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ORIGINAL ARTICLE

Effects of rosiglitazone on ovarian function and fertility in animals with reduced fertility following fetal and neonatal exposure to nicotine

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Abstract We have previously shown that in utero nicotine exposure causes impaired fertility, follicle immaturity, and ovarian dysfunction in adult female rat offspring. These characteristics overtly resemble the clinical profile of polycystic ovarian syndrome (PCOS) and recent studies have shown that thiazolidinediones such as rosiglitazone improve fertility in women with PCOS but the mechanism is not well defined. Our goal was to examine whether rosiglitazone would (1) ameliorate the altered ovarian physiology that occurs following fetal and neonatal exposure to nicotine and (2) to examine whether this could be due to normalization of ovarian vascularization. At weaning, offspring of nicotine-exposed dams were given either vehicle (NV) or rosiglitazone (3 mg kg^{-1} day^{-1}; NR). Offspring of saline-exposed dams received vehicle (SV). Tissues were collected when the female offspring reached 26 weeks of age. NV animals had reduced granulosa cell proliferation and increased ovarian cell apoptosis. Treatment with rosiglitazone increased proliferation, and decreased apoptosis, compared NV animals. NV animals had decreased ovarian vascularity relative to controls, whereas NR animals had an intermediate level of ovarian vessel density. Moreover, ovaries from NV animals had decreased levels of the pro-angiogenic growth factors vascular endothelial growth factor (VEGF) and endocrine gland-derived VEGF both of which were increased with rosiglitazone treatment. Rosiglitazone reversed some of the nicotine effects in the ovary and increased ovarian vascularization, follicle maturation and improved oocyte competence. Rosiglitazone may be an important treatment option for PCOS and the present study provides a potential mechanism by which rosiglitazone may have beneficial effects on fertility in these patients.

Keywords Nicotine · Fertility · Rosiglitazone · Ovary

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Introduction

Although it has been well documented that cigarette smoking is associated with decreased fertility in adults [1–3] there also is evidence that in utero exposure to cigarette smoke has long-term consequences on the fertility of the offspring. Indeed, sons born to mothers who smoked during pregnancy have lower sperm counts, decreased fecundability, and reduced numbers of morphologically normal sperm cells [4–7] and daughters of mothers who smoked during pregnancy have an earlier menopause, shorter reproductive lifespan, and reduced fecundability [5, 8].

Cigarette smoke is estimated to contain as many as 4,000 chemicals [9, 10] including the addictive component, nicotine. Data from animal studies suggests that nicotine exposure may be a critical component in the development

of adverse reproductive effects in women who smoke [11, 12]. Moreover, we have previously shown that in utero nicotine exposure causes impaired fertility, altered ovarian steroid hormone and protein levels, and increased numbers of atretic follicles in adult female rat offspring [13, 14]. These characteristics overtly resemble the clinical profile of polycystic ovarian syndrome (PCOS), suggesting that fetal and neonatal exposure to nicotine may contribute to ovarian dysfunction in adult life.

The adult ovary is a somewhat unique structure in that it is one of the few organs that undergoes cyclic angiogenic processes. Angiogenesis in the ovary is regulated by a balance between angiogenic stimulators, including vascular endothelial growth factor (VEGF) and endocrine gland-derived VEGF (EG-VEGF) and inhibitors such as thrombospondin (TSP-1) [15–17]. Numerous studies have demonstrated that normal vessel formation is essential for follicular and luteal development, and any alteration in ovarian angiogenesis can have a significant influence on reproduction [18, 19]. Indeed, a number of reproductive disorders, including PCOS, are characterized by altered expression of angiogenic factors [15, 20]. We therefore wished to examine if the reduced fertility in adult rats exposed to nicotine during fetal and neonatal development reported by our group [13] can be attributed to altered ovarian angiogenesis. Furthermore, since recent studies have shown that thiazolidinediones (TZDs), synthetic peroxisome proliferator-activated receptor g (PPARg) agonists, improve pregnancy and ovulation rates in women with PCOS reviewed in [21], we wanted to determine whether the TZD rosiglitazone could affect ovarian angiogenesis. In vitro, we evaluated the mechanisms by which nicotine and rosglitazone could interact to affect markers of angiogenesis and ovarian cell health.

Methods

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 200–250 g female Wistar rats (Harlan, Indianapolis, IN, USA) were maintained under controlled lighting (12:12 L:D) and temperature (22°C) with ad libitum access to food and water. Two weeks prior to mating the dams were randomly assigned to receive either saline (vehicle) or nicotine ($n = 20$ per group). Dams were injected with 1 mg kg^{-1} day^{-1} nicotine bitartrate (Sigma Aldrich, St. Louis, MO, USA) or saline subcutaneously for 14 days prior to mating, and during pregnancy until weaning. The maternal steady state levels of serum cotinine (the major metabolite of nicotine) resulting from this exposure of 135.9 ± 7.86 ng/ml [13] are within the range of cotinine concentrations recently reported by George et al. [22] in pregnant smokers. Pups were weighed after birth (postnatal day 1; PND1) and litter size was culled to eight at birth. After weaning at PND21, female offspring whose mothers had been exposed to nicotine were randomly assigned to receive either vehicle (NV) or rosiglitazone (3 mg kg^{-1} day^{-1} orally, provided by GlaxoSmithKline, Canada; NR), and female offspring whose mothers were exposed to saline were given vehicle (SV) daily.

