which in rodents leads to five phenotypically distinct cell types: trophoblast giant cells, spongiosotrophoblast cells, invasive extraplacental trophoblast cells, glycogen cells, and syncytiotrophoblast cells. Recent work from Ain et al. (1) and Verocyysse et al. (57) showed that rat placenta proceeds along two types of invasion, the endovascular pathway that occurs between gestational days (GD) 8 and 10 and involves mainly giant trophoblast cells and the interstitial pathway that involves glycogen cells and occurs around GD 15. Considering the greater depth of both endovascular and interstitial trophoblast invasion in the rat compared with the mouse, the former species seemed more appropriate as an animal model to study...
trophoblast invasion (57). In cultured human placental explants, Zdravkovic et al. (63) have shown that nicotine inhibits trophoblast migration, a key process in the establishment of the fetomaternal circulation (FC). However, neither a mechanism of nicotine inhibition of trophoblast invasion nor an in vivo study confirming these findings has been performed to date.

The aim of this study was to investigate the effects of nicotine on the key processes of trophoblast invasion and differentiation. Using both in vitro and in vivo models, we examined the impact of nicotine on 1) trophoblast proliferation, migration, and invasion, 2) placental vascularization and degree of hypoxia, 3) expression of key genes of placental development, and 4) expression of circulating and placental angiogenic factors. The cellular model, the RCHO-1 cell line, was used to determine the mechanism(s) by which nicotine affects these processes.

**MATERIALS AND METHODS**

Two models to study the effects of nicotine on trophoblast invasion and cell differentiation were used. For in vivo studies, rats were exposed to nicotine before mating and during placental development. For in vitro studies, we used the rat trophoblast cell line RCHO-1. The RCHO-1 cell line provides an effective in vitro model system for dissecting the trophoblast cell differentiation pathway (23, 51), as these cells exhibit many characteristics of trophoblast stem cells (15, 23, 44). More importantly, the RCHO-1 trophoblast stem cells can be manipulated to proliferate or differentiate by altering their culture conditions (44).

**Maintenance and Treatment of Animals**

All animal experiments were approved by the Animal Research Ethics Board at McMaster University in accordance with the guidelines of the Canadian Council for Animal Care. Nulliparous 200- to 250-g female Wistar rats (Harlan, Indianapolis, IN) were maintained under controlled lighting (12:12-h light-dark cycle) and temperature (22°C) with ad libitum access to food and water. Dams were randomly assigned to receive saline (n = 6) or nicotine bitartrate (1 mg·kg\(^{-1}\)·day\(^{-1}\); Sigma-Aldrich, St. Louis, MO) (n = 5) via daily subcutaneous injection starting 2 wk prior to mating. We have demonstrated previously that this dose of nicotine results in cotinine concentrations in maternal and neonatal serum that are similar to those found in female smokers and their infants (20, 32, 39). Copulation (GD 0) was confirmed by the presence of sperm in a vaginal flush. At necropsy (GD 15) each fetus and its corresponding placenta were separated and weighted, and serum (maternal) was collected. Placental tissue samples were snap-frozen in liquid nitrogen and stored at −80°C until analysis or immersed in Accustain formalin-free fixative (Sigma-Aldrich).

**Serum VEGF and Endocrine Gland-Derived VEGF**

VEGF and endocrine gland-derived VEGF (EG-VEGF) concentrations in the serum of saline- and nicotine-treated dams were determined using commercially available ELISA kits; we used murine VEGF and human EG-VEGF kits, respectively (PeproTech). For each assay, two separate standard curves were constructed to allow accurate readings of samples at upper and lower ranges of the assay. All samples were in the linear range of the standard curves. The detection limit of the assay was 16 pg/ml for EG-VEGF and 63 pg/ml for VEGF.

**Placental Histology and Immunohistochemistry**

Placental histomorphometry. Following overnight immersion in 10% (vol/vol) neutral buffered formalin (EM Science, Gibbstown, NJ) at 4°C, two placentas from each dam were washed in water, fixed in PFA or Accustain formalin-free fixative, and embedded in paraffin and sectioned (5 μm). Sections were stained with hematoxylin and eosin (H & E) for general histological analysis or periodic acid-Schiff (PAS; Sigma Aldrich) for the identification of glycogen cells. The relative cross-sectional areas of GD 15 placentas were determined from H & E-stained sections. Pictures were made with a digital camera (Spot-RT; Diagnostic Instruments, Sterling Heights, MI) and used to calculate the layer surface of the three placental zones (decidua, junctional zone, and labyrinth) with Image J software (National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/). The sections to be analyzed were determined based on the site of the umbilical attachment. At least three sections per placenta were analyzed, but to control for maternal effects only one placenta per dam was used for each outcome measure. To quantify capillary length, images were processed for morphometric analysis with Image J software. A macro command was edited to give the total vessel length after binarization, skeletonization, and pixel count of the CD31 staining (46).