Tissue collection

When the female offspring reached 26 weeks of age, vaginal swabs were performed daily ($n = 6$ per group) to determine the time of estrous. The morning of estrus, rats were euthanized via CO_2 asphyxiation and ovaries were collected to determine whether rosiglitazone reversed nicotine-induced changes in ovarian structure and function. One ovary was fixed by immersion in 10% neutral buffered formalin (EM Science, Gibbstown, NJ) at 4°C overnight, washed in water and embedded in paraffin for immunohistochemical analysis, and the other ovary was snap frozen in liquid nitrogen and stored at −80°C for analysis of protein expression.

Blood vessel parameters

Immunohistochemical determination of CD31, a marker for vessel endothelial cells, was used for quantification of blood vessel morphology in ovaries collected from female offspring in all 3 treatment groups (SV, NV, and NR). Briefly, tissues were deparaffinized and rehydrated and antigen retrieval was performed by immersion in 10 mM citrate buffer (90°C) for 12 min. After inhibition of endogenous peroxidase activity with 2% (vol/vol) hydrogen peroxide, tissues were blocked in 5% (wt/vol) bovine serum albumin in PBS for 10 min. Tissues were then incubated overnight at 4°C in a humidified chamber with an anti-CD31 antibody (1:500 dilution; Pharmingen). Anti-sera was diluted in 0.01 M PBS (pH 7.5) containing 2% (wt/vol) BSA and 0.01% (wt/vol) sodium azide (100 µl/slide). All subsequent incubations were at room temperature. Biotinylated secondary antibody (1:100 dilution; Sigma) was diluted in the same buffer and incubated for 1 h. The slides were then washed in PBS and incubated with avidin and biotinylated horseradish peroxidase (1:30 dilution) (Extravidin, Sigma Chemical Co.). Peptide immunoreactivity was localized by incubation in fresh diaminobenzidine tetrahydrochloride (DAB tablets, 10 mg, Sigma) with 0.03% (vol/vol) hydrogen peroxide for 2 min. Tissue sections were counterstained with Carazzi's Hematoxylin for 1 min. Tissues were

dehydrated and placed under a coverslip with Permount (Fisher). Sections were imaged using an Olympus BX-61 fluorescent microscope vessel morphology was evaluated using image analysis software (Metamorph, Universal Imaging Corp., CA). Microvessel density (MVD; the total count of microvessels per mm^2 ovarian tissue) and total vascular area (TVA; the total area occupied by microvessels per optical field) was determined on 5 mm sections, separated by an average of 10 mm. For these measurements six sections per group were used for quantification and a minimum of five fields per section were randomly selected for each tissue.

Expression of the VEGF, TSP-1, and EG-VEGF family members

Immunohistochemistry and immunofluorescence

Paraffin embedded sections were used for immunohistochemical localization of the proliferation marker PCNA, VEGF and its receptor VEGFR-2, EG-VEGF, and TSP-1 and its receptor CD36. Tissues were prepared for immunohistochemistry as described above and incubated with anti-VEGF antibody (1:600 dilution; Santa Cruz, CA), anti-PCNA antibody (1:1000 dilution; Sigma), anti-VEGFR-2 (1:600 dilution; Santa Cruz), anti-EG-VEGF (generated by N. Alfaidy; 0.33 μg/ml), anti-TSP-1 (1:500 dilution; Pharmingen, ON), or anti-CD36 (1:400 dilution; Pharmingen), overnight at 4°C. Biotinylated anti-rabbit or anti-mouse IgGs (both 1:100 dilution; Sigma) were diluted in the same buffer and incubated for 1 h. Following antibody incubation, tissues were processed as above and were imaged using an Olympus BX-61 fluorescent microscope and cell counts were performed using image analysis software (Metamorph, Universal Imaging Corp., CA). VEGF, EG-VEGF, VEGFR-2, TSP-1, and CD36 staining was quantified as the percentage of immunopositive ovarian tissue while PCNA staining was quantified as the percentage of immunopositive ovarian cells in six fields of view per section, with a minimum of four animals per group.

Immunoblot analysis

Total protein from ovarian tissue was liberated in lysis buffer (250 mg sodium deoxycholate, 5 mg phenylmethyl sulfonyl fluoride (PMSF), 5 mg aprotinin, 500 μl nonidet P-40, 500 μl 10% (wt/vol) SDS). Protein lysates (10 μg) were separated by SDS PAGE on 12% gels. Proteins were blotted onto PVDF (Millipore, MA, USA) membranes then blocked 1 h at room temperature in 5% (wt/vol) skim milk. Primary antisera (VEGF, 1:500—Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:600, PCNA—Sigma Chemical Co, St. Louis, MO, USA; TSP-1 Santa Cruz, CA; VEGFR-2, 1:500, Santa Cruz; CD36, 1:600, Pharmingen) was applied overnight at 4°C on a rocking platform. Blots were washed in TBST (TBS, 1% Tween 20) and incubated with the appropriate peroxidase conjugated secondary antibody for 1 h at room temperature. Reactive protein was detected with ECL chemiluminescence (Boehringer Manheim, MD, USA) and Konica medical X-ray film (Wayne, NJ, USA). All blots were probed for α-tubulin (1:1000 dilution; Abcam) to ensure equal protein loading. Densitometry was performed and protein values were expressed as a ratio with α-tubulin.

Follicle morphometry

To evaluate follicle morphology, ovarian tissue from animals in all three groups was stained with hematoxylin and eosin. Follicles were classified as pre-antral when there was more than one layer of granulosa cells and an absence of an antral space. Once an antral space was formed, follicles were classified as antral. For data collection, tissue sections were obtained every 30 μm throughout the depth of the tissue. The number of pre-antral and antral follicles was counted for each ovary section, with a minimum of six fields of view per section, four animals per group.

Cell culture

Spontaneously immortalized rat granulosa cells (SIGC, a generous gift from Dr. Robert Burghardt, Texas A&M University, College Station, TX) were maintained in DMEM/F12 supplemented with 10% FBS and 1% antibiotic/antimycotic (Gibco BRL, Burlington, ON). To determine the effect of nicotine exposure, SIGC were first switched to serum-free DMEM/F12 for 6 h and then either left in serum-free media (S), or exposed to nicotine bitartrate (N; 4.5 ng/ml), or nicotine (4.5 ng/ml) and rosiglitazone (NR; 10^{-4} M) for 12 h. Cells were either fixed in 10% neutral buffered formalin for TUNEL detection of apoptosis or were lysed for protein collection and immunoblot analysis.

Identification of nicotine receptor expression on ovarian cells

In order to determine whether nicotine could have a direct effect on ovarian cell function, we first needed to examine whether the nicotine receptors (nicotinic acetylcholine receptors; nAChRs) were expressed on these cells. To this end, SIGC were cultured to approximately 80% confluence and fixed for 1 h with 10% neutral buffered formalin. Cells were permeabilized with 0.1% Triton X-100 (Sigma) for 15 min at room temperature and incubated with primary antibodies for nAChR-2 and nAChR-7 receptors (1:400

dilution) overnight at 4°C. Cells were then incubated with secondary antibodies conjugated with AlexaFluor 488 (α2) and AlexaFluor 594 (α7) for 1 h at room temperature. Cells were visualized with a fluorescence microscope and co-localization was determined with integrated morphometry software (Metamorph, Universal Imaging Corp, CA).

Apoptosis

Detection of apoptosis was performed using a TUNEL assay according to manufacturer's instructions (Roche Applied Science, Laval, Quebec). Briefly, tissue sections or SIGC were permeabilized in 0.1% (vol/vol) Triton X-100 (Sigma), washed in PBS and incubated with the FITC-conjugated TUNEL enzyme for 60 min to detect DNA fragmentation. Nuclei were counterstained with DAPI and imaged with an Olympus BX-61 microscope and integrated morphometry software (Metamorph). For analysis, six fields of view at 250× magnification were quantified in each experiment ($n = 3$). Apoptosis was quantified as the percentage of TUNEL-positive cells.

Statistical analysis

All statistical analyses were performed using SigmaStat (v.2.03, SPSS, Chicago, IL) and one-way analysis of variance (ANOVA) followed by post hoc multiple comparisons when significance was indicated ($\alpha = 0.05$). Data were tested for normality as well as equal variance, and when normality or variance tests failed, data were analyzed using Kruskal–Wallis one-way ANOVA on ranks.

Results

Blood vessel parameters

At 26 weeks of age, nicotine-exposed animals exhibited a reduction in blood vessel area and density compared to saline-treated controls (Fig. 1) and this effect was ameliorated in rosiglitazone treated animals who had a higher vessel area density compared to nicotine-exposed animals (Fig. 1). Rosiglitazone treatment resulted in blood vessel area and blood vessel density that were not statistically different from SV controls (Fig. 1).

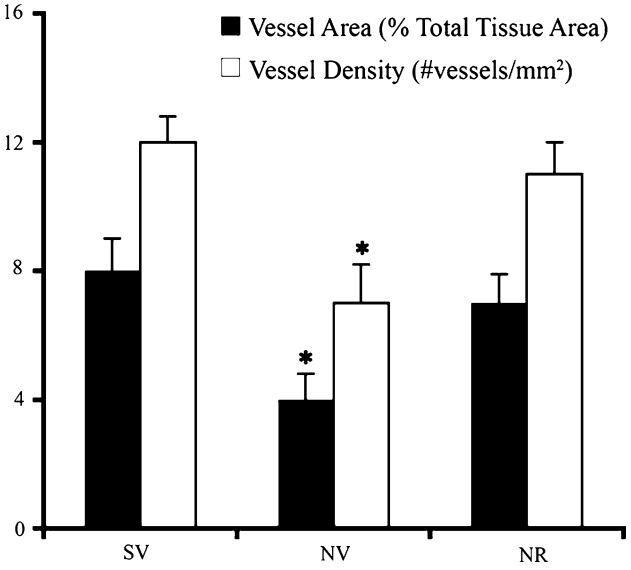

Fig. 1 Blood vessel parameters following in utero exposure to nicotine in the presence or absence of postnatal treatment with rosiglitazone. Rats exposed to nicotine and treated with vehicle (NV) had a decrease in blood vessel area and blood vessel density, compared to offspring from dams treated with saline and vehicle postnatally (SV). Blood vessel area and density in offspring exposed to nicotine in utero and treated postnatally with rosiglitazone (NR) was not different from the SV control group. * Denotes statistical difference between groups ($P < 0.05$). $N = 5$ animals per group