**Immunohistochemistry and immunocytochemistry.** Immunohistochemistry was performed as described previously (2, 7). For antigen detection, 5-μm sections were incubated with the following antibodies: anti-cytokeratin (Abcam), anti-VEGF (Abcam), anti-EG-VEGF (Covalab), anti-CD31 (DAKO), anti-carboxic anhydrase IX (CA-IX; Novus Biological), anti-proliferating cell nuclear antigen (PCNA; DAKO), and anti-cytokeratin. Immunopositive staining was detected using a Vectastain ABC kit, using 3,3-diaminobenzidine as the chromagen (Vector Laboratories). Slides were counterstained using H & E (Sigma-Aldrich). At least three sections per placenta collected from each animal were analyzed. Immunocytochemistry was performed for RCHO-1 cells using desmoplakin antibody (Abcam). Desmoplakin staining was performed as described previously (9). Desmoplakin was used to visualize the cell’s edges. RCHO-1 cells were washed, fixed, and permeabilized in methanol at −20°C for 25 min. Immunopositive staining was detected using a Vectastain ABC kit, using 3,3-diaminobenzidine-tetrahydrochloride as the chromagen.

**Western blotting.** Frozen placental samples (n = 6 saline and n = 5 nicotine) collected from different animals were homogenized on ice for 1 min in RIPA lysis buffer [50 mm Tris·HCl (pH 7.5), 150 mM NaCl, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 10% Triton X-100, 1 mM phenylmethylsulfonylfluoride, 5 μg/ml leupeptin, and 5 μg/ml aprotinin], as previously described (30). The homogenates were centrifuged (15,000 g at 4°C) for 15 min, and the supernatants were collected. Protein concentrations were determined using the Bradford assay. Twenty to forty micrograms of protein extracts was electrophoretically separated on SDS-PAGE (12%) for immunoblot analysis using the following antibodies: anti-CD31, anti-PCNA, anti-CA-IX, anti-4HNE (R & D Systems, Minneapolis, MN), anti-VEGF, anti-FLT-1 (fms-like tyrosine kinase-1; Santa Cruz Biotechnology), and anti-EG-VEGF. As described previously (9), a specific Western blot protocol was set up to detect EG-VEGF protein (10–12 kDa). Briefly, we used 100 μg of placental protein that was separated on 0.1% SDS-17% polyacrylamide gels in Tris-tricine-SDS buffer (Sigma-Aldrich) and electrically transferred onto 0.2-μm polyvinylidine difluoride membranes (Millipore, Bedford, MA). The transfer of the proteins was reduced to 30 min at 90 V. The blots were washed with PBS-Tween 0.1% and incubated overnight in blocking solution (2.5% skimmed milk in PBST). Subsequently, membranes were immunoblotted with a rabbit antibody against EG-VEGF (0.48 μg/ml; Covalab Lyon) for 2 h. Blots were then rinsed with PBST and incubated with biotinylated goat anti-rabbit IgG (500 ng/ml, 1:2,000, dilution in blocking solution; DakoCytonation) for 30 min. After 3 PBST washes, the membrane was incubated with a peroxidase-conjugated extravidin (1:2,000 dilution in blocking solution; Sigma Aldrich) for 30 min. Blots were washed six times with PBST, and the antibody-antigen complex was detected using the ECL Plus detection system (Amersham Pharmacia Biotech). β-Actin was used to stan-
dardize the loading. For quantification of 4HNE, we determined the total intensity of all the bands in each of the sample lanes following immunostaining with anti-4HNE. The total 4HNE intensity/lane was normalized to the intensity of total protein per lane.

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction Analysis

Total RNA was extracted from RCHO-1 cells, GD 15 placental tissue samples of saline, and nicotine-treated animals (n = 5 saline and n = 6 nicotine) using a rapid RNA isolation system (Qiagen, Courtaboeuf, France). Reverse transcription was performed on 1 μg of total RNA with Superscript II-RnaseH reverse transcriptase (Invitrogen, Cergy Pontoise, France) under conditions recommended by the manufacturer.

Real-Time Polymerase Chain Reaction Analysis

Proliferin, Hand1, Hand2, Mash2, Gcm1, nAch7, nAch4, and RPL13 expression were quantified by real-time RT-PCR using a Light Cycler apparatus (Roche Diagnostics, Meylan, France). The PCR was performed using the primers shown in Table 1 and SYBR green PCR core reagents (Light Cycler-FastStart Master SYBR Green I; Roche Diagnostics). The results were normalized to RPL13 mRNA expression levels. To assess linearity and efficiency of PCR amplification, standard curves for all transcripts were generated by using serial dilutions of cDNA. The RealQuant analysis software (Roche Diagnostics) was used to quantify relative levels of expression.