Expression of EG-VEGF and members of the VEGF and TSP-1 families

Immunostaining for VEGF and EG-VEGF demonstrated a significant reduction ($P < 0.05$) in the percent of immunopositive ovarian cells following fetal and neonatal exposure to nicotine (NV) compared to SV controls (Fig. 2). Compared to the vehicle-treated nicotine-exposed offspring (NV), rosiglitazone treatment of nicotine-exposed offspring (NR) significantly ($P < 0.05$) increased ovarian VEGF and EG-VEGF expression. The ovaries from NR females had significantly lower VEGF expression ($P < 0.05$) compared to saline-treated controls while EG-VEGF levels were similar to SV controls (Fig. 2). Expression of the kinase receptor VEGFR-2 mirrored expression of the ligand (Fig. 2c). Ovaries from NV females had a significant decrease in VEGFR-2 expression at 26 weeks of age compared to that seen in the SV females (Fig. 2c). Treatment of nicotine-exposed animals with rosiglitazone (NR) significantly ($P < 0.05$) increased the expression of VEGFR-2 compared to that seen in vehicle-treated nicotine-exposed (NV) animals (Fig. 2c). When tissue lysates were probed for expression of TSP-1, an angiostatic factor and its receptor CD36, we found that fetal and neonatal exposure to nicotine (NV) caused a significant ($P < 0.05$) increase in expression of both ligand and receptor at 26 weeks of age compared to offspring that were born to saline-exposed mothers (Fig. 3). Treatment of nicotine-exposed offspring with rosiglitazone (NR) significantly ($P < 0.05$) decreased expression of TSP-1 and CD36 compared with the NV group and resulted in an expression similar to SV controls (Fig. 3).

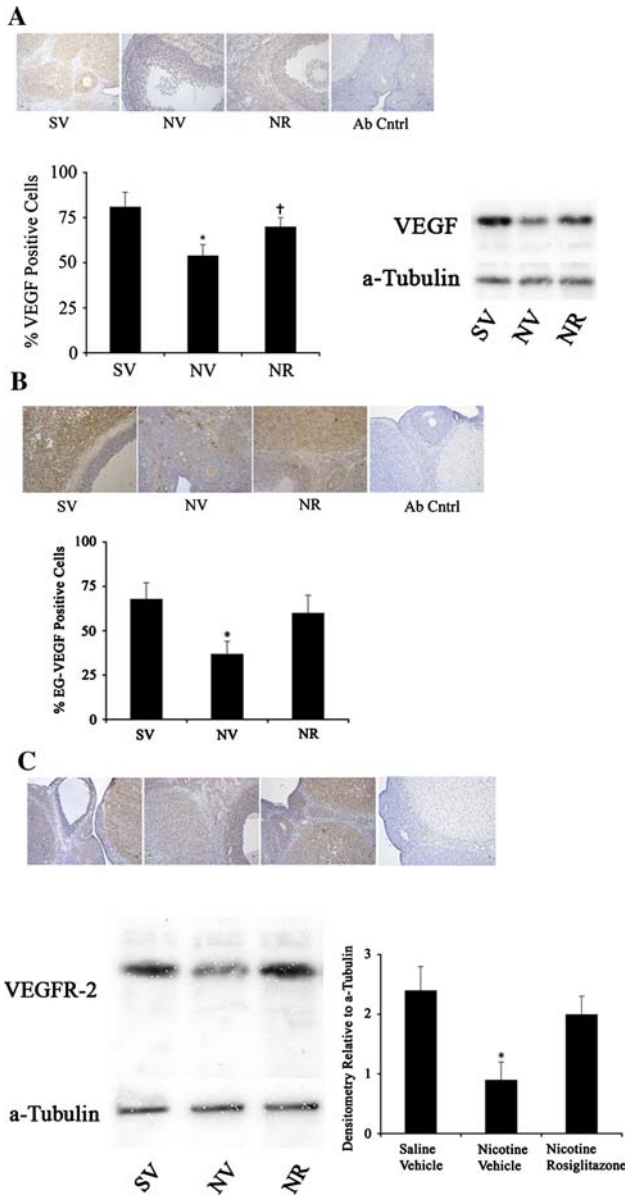

Fig. 2 Effect of nicotine exposure and rosiglitazone treatment on expression of VEGF, EG-VEGF, and VEGFR-2. **a** Ovaries from NV rats had fewer cells immunopositive for VEGF and lower VEGF protein compared to SV controls. Treatment of nicotine-exposed animals with rosiglitazone (NR) increased VEGF expression compared to the NV group, but the VEGF expression was still lower than the SV control group. **b** Nicotine treatment caused a significant reduction in the percentage EG-VEGF immunopositive ovarian cells compared to saline or nicotine/rosiglitazone treated animals. * Values are significantly ($P < 0.05$) different from saline controls (SV). † Values are statistically different ($P < 0.05$) from nicotine-exposed animals randomized to receive vehicle postnatally (NV). $N = 5$ animals per group. **c** VEGFR-2 was localized to granulosa, theca, and endothelial cells in ovaries of SV, NV, and NR groups. **b** Immunoblot analysis of VEGFR-2 protein in whole-ovary homogenates from rats from SV, NV, and NR treatment groups. Nicotine exposure reduced VEGFR-2 levels compared to SV controls and treatment with rosiglitazone returned VEGFR-2 levels to control values. * Denotes a statistical difference between groups ($P < 0.05$). $N = 5$ animals per group