Mitochondrial Electron Transport Chain Activity

Frozen GD 15 placental tissue was homogenized in homogenization buffer (5 mM HEPES, pH 7.4, 100 mM KCl, 70 mM sucrose, 220 mM mannitol, and 1 mM EGTA) with Complete Mini EDTA-free protease inhibitors (Roche Applied Science, Laval, QC, Canada), using Tenbroeck tissue grinders. Homogenates were spun for 10 min at 600 g, and the supernatant was removed, flash-frozen in liquid nitrogen, and stored at −80°C until use. Citrate synthase activity (an indicator of total mitochondrial mass) was measured using the thiol reagent 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma Chemical, St. Louis, MO). Complex IV (cytochrome c oxidase) activity was assessed by measuring the rate of cytochrome c (from equine heart; Sigma Chemical) oxidation. Both activity assays were performed using UV-spectrophotometry (Varian, Palo Alto, CA) as described previously (41). Data are expressed as the mean enzyme activity relative to the wet weight of tissue.

Table 1. Primers used for real-time RT-PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward 5′ to 3′</th>
<th>Reverse 5′ to 3′</th>
</tr>
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<tbody>
<tr>
<td>Hand1</td>
<td>CACCAAGCTGCTGCTACTTTGTTAATCCTCTCT</td>
<td>AGCCAAGTGCGTGCTTCTTTAAATCCTCTCT</td>
</tr>
<tr>
<td>Hand2</td>
<td>AGCTGATGGCCACACTCTGAGAT</td>
<td>TCTCGCTCTCTCTGAGCGGTCTCTCT</td>
</tr>
<tr>
<td>Mash2</td>
<td>GCTGAGCTGCGCTGACGCTA</td>
<td>TCGCGAACAGGAGGAGGGTGTC</td>
</tr>
<tr>
<td>Gcm1</td>
<td>CGAGGCTGCAATGCGCTCATCA</td>
<td>CTCTCTCTGAGGACGTCGCT</td>
</tr>
<tr>
<td>Proliferin</td>
<td>GGTTGCGAAATGGAGCTTCA</td>
<td>TAGTGTGTAGCCGCTCGCTG</td>
</tr>
<tr>
<td>CD31</td>
<td>GCTCGAGCAATGGTCTCAGA</td>
<td>GTCATGTTCCGGGAACATCGC</td>
</tr>
<tr>
<td>nACh4</td>
<td>CCAAGCTGACATCTTGAGGAAA</td>
<td>GCCATAGGGTGATCGATGATAG</td>
</tr>
<tr>
<td>nACh7</td>
<td>GTACAAGGAGGCTGCTAAAGAA</td>
<td>CAGGAGACTGAGGGAGAAAGTA</td>
</tr>
<tr>
<td>RPL13</td>
<td>AGGGTGCTGCTATGCTGAGAAGAAA</td>
<td>AGTSACTCCGCGATTGGATTTCGSC</td>
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nAChr, nicotinic acetylcholine receptor.
RCHO Cell Culture

The RCHO-1 cells were cultured as indicated previously (23, 44). The RCHO-1 cell line was established from a rat-transplantable choriocarcinoma (23). The cells exhibit many characteristics of trophoblast stem cells (15, 54).

There are two strong advantages to using these cells. First, RCHO-1 is a rat cell line, and second, this cell line can be maintained in a proliferative (i.e., stem cells) or differentiated state (i.e., giant cells). RCHO-1 cells are in a proliferative state when cultured in RPMI 1640 medium and supplemented with 20% fetal bovine serum (heat inactivated), 100 mg/ml penicillin-streptomycin, 1 mM sodium pyruvate, and 50 mM 2-mercaptoethanol in a 37°C incubator under 95% air-5% CO₂. Three days after the cells were cultured under proliferative conditions, a differentiated state could be obtained by switching to RPMI 1640 medium containing 10% horse serum (29). Trophoblast giant cell differentiation was verified by the morphological detection of trophoblast giant cells and/or the expression of placental lactogen-I (44).

Cell Proliferation

The effect of nicotine on cell proliferation was assessed on proliferative RCHO-1 cells using [3H]thymidine incorporation. Nicotine concentrations ranged from 10⁻⁹ to 10⁻³ M. This range encompasses the EC₅₀ of nicotine for all of its receptor subtypes (0.85–113 μM) (13, 18, 26) and the maximum (C_max, 1.8–112nM) and average (AUC₀₋∞) range of nicotine concentrations that have been reported in pharmacokinetic studies of nicotine replacement therapies (10, 17, 28, 36). Proliferative cells were cultured overnight (7 × 10⁴ cells/well, 37°C, 5% CO₂). The cells were incubated for 24 h in the absence or presence of nicotine, labeled with 0.5 μCi/ml [3H]thymidine (Amersham, France), and subsequently washed in HBSS and incubated in 2 ml of ice-cold 5% trichloroacetic acid for 20 min at RT. After washing, 0.4 ml of 0.1 M NaOH and 0.1% SDS were added; the lysates were transferred into a vial containing scintillation liquid, and the radioactivity was counted in a β-counter (Beckman).