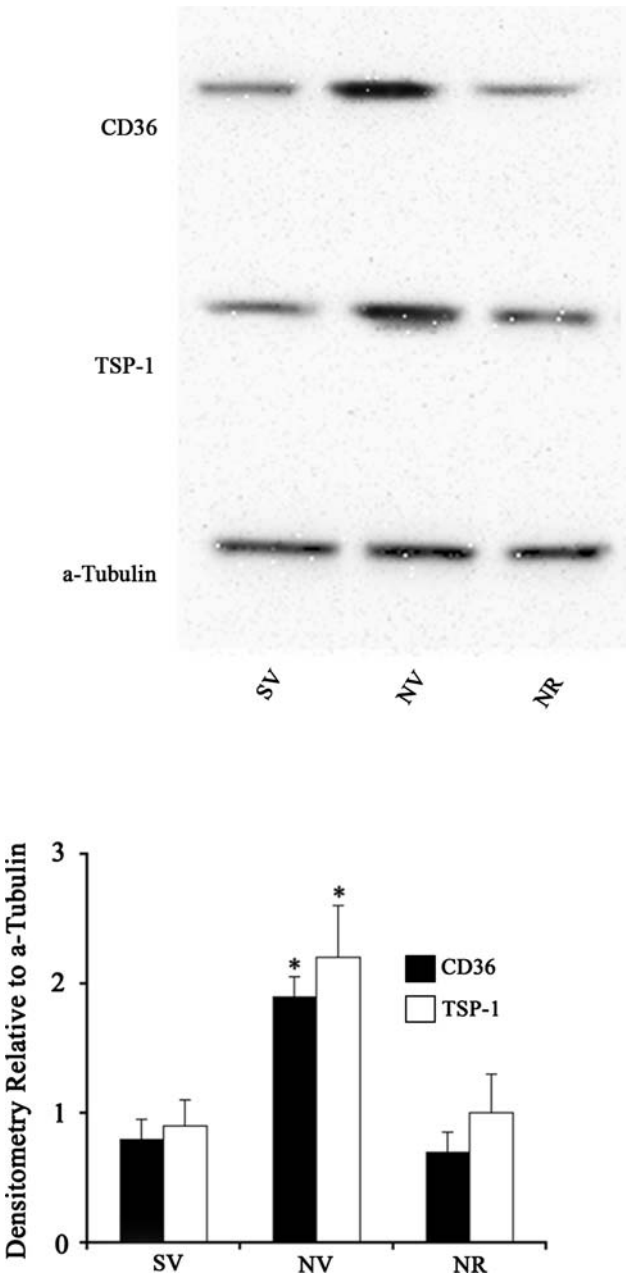

Fig. 3 CD36 and TSP-1 protein in ovaries from SV, NV, and NR animals. Immunoblot analysis was performed on whole-ovary homogenates from animals from each group. The NV group had an increase in expression of TSP-1 and its receptor CD36 compared to other groups. Rosiglitazone treatment (NR) reversed the effects of nicotine and returned the protein levels of CD36 and TSP-1 to control levels. * Values are significantly ($P < 0.05$) different from saline controls (SV). $N = 5$ animals per group

Follicle dynamics

Compared to saline-treated controls, NV animals had a significant ($P < 0.05$) increase in the proportion of immature follicles, with a concomitant reduction in the number

Table 1 Follicle morphology

	Saline		Nicotine		Rosiglitazone	
	Pre-antral	Antral	Pre-antral	Antral	Pre-antral	Antral
26 Week	21 ± 5	11 ± 4	30 ± 6*	8 ± 3*	22 ± 4	11 ± 5

Ovaries were collected and scored using integrated morphometry software and the numbers of pre-antral and antral follicles were counted for each group. Data presented as mean ± SEM

* Denotes statistical difference ($P < 0.05$)

of mature antral follicles (Table 1). Rosiglitazone reversed this effect and resulted in follicle parameters that were similar to saline-treated controls (Table 1).

Expression of nicotine receptors

To determine whether nicotine could have a direct effect on ovarian cells, we probed SIGC for the nAChR subunits, α2 and α7. Both the α2 and α7 subunits were expressed in these cells (Fig. 4a). Furthermore, SIGC treated in vitro with 4.5 ng/ml nicotine bitartrate had a significant ($P < 0.05$) increase in the production of CD36 and TSP-1 protein compared to serum-free controls as measured by immunoblotting. Conversely, nicotine treatment reduced ($P < 0.05$) the levels of VEGF (Fig. 4b). Compared to nicotine-treated cells, SIGC co-treated with nicotine and 10^{-4} M rosiglitazone had a significant ($P < 0.05$) decrease in CD36 and TSP-1 expression and an increase in VEGF levels (Fig. 4b). Although we used a pan-VEGF antibody that detects all VEGF antibodies the SIGC appeared to express VEGF165 specifically, as determined using isoform-specific antibodies (data not shown). In vitro, nicotine

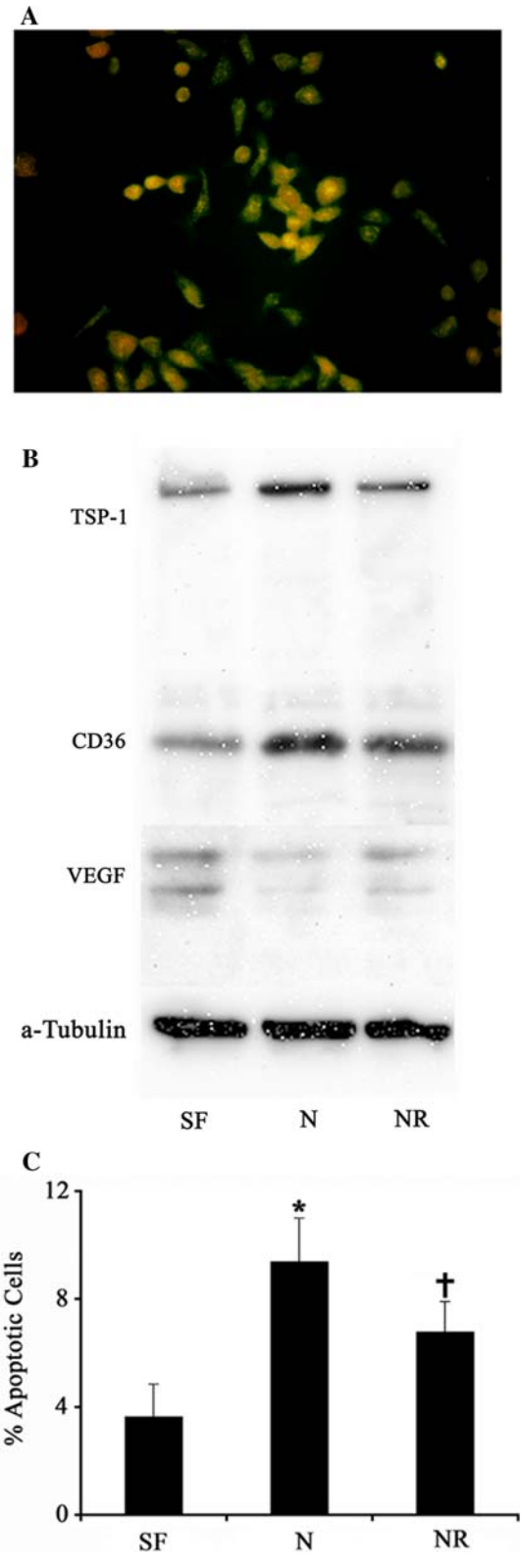

Fig. 4 **a** Expression of nicotine receptors nAChα2 (*green*) and nAChα7 (*red*) in cultured SIGC. Co-localization of both receptors is indicated by *yellow* staining. **b** Immunoblot analysis of TSP-1, CD36, and VEGF protein in immortalized rat granulosa cells (SIGC) in serum-free media (SF) following treatment with nicotine (4.5 ng/ml; N) or nicotine (4.5 ng/ml) and rosiglitazone (10^{-4} M; NR). Nicotine treatment caused an increase in protein levels of both TSP-1 and CD36 and a decrease in VEGF compared to saline-treated controls. Co-treatment with rosiglitaone inhibited the effects of nicotine and caused a reduction in TSP-1 and CD36 and an increase in VEGF expression compared to N group. **c** Nicotine induces apoptosis in immortalized granulosa cells in vitro and rositlitazone reduces this effect. Spontaneously immortalized rat granulosa cells (SIGC) were treated in serum-free media alone or with nicotine (4.5 ng/ml) or nicotine (4.5 ng/ml) and rosiglitazone (10^{-4} M). Nicotine caused an increase in SIGC apoptosis compared to serum-free controls. Rosiglitazone combined with nicotine caused a reduction in SIGC apoptosis, although the percentage of apoptotic cells was higher than serum-free controls. Bars with *different symbols* are statistically different ($P < 0.05$). * Values are significantly ($P < 0.05$) different from saline controls (SV). † Values are statistically different ($P < 0.05$) from nicotine-exposed animals randomized to receive vehicle postnatally (NV). $N = 4$ independent culture experiments

treatment significantly ($P < 0.05$) increased SIGC apoptosis compared to SV controls (Fig. 4c). Co-treatment with rosiglitazone caused a significant ($P < 0.05$) reduction in

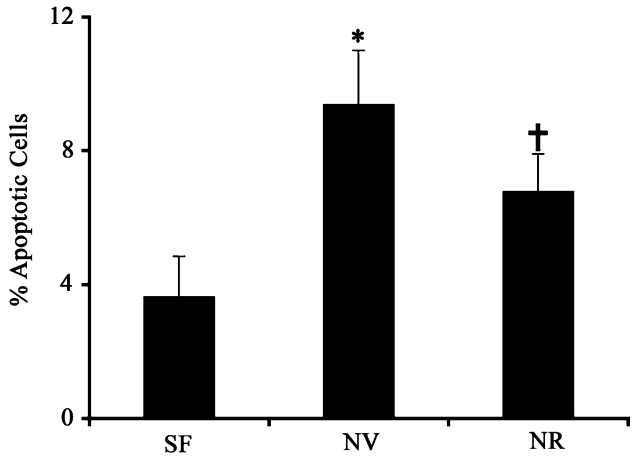

Fig. 5 Ovary cell apoptosis following exposure to nicotine in utero with or without postnatal treatment with rosiglitazone. Exposure to nicotine (NV) increased the percentage of apoptotic cells compared to saline-exposed (SV) controls. Nicotine exposure combined with postnatal rosiglitazone treatment (NR) decreased the pro-apoptotic effect of nicotine. * Values are significantly ($P < 0.05$) different from saline controls (SV). † Values are statistically different ($P < 0.05$) from nicotine-exposed animals randomized to receive vehicle postnatally (NV). $N = 5$ animals per group

apoptosis, compared to SIGC treated with nicotine alone (Fig. 4c).