Matrigel Invasion Assay

The invasive properties of nicotine-treated RCHO-1 cells were measured using Matrigel-coated HTS FluoroBlok transwell inserts (Becton-Dickinson). Matrigel (100 μl diluted at 1:25 with RPMI 1640) was added to the transwell inserts and allowed to set for 2 h at 37°C. RCHO-1 cell monolayers were prelabeled in situ for 1 h with 10 μg/ml acetylated low-density lipoprotein (DiI-Ac-LDL).
labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate. When cells are labeled with DiI-Ac-LDL, the lipoprotein is degraded by lysosomal enzymes and the DiI (fluorescent probe) accumulates in the intracellular membranes. Labeling cells with DiI-Ac-LDL has no effect on cell viability (58).

Cells were then trypsinized and resuspended to the apical chamber of Matrigel precoated FluoroBlok transwell (5 × 10⁴ cells) in the absence or presence of nicotine (10⁻⁹ to 10⁻³ M). Uncoated fluoroBlok transwell inserts were also used to determine the number of cells that invaded toward the chemoattractant. The chemoattractant (10% serum) is added to the basal chamber. The FluoroBlok transwell is incubated for 24 h at 37°C and 5% CO₂. The fluorescence of invaded cells is read at wavelengths of 540/570 nm. Data are expressed as the following equation [%invasion = (mean fluorescence of cells invaded through the Matrigel coated membrane toward chemoattractant/mean fluorescence of cells invaded through uncoated membrane toward chemoattractant) × 100%].

Wound Healing Assay

To evaluate the effect of nicotine on RCHO-1 migration capacity, wound healing assays were performed as reported previously (7, 31). At confluence, differentiated RCHO-1 cells (i.e., giant cells) were scraped with a sterile tip to create an artificial wound that was allowed to heal for the next 18 h. The cells were treated at time 0 by nicotine at 10⁻⁹ to 10⁻³ M. The treatment lasted for 18 h. Preselected fields were photographed at regular intervals (0–18 h). The width of denuded area was measured using an electronic grid, and the distances crossed by cells were determined using Scion Image software (version 4.0.2; Scion). Results of at least three separate experiments were expressed as percent of control (the initial wound).

Fig. 3. Nicotine effect on placental and on circulating VEGF and endocrine gland-derived VEGF (EG-VEGF) in saline- and nicotine-treated dams. A: VEGF staining in placental sections of saline (image a) and nicotine placentas (image b). B: comparison of VEGF protein levels in saline and in nicotine placental extracts. C: EG-VEGF staining in placental sections of saline (images a and c) and nicotine placentas (images b and d). D: comparisons of EG-VEGF protein levels in saline (n = 6) and nicotine (n = 5) placental extracts. E and F: comparisons of VEGF and EG-VEGF serum levels in saline- and nicotine-treated dams, respectively. VEGF and EG-VEGF levels were measured by ELISA. *P < 0.05. D, decidua; Jz, junctional zone; Gc, glycogen cell; Gic, giant trophoblast cell; L, labyrinth.
Zymography

About 20 μl of harvested RCHO-1 culture medium was electrophoresed under nonreducing conditions in a 10% acrylamide gel containing 1 mg/ml gelatin (Sigma Aldrich) according to the method of Xu et al. (62). After electrophoresis, the gels were washed at room temperature for 1 h in 2.5% Triton X-100 and 50 mM Tris-HCl, pH 7.5, and then incubated at 37°C overnight in buffer containing 150 mM NaCl, 5 mM CaCl2, and 50 mM Tris-HCl, pH 7.6. Thereafter, gels were stained with 0.1% (wt/vol) Coomassie brilliant blue R-250 in 30% (vol/vol) isopropyl alcohol and 10% glacial acetic acid for 60 min and destained in 10% (vol/vol) methanol and 5% (vol/vol) glacial acetic acid. Semiquantification of the bands corresponding to 72- and 92-kDa gelatinases was performed using a densitometer.

Fig. 4. A and B: nicotine effects on circulating soluble fms-like tyrosine kinase-1 (sFLT-1) and on VEGF/sFLT-1 ratio, respectively. C and D: nicotine effects on placental FLT-1 protein expression and VEGF/fms-like tyrosine kinase-1 (FLT-1) ratio, respectively. Blot shows comparisons of FLT-1 protein levels in saline (n = 6) and nicotine (n = 5) placental extracts. sFLT-1 levels were measured by ELISA.

Fig. 5. Nicotine treatment effect on the mRNA expression of Hand1, Hand2, Mash2, and GCm1 mRNA in the placenta. mRNA was isolated from whole placenta (saline: n = 6; nicotine: n = 5). Expression of the indicated genes was analyzed by quantitative RT-PCR, plotted in arbitrary units, and normalized to RPL13. Data are presented as means ± SE. *P < 0.05.
Statistical Analysis

All statistical analyses were performed using SigmaStat (Jandel Scientific Software, SanRafael, CA). Data were analyzed by Student’s t-test and one-way analysis of variance (ANOVA), followed by comparison with the controls (Bonferroni’s t-test, \( \alpha = 0.05 \)). Data were tested for normality as well as equal variance, and when normality or variance tests failed, data were analyzed using Mann-Whitney Rank Sum test or Kruskal-Wallis one-way ANOVA on ranks.

RESULTS

Nicotine Treatment Effects on Fetal Weight, Placental Weight, and Placental Efficiency

Fetal and placental weights were measured at GD 15, and placental efficiency was determined (fetal weight/placental weight). There was no effect of nicotine treatment on fetal weight, placental weight, or placental efficiency (all \( P > 0.05 \); data not shown).

Structural Changes in Rat Placentas Following Nicotine Treatment

At GD 15, placentas from nicotine-exposed dams had significantly decreased decidua and junctional zone area and seemed more compacted (Fig. 1, A and B). There was no effect of nicotine exposure on the size of the labyrinth zone (Fig. 1B).

Nicotine treatment affects placental vascularization and development. To get more insight into the effects of the nicotine treatment on placental development, we examined the expression levels of PCNA, a marker of cell proliferation, and CD31, a marker of vascularization. We have also examined the level of expression of a marker of hypoxia, CA-IX (37). Nicotine exposure caused a significant reduction in PCNA expression in the labyrinth zone (Fig. 2, A vs. B) and in whole placenta (Fig. 2, C and D). Also, a quantification of the PCNA-stained nuclei in the labyrinth zone showed a significant reduction in the number of positive cells in the placenta of nicotine-treated dams (data not shown). Exposure to nicotine caused a disorganization of the vascular tree in the labyrinth layer (the major site of nutrient and gas exchange), with less branching in the nicotine group (Fig. 2, E and E’ vs. F and F’). There was a significant decrease in the total capillary length in the labyrinth of nicotine-treated animals compared with the saline controls (Fig. 2G).

This disorganization occurred in association with a significant reduction in CD31 expression levels in whole placental homogenates from nicotine-exposed animals (Fig. 2H).

![Fig. 6. Nicotine effect on interstitial invasion. A and B: representative photographs of rat placental sections stained with cytokeratin (brown). Saline placenta (A) and nicotine placenta (B) are shown. Note the presence of invasive Gc in saline and not in the nicotine placenta. C and D: placental sections with higher magnification of sections shown in A and B. E and F: placental sections stained, with periodic acid-Schiff (purple) confirming the identity of Gc. Scale bar, 50 μm.](http://ajpendo.physiology.org/doi/abs/10.1152/ajpendo.00478.2013)
The reduction in the labyrinth vascularization occurred in association with an increase in hypoxia (i.e., increased expression of carbonic anhydrase in the labyrinth and whole placenta; Fig. 2, I–L).

Nicotine effect on placental angiogenic factors. It is well established that changes in placental vasculature that result in increased placental hypoxia are often associated with alteration in the expression and production of angiogenic factors by the placenta. We compared the local and circulating levels of VEGF, a key placental angiogenic factor that was reported to be affected by smoking in other systems (34, 47). There was no effect of nicotine treatment on the expression of VEGF in the placenta (Fig. 3, A and B) or on serum levels of VEGF (Fig. 3E). Because VEGF needs to be considered in relation to its receptor VEGFR1 (FLT-1), we have compared both placental VEGFR1 levels and the levels of its circulating form, the soluble fms-like tyrosine kinase-1 (sFLT-1), an antiangiogenic factor, in the saline and nicotine groups. There were no changes in the levels of placental FLT-1 or the circulating sFLT-1 (Fig. 4). Because changes in the placental (VEGF to FLT-1) ratio or in the circulating (VEGF to sFLT-1) ratio might explain differences in the angiogenic status of a given organ, we compared these ratios in both groups. No differences could be observed between the saline and nicotine group (Fig. 4).

We have also compared the levels of expression of the new placental angiogenic factor EG-VEGF. EG-VEGF plays a major role in placental growth and angiogenesis (7–9, 31) and was recently reported to be increased in placental pathologies such as preeclampsia and intrauterine growth restriction (8, 9, 31). The placental expression of EG-VEGF in the placenta was significantly decreased in nicotine-exposed animals compared with the saline group (Fig. 3C). This was confirmed in whole placental homogenates by Western blotting analysis (Fig. 3D). A comparison of the circulating levels of EG-VEGF showed a trend toward a decrease in the nicotine group; however, this did not reach statistical significance (P = 0.056; Fig. 3D).

Nicotine effect on mitochondrial electron transport chain activity and oxidative damage. There was no effect of maternal nicotine administration on the activities of placental complex IV or citrate synthase (data not shown). Moreover, there was no evidence of increased oxidative damage (i.e., 4HNE expression) in the placentas of nicotine-exposed dams compared with controls (data not shown).