Apoptosis and cell proliferation

Compared to saline controls, NV animals had reduced ovarian cell apoptosis and NR animals had levels intermediate between saline and NV animals (Fig. 5). To determine the incidence of ovarian cell proliferation, tissue from saline, nicotine, and rosiglitazone treated animals was stained for PCNA, which detects cells in the S-phase of the cycle. At 26 weeks of age, PCNA localized primarily to granulosa cells, with some immunopositive theca, stromal, and endothelial cells (Fig. 6). When the percentage of immunopositive cells was quantified, nicotine-exposed offspring (NV) had significantly decreased ($P < 0.01$) cell proliferation compared to saline-treated controls (SV) (Fig. 6). Rosiglitazone treatment (NR) resulted in an intermediate rate of proliferation compared to SV and NV animals (Fig. 6).

Discussion

There is a plethora of evidence linking smoking to a host of adverse health outcomes including infertility [1–3]. More recently there has been data showing that daughters of women who smoked during pregnancy also have impaired fertility. However, the mechanisms underlying this reproductive dysfunction have not been well studied. In an animal model, we have demonstrated reduced fertility and altered ovarian function following fetal and neonatal exposure to nicotine [13]. In the present study, we have demonstrated that impaired fertility in nicotine-exposed offspring is associated with reduced ovarian vascularization and angiogenic factor expression, as well as altered follicle dynamics and increased apoptosis: abnormalities that were

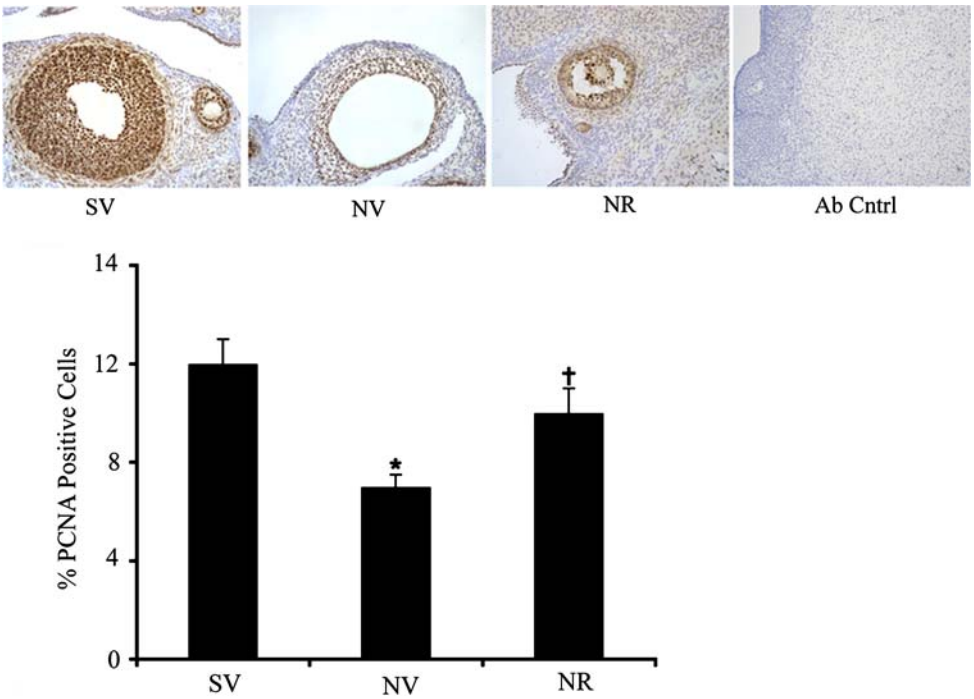

Fig. 6 Cell proliferation in SV, NV, and NR groups. Nicotine-exposed, vehicle-treated (NV) rats had reduced ovarian cell proliferation, as determined by quantification of PCNA immunohistochemistry. NR rats had an increase in ovary cell proliferation compared to NV animals, but less proliferation than the SV group. * Values are significantly ($P < 0.05$) different from saline controls (SV). † Values are statistically different ($P < 0.05$) from nicotine-exposed animals randomized to receive vehicle postnatally (NV). $N = 5$ animals per group

ameliorated by rosiglitazone treatment. Although it is possible that rosiglitazone improves fertility by improving glucose homeostasis and insulin sensitivity, as has been suggested in other studies [23, 24], results from the present study suggest that it also acts directly at the level of the ovary to improve reproductive outcomes and ovarian health. However, we have not determined whether rosiglitazone treatment reversed nicotine-induced damage to the ovary or whether it prevented the decline in ovarian health seen with aging in the nicotine-exposed animals [13]. It is interesting to note, that rosiglitazone treatment also improved measures of fertility following fetal and neonatal exposure to nicotine. Specifically, nicotine-exposed animals (NV) had an increased time to pregnancy and an increased proportion of mothers delivering stillborn offspring relative to saline controls [13]. Both of these parameters were improved in the nicotine-exposed animals given rosiglitazone postnatally (time to pregnancy SV: 2.6 ± 0.51 days: NV: 9.2 ± 1.78 days; NR: 2.2 ± 0.37 days) and decreased proportion of mothers delivering stillborn offspring (% of dams with stillborn pups: SV: 17%; NV: 67%; NR 50%) [25].