![Nicotine effect on proliferation and differentiation of RCHO-1 cells](http://ajpendo.physiology.org/)

**Fig. 7.** Nicotine effect on the proliferation and differentiation of RCHO-1 cells. **A:** dose-response effect of nicotine (10⁻⁹, 10⁻⁶, and 10⁻³ M) on RCHO-1 stem cell differentiation. Images a–d show photographs of RCHO-1 cells stained with desmoplakin. Images e–h show photographs of RCHO-1 cells taken under phase contrast. **B:** [³H]thymidine incorporation into RCHO-1 stem cells in the absence (black bar) or presence of nicotine at indicated concentrations (dashed bars). Data are presented as the mean ± SE of 3 independent experiments. *P < 0.05.
Correlation between structural changes and placental gene expression. To determine whether the architectural abnormalities observed in placentas from nicotine-exposed animals are linked to quantitative changes in gene expression, real-time RT-PCR analyses were performed for several key trophoblast genes with specific roles in the formation of placental structures. We determined the expression of Hand1, a protein that promotes differentiation of trophoblast giant cells, and Mash2, a marker for spongiotrophoblast and a protein required for the maintenance of giant cell precursors (50). We also determined the expression levels of Gcm1, an important protein of labyrinth branching. Nicotine exposure had no effect on the placental expression of Hand2 or Mash2. Both Hand1 and Gcm1 expression were significantly decreased in the placentas from nicotine-treated dams (Fig. 5, A and D). Taken together, these data suggest that nicotine treatment affects trophoblast differentiation toward an invasive phenotype and negatively impacts labyrinth development.

Nicotine Treatment Inhibits Interstitial Trophoblast Invasion

Because the decidua and the junctional zones constitute the main sites that govern interstitial trophoblast invasion, we further analyzed the invasive process in the placenta of saline- and nicotine-treated rats. In Fig. 6, glycogen cell staining by cytokeratin is compared in the saline (Fig. 6, A and C) and in the nicotine placentas (Fig. 6, B and D). Photographs in Fig. 6, E and F, report PAS staining in the saline and in the treated placentas, respectively. Both cytokeratin and PAS staining show glycogen cells that migrate from the thick trophoblastic wall into the decidua (see arrows for glycogen columns in Fig. 6, A and C). In contrast, in the treated placentas this migration was not observed, and glycogen cells remained blocked within the junctional zones by a barrier of trophoblast giant cells.

These data strongly suggest that nicotine treatment affects interstitial invasion in rat placentas.

Because in vivo data strongly suggested that nicotine treatment affected trophoblast invasion and hence, their differentiation, we carried out an in vitro study to examine the direct effects of nicotine on trophoblast stem cell differentiation. We used RCHO-1 cells, a rat cell line. These cells have the advantage of being able to differentiate from trophoblast stem cells to giant trophoblast cells under specific culture conditions.

Effect of nicotine on the differentiation of trophoblast stem cells to giant trophoblast cells. Under high-serum conditions (20%), RCHO-1 cells proliferate and maintain their undifferentiated state. A switch to 10% horse serum allows these cells to differentiate. Under these conditions, the number of proliferating cells decreases significantly, and the stem cells are replaced by differentiated cells, i.e., the giant trophoblast cells. To determine whether nicotine directly affects this process of differentiation, we tested its effect over a large range of nicotine concentrations (10^{-9} to 10^{-3} M). In the absence of nicotine (i.e., control) almost all of the RCHO-1 cells differentiated to giant cells; this differentiation process was inhibited by nicotine treatment, resulting in significantly enhanced proliferation in nicotine-exposed cells (Fig. 7).

Nicotine effects on the expression of genes involved in cell differentiation. Nicotine treatment of RCHO-1 cells resulted in a significant reduction in Hand1 and proliferin mRNA expression (Fig. 8, A and B). Hand1 has been shown to be pivotal to the differentiation of trophoblast giant cells (15, 24), and its overexpression in RCHO-1 trophoblast cells has been shown to promote their differentiation to trophoblast giant cells (15). Conversely, nicotine treatment increased the expression of Mash2 significantly (Fig. 8D). There was no effect of nicotine

![Fig. 8. Nicotine effect on the mRNA expression of proliferin, Mash2, Hand1, and Hand2 in control and nicotine-treated RCHO-1 cells. Cells were treated for 24 h with nicotine (10^{-9}, 10^{-6}, and 10^{-3} M). Expression of the indicated genes was analyzed by quantitative RT-PCR, plotted in arbitrary units, and normalized to RPL13. Data are presented as means + SE. *P < 0.05.](http://ajpendo.physiology.org/)
on the expression of Hand2 at any dose tested (Fig. 8C). These data demonstrate at the cellular level that nicotine directly affects key genes of the cell differentiation toward an invasive phenotype, i.e., giant trophoblast cells.

Nicotine effects on RCHO cell migration. Trophoblast migration is an important process that accompanies trophoblast differentiation and invasion into the maternal decidua. We examined the effect of nicotine (10⁻⁹ to 10⁻³ M) on the migration of RCHO-1 giant cells (i.e., differentiated cells) using the wound healing assay. Treatment with nicotine significantly delayed the wound healing of RCHO-1 giant cells (Fig. 9, A and B).

Nicotine effect on RCHO-1 invasion. To further investigate the effect of nicotine on the process of invasion of RCHO-1 cells, we determined their percentage of invasion through Matrigel in the absence or presence of nicotine using the BD FluoroBlok cell culture inserts. At the highest concentration tested, there was a significant decrease in the invasion of RCHO-1 cells (Fig. 9C). The migration of trophoblast cells through the maternal decidua is mediated via the activity of matrix metalloproteinases (MMPs). MMP2 and MMP9 are the main MMPs known to be expressed in the placenta (5). MMP2 was weakly detected in RCHO-1 cells, in accord with previous reports (43). MMP9 was expressed abundantly in these cells and was significantly reduced by nicotine treatment (Fig. 9D).

Effect of Nicotine on Nicotinic Acetylcholine Receptor Expression

Studies on nicotinic acetylcholine receptors (nAChRs) in the placenta have reported that this tissue expresses different forms of the nAChR. The α4- and α7-subunits are highly expressed in the placenta (38). Nicotine treatment both in vivo (rats) and in vitro (RCHO-1 cells) did not significantly alter the expression of the nAChR α7-subunit (Fig. 10, A and C). However, nicotine treatment in vitro increased the expression of the nAChR α4-subunit in a dose-dependent manner (Fig. 10D). There was also a trend toward an increase in the expression of this subunit in the placenta of nicotine-treated dams (Fig. 10B).

Fig. 9. Nicotine effect on RCHO-1 cell migration and invasion. A: photographs of wounded RCHO-1 cells at 0 (T0) and 18 h (T18h) post-wounding. The cells were treated at T0 with nicotine (10⁻⁹, 10⁻⁶, and 10⁻³ M). B: graph that reports the percentages of wound closure after 18 h of treatment. C: effect of nicotine on RCHO-1 giant cell invasion using Matrigel-coated HTS FluroBlok transwell inserts. Top: photograph of RCHO-1 cells prelabeled with acetylated low-density lipoprotein. Bottom: % invading cells upon treatment with nicotine (10⁻⁹, 10⁻⁶, and 10⁻³ M). Data are presented as means ± SE. D: effect of nicotine on matrix metallopeptidase (MMP)9 and MMP2 activities in RCHO-1 cells. Top: representative zymography of cells treated or not with nicotine for 24 h at indicated concentrations. Bottom: densitometric analysis of zymographies of 3 independent experiments. Each bar represents the amounts of MMP9 as arbitrary units. Data are presented as means ± SE.

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EVIDENCE FOR ADVERSE EFFECTS OF NICOTINE ON PREGNANCY OUTCOME

DISCUSSION

Here, both in vitro and in vivo studies demonstrate that nicotine 1) acts directly within the placenta, 2) exerts harmful effects on placental development, 3) is involved in the dysregulation of the establishment of fetomaternal circulation, and 4) impacts the mechanisms underlying trophoblast invasion.

These statements are based on several key findings from this study. First, nicotine receptors are expressed in the placenta, and their levels are affected by nicotine treatment. Second, both in vitro and in vivo data showed that nicotine inhibits trophoblast differentiation by affecting key placental transcription factors involved in the differentiation of trophoblast stem cells, an effect that leads to an increase in their proliferation at the expense of their differentiation toward an invasive phenotype. Third, nicotine inhibited trophoblast cell migration and invasion, an effect that may be due in part to the reduction in MMP9 activity. Fourth, nicotine affected the labyrinth zone of the placenta by decreasing its vascularization and cell proliferation and by increasing its hypoxic environment, all aspects that might be the result of a failure in trophoblast invasion and establishment of appropriate fetomaternal circulation.

In rodents, trophoblast invasion proceeds along two pathways: endovascular trophoblast invasion involving mainly giant trophoblast cells and interstitial trophoblast invasion involving glycogen cells (1, 57). In this study, we investigated the effects of nicotine on both types of invasion by using the RCHO-1 cell, a cell line that models trophoblast stem cells that differentiate to giant trophoblast cells (i.e., endovascular trophoblast invasion), and an animal model in which we studied glycogen cell invasion (i.e., interstitial trophoblast invasion).

We have demonstrated that nicotine treatment both in vivo and in vitro inhibits trophoblast invasion and differentiation. An inhibitory effect of nicotine on cell migration has previously been reported in other systems such as primary human fibroblasts from gingival tissues (21, 22). However, the effect of nicotine on trophoblast cell invasion and differentiation has not been reported.