Nicotine exposure in utero may also cause impaired ovarian function via altered ovarian health. For example, ovaries of rats exposed to nicotine in utero had a significant increase in the percentage of apoptotic ovarian cells compared to saline-treated controls. Nicotine, through its interaction with specific nAChRs has been shown to induce apoptosis in multiple tissues [26–30]. Apoptotic mechanisms exploited by nicotine include altering the bcl-2:bax ratio and inducing activation of the caspase-3 pathway [31]. We identified expression of nAChR-2 and -7 in fixed ovarian tissue and in isolated granulosa cells. Therefore, one mechanism by which nicotine may have initiated a change in ovarian morphology and function is by directly causing ovarian cell apoptosis via the nAChRs. Through this mechanism, nicotine would affect growing and developing follicles, but would also likely negatively affect the pool of primary follicles, reducing subsequent ovulation events. Concurrent with this receptor-mediated effect, nicotine exposure also increased expression of the extracellular matrix glycoprotein TSP-1 and its receptor CD36. TSP-1 is a potent inhibitor of angiogenesis [32, 33] and we have shown that at least one of its anti-angiogenic mechanisms involves the inhibition of VEGF expression [18]. Both in vitro and in vivo, exposure to nicotine increased expression of TSP-1 and CD36, while rosiglitazone treatment reversed this effect. Conversely, exposure to nicotine in vitro and in vivo caused a significant reduction in the expression of VEGF and its receptor VEGFR-2. In addition to its angiogenic role, VEGF is a potent survival factor for ovarian cells, and loss of VEGF expression is associated with increased apoptosis [16, 17]. The increased expression of TSP-1 caused by nicotine likely contributed to the increased ovarian cell apoptosis and decreased ovarian blood vessel density as TSP-1 is also a direct inhibitor of VEGF [17]. Therefore, in addition to its direct apoptotic effect through interaction with the nAChRs, nicotine also likely contributed to ovarian cell death by inhibiting the expression of survival factors. Of interest, exposure to nicotine in utero caused a significant change in the expression of TSP-1 and VEGF family members, and this change was evident in adulthood past the exposure period. Although the mechanisms for this apparent reprogramming are unclear, these results mirror those we have seen in our mouse model of epithelial ovarian cancer where epithelial-stromal cell interaction causes a significant and sustained change in expression of VEGF and TSP-1 [18].

Both in vitro and in vivo, rosiglitazone was able to partially reverse the effects of nicotine on granulosa cell protein expression, ovarian morphology, and ovarian angiogenesis. Recent data has suggested that rosiglitazone may enhance fertility in patients with PCOS. Although we evaluated fertility in offspring treated in utero, it has been shown that nicotine exposure has a direct effect on the ovarian morphology of the treated female [12]. Nicotine exposure caused a significant decrease in ovarian weight with a reduction in the number and size of graffian follicles, corpora lutei, and increase in atretic follicles, as well as an increase in total cholesterol content of the ovary. These morphological changes mimic some of those seen in women with PCOS. Although the mechanisms by which rosiglitazone improves fertility in women with PCOS are not fully understood, it is thought to reduce androgen production [24, 34], decrease oxidative stress, and increase insulin sensitivity [35]. The more recently discovered EG-VEGF has also been linked to PCOS, and is thought to synergize with VEGF in stimulating new ovarian vessel growth in this disease [15]. The reduced EG-VEGF expression caused by nicotine exposure may be an important mechanism by which nicotine decreases blood vessel density. Interestingly, EG-VEGF has been shown to be an important regulator of placentation [36] which may partially explain why we see an increased number of stillborn pups in the nicotine-treated group. By inhibiting the expression of TSP-1 and thus increasing bioavailable VEGF, rosiglitazone may also improve ovarian function by decreasing ovarian cell apoptosis and increasing ovarian vessel density. Rosiglitazone has been shown to be a survival factor in neurons by increasing the expression of the bcl-2 protein and protecting against apoptotic stress [37]. VEGF promotes the expression of bcl-2 and direct intraovarian injection of VEGF causes increased bcl-2 expression, increased ovarian angiogenesis, reduced apoptosis, and improved fertility [38]. Rosiglitazone has been shown to have other pro-angiogenic effects, such as stimulating an increase in the number and migratory activity of endothelial cells [39], and stimulating

the differentiation of angiogenic progenitor cells to an endothelial lineage [40]. Rosiglitazone therefore appears to affect ovarian physiology and improve fertility in a number of ways. By increasing expression of pro-angiogenic and cytoprotective factors, rosiglitazone improved ovarian vascularity, decreased granulosa cell apoptosis and returned the ovary to a more functional morphology, combating the deleterious effect of nicotine exposure. Also, through its actions on various cytokines and growth factors, rosiglitazone may be able to combat some of the clinical symptoms of ovarian dysfunction such as PCOS.

The results from the present study suggest that the impaired fertility in animals exposed to nicotine in utero may be due to altered ovarian angiogenesis in adult life. This re-programming of adult ovarian vasculature as a result of an in utero challenge has not been described before. We have also shown for the first time that rosiglitazone has important pro-angiogenic functions in the ovary and it may be through this mechanism that it can restore fertility in offspring exposed to nicotine in utero.

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