In the two models used in this study, nicotine reduced the expression of Hand1, and we know that overexpression of Hand1 in RCHO-1 trophoblast cells promotes their differentiation to invading trophoblast giant cells (15, 24). This suggests that the substantial invasion of giant trophoblast that still persists at GD 15 might also be affected by the nicotine treatment in vivo.

Trophoblast invasion is accompanied by the remodeling of extracellular matrix by MMPs. We demonstrated that nicotine inhibits the activity of MMP9, the main MMP expressed by trophoblast cells during early pregnancy (5, 62). This inhibitory effect suggests that nicotine affects the main enzyme involved in the remodeling of the decidua.

The effects of nicotine on placental development are likely to be mediated via nACh receptors in the placenta. Indeed, we and others have identified the presence of nAChRs in the placenta (38). Moreover, we have identified that nicotine exposure increased the levels of the nAChR α4-subunit both in vitro and in vivo. Importantly, the nAChR α4-subunit is the receptor that exhibits the highest affinity for nicotine, and it is highly expressed in trophoblast cells (14, 40).

In other systems, it has been reported that under stressful conditions trophoblast cells release antiangiogenic factors lo-

Fig. 10. Nicotine effect on the mRNA expression of nicotinic acetylcholine receptors (nAChRs) nACh7α and nACh4α. A and B: mRNA expression of nACh7α and nACh4α in the placenta of saline- and nicotine-treated dams, respectively. C and D: expression of the same genes in RCHO-1 cells treated with nicotine for 24 h (10^-9, 10^-6, and 10^-3 M). Expression of the indicated genes was analyzed by quantitative RT-PCR, plotted in arbitrary units, and normalized to RPL13. Data are presented as means ± SE. *P < 0.05. Ctl, control.
cally in the placenta and in the maternal circulation, causing a failure in placental development along with harmful effects on the maternal vascular system (11). In the present study, nicotine caused an increase in carbonic anhydrase, suggesting hypoxia, but since there was no increase in 4HNE, this hypoxia was not sufficient to result in oxidative damage in the placenta. In contrast, we observed a significant decrease in the levels of placental EG-VEGF expression and a trend toward a decrease in its circulating levels in the nicotine-treated rats. This finding corroborates with the one reported by our group showing a decrease in the EG-VEGF levels in the ovary of rats whose mothers were exposed to nicotine during gestation (45). However, the mechanism by which nicotine decreases the production of this key placental factor is still to be determined.

In a previous study (33), we have shown that, compared with control animals, nicotine treatment throughout the gestational period was associated with a higher proportion of females who delivered at least one stillborn pup. In our study, we have demonstrated that nicotine administration to pregnant rats resulted in structural abnormalities in the placenta at GD 15. Importantly, these structural changes precede any deficits in fetal growth that we have previously demonstrated were not observable until GD 21 in this model (27). These data suggest that the observed nicotine effects on fetal growth might well be a consequence of a failure in placental development occurring at the time of invasion and differentiation of trophoblast cells. In fact, we have observed a significant decrease in the vascularization along with a decrease in the levels of proliferating cells within the labyrinth zone, the key exchanging zone. The nicotine effects in this zone might well be explained by the reduced expression of EG-VEGF, as this factor has been shown to be an important regulator of placentation by regulating trophoblast proliferation and intraplacental vascularization (7–9). Further studies will determine the mechanism by which nicotine regulates EG-VEGF expression.

Although it is well established that smoking during pregnancy reduces the risk of preeclampsia (PE) (19), studies of smokeless tobacco use during pregnancy argue against nicotine as being protective against the development of PE (19, 61). Indeed, cigarette smoking is thought to protect against the development of PE by decreasing levels of the circulating factor sFLT-1 and hence, increasing the VEGF/sFLT-1 ratio, which favors an improved placental angiogenesis (56). However, in our model, nicotine did not affect either VEGF and FLT-1 or VEGF/FLT-1 in either the circulation or the placenta. Hence, these data substantiate previous studies suggesting that nicotine is not the agent that confers PE protection in pregnant women and suggest that it is more likely that tobacco combustion products and not nicotine are the ingredients in cigarette smoke that protect against PE (56, 61).

In conclusion, we have demonstrated that nicotine has direct and local harmful effects on several main processes of placental development, and we propose a mechanism by which this might occur at both the animal and cellular levels. This study also suggests molecular explanations for why maternal smoking has a negative effect on pregnancy outcome.

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DISCLOSURES

No conflict of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.C.H., M.J.S., S.R., J.J.F., and N.A. contributed to the conception and design of the research; A.C.H., A.S., V. G., S.R., F.S., C.J.N., and M.B. performed the experiments; A.C.H. and N.A. interpreted the results of the experiments; A.C.H., M.J.S., S.R., J.J.F., M.B., and N.A. edited and revised the manuscript; A.C.H., M.J.S., and N.A. approved the final version of the manuscript; J.J.F. and N.A. drafted the manuscript; N.A. analyzed data; N.A. prepared the figures.

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