Defective Uterine Spiral Artery Remodeling and Placental Senescence in a Pregnant Rat Model of Polycystic Ovary Syndrome

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Pregnancy-related problems have been linked to impairments in maternal uterine spiral artery (SpA) remodeling. The mechanisms underlying this association are still unclear. It is also unclear whether hyperandrogenism and insulin resistance, the two common manifestations of polycystic ovary syndrome, affect uterine SpA remodeling. We verified previous work in which exposure to 5-dihydrotestosterone (DHT) and insulin (INS) in rats during pregnancy resulted in hyperandrogenism, insulin intolerance, and higher fetal mortality. Exposure to DHT and INS dysregulated the expression of angiogenesis-related genes in the uterus and placenta and also decreased expression of endothelial nitric oxide synthase and matrix metalloproteinases 2 and 9, increased angiogenesis-related genes in the uterus and placenta and also decreased expression of endothelial nitric oxide synthase and matrix metalloproteinases 2 and 9, increased b-fibrotic collagen deposits in the uterus, and reduced expression of marker genes for SpA-associated trophoblast giant cells. These changes were related to a greater proportion of unremodeled uterine SpAs and a smaller proportion of highly remodeled arteries in DHT þ INS–exposed rats. Placentas from DHT þ INS–exposed rats exhibited decreased basal and labyrinth zone regions, reduced maternal blood spaces, diminished labyrinth vascularity, and an imbalance in the abundance of vascular and smooth muscle proteins. Furthermore, placenta exposed to DHT þ INS–exposed rats showed expression of placental insufficiency markers and a significant increase in cell senescence–associated protein levels. Altogether, this work demonstrates that increased pregnancy complications in polycystic ovary syndrome may be mediated by problems with uterine SpA remodeling, placental functionality, and placental senescence. (Am J Pathol 2023, 193: 1916–1935; https://doi.org/10.1016/j.ajpath.2023.08.008)

Polycystic ovary syndrome (PCOS) is a syndrome of reproductive, metabolic, and psychological aberrations and affects up to 18% of adult women worldwide.1 Although no single etiological factor can fully account for the spectrum of abnormalities in PCOS, hyperandrogenism androgen excess and insulin (INS) resistance have emerged as key clinical hallmarks that are attributed to the development and progression of PCOS.2 Women with PCOS are at high risk...
for several pregnancy-related complications and adverse obstetric outcomes, such as miscarriage and pre-eclampsia. There is currently no effective prevention or cure for patients with PCOS with pregnancy complications. Despite findings showing uterine and placental dysfunction, the precise mechanisms leading to PCOS-induced pregnancy-related complications are not fully understood.

Maternal uterine spiral artery (SpA) remodeling is a prerequisite for successful pregnancy, and impaired or excessive SpA remodeling often causes pregnancy-related complications in humans. In healthy pregnancies, during early to middle gestation, remodeled SpAs demonstrate endothelial cell (EC) re-endothelialization and vascular smooth muscle cell (VSMC) dedifferentiation, which can be monitored using VSMC contractile markers, such as α-smooth muscle actin (α-SMA), smooth muscle protein 22α (SM22α), and calponin 1. Through the production of angiogenic and vasodilatory factors and matrix metalloproteinase (MMP) enzymes, maternal decidual immune cells and invasive interstitial and endovascular extravillous trophoblasts contribute to the SpA remodeling process. Several lines of evidence also indicate that at the time of embryo implantation, estrogen and progesterone are involved in the regulation of angiogenesis-mediated endometrial vascular development, and abnormal endometrial angiogenesis is associated with recurrent pregnancy loss. Systemic microvascular endothelial function is impaired in patients with PCOS with hyperandrogenism and in 5α-dihydrotestosterone (DHT)−induced PCOS-like rats. Furthermore, impaired endothelial function is associated with PCOS-related infertility, demonstrating a crucial link between maternal vascular abnormalities and PCOS-induced reproductive failure. Midgestational exposure to DHT and INS in rats mimics typical PCOS features, including hyperandrogenism, INS intolerance, and increased fetal loss. A close association has been reported between uteroplacental developmental defects and the aberrant expression of angiogenesis- and vascular function−related genes in PCOS-like rodents. Evidence from clinical studies indicates that women with PCOS display a reduced depth of endovascular trophoblast invasion and abnormal blood flow in uterine arteries. These findings led us to postulate that in PCOS hyperandrogenism and INS resistance-induced pregnancy, loss is due in part to impaired maternal SpA remodeling through dysregulation of EC re-endothelialization and VSMC dedifferentiation.

Cellular senescence is a permanent growth arrest state that can be triggered by numerous environmental and cellular stresses, and when appropriately regulated, this permits normal uterine decidualization and placental development. For example, induction of cellular senescence causes defective endometrial decidualization, which is linked to implantation failure and recurrent pregnancy loss, whereas appropriate activation of cellular senescence contributes to the controlled invasion of extravillous trophoblasts into the endometrium during pregnancy. Recent studies have demonstrated that the activation of senescence in ovarian granulosa cells is involved in the pathophysiology of PCOS. However, whether the hyperandrogenism and INS resistance seen in PCOS regulate uterine and placental cellular senescence has not been determined.

Using a pregnant PCOS-like rat model, the present study was performed to investigate whether the remodeling of maternal SpA and molecular markers for cellular senescence in the gravid uterus and placenta was altered by hyperandrogenism and INS resistance during pregnancy. The cellular and molecular data from this study provide insights into the impacts of hyperandrogenism and INS resistance on maternal uterine SpA remodeling and placental senescence during pregnancy.

Materials and Methods

Animal Care and Ethics Statement

Sprague-Dawley rats of both sexes were 70 days of age and obtained from the Laboratory Animal Centre of Harbin Medical University (Harbin, China). Animals were housed under standard environmental conditions [a 12-hour light/dark cycle (light on at 8 AM) at 22°C ± 2°C and 55% to 65% humidity] with water and normal diet available ad libitum. Female rats on the night of proestrus were mated with fertile males of the same strain at a 2:1 female/male ratio. The presence of a vaginal plug was considered as pregnancy achievement, and this was designated as gestational day (GD) 0.5. All experiments complied with the Animal Research Reporting of In Vivo Experiments (ARRIVE) 2.0 guidelines for reporting animal research.
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(table continues)
pregnant rats, as described previously. Animals were sacrificed at GD 14.5, which is when maternal SpA remodeling is complete and uteroplacental blood flow is increased to support the oxygen and nutrient requirements for the growing fetus in pregnant rats. Trunk blood was collected directly from the heart under anesthesia and maintained at room temperature for 1 hour before the isolation of serum. The maternal uterus and placenta, as well as fetuses, were collected and were either fixed for immunolabeling-enabled three-dimensional imaging of solvent-cleared organs (iDISCO), transmission electron microscopy, and immunohistochemical and immunofluorescence analyses or immediately frozen in liquid nitrogen and stored at −70°C for quantitative real-time PCR (qPCR) and Western blot analyses.

qPCR Analysis

The procedure of cellular RNA extraction and qPCR was performed with a Roche Light Cycler 480 sequence detection system (Roche Diagnostics Ltd, Rotkreuz, Switzerland), as previously described. The primers are summarized in Table 1. All reactions were performed in duplicate, and each reaction included a nontemplate control. The results for target genes were expressed as the amount relative to the average cycle threshold (CT) values of β-actin (Actb) and Gapdh expression in each sample. The expression of Actb and Gapdh was not significantly different from the study groups. Relative gene expression was determined with the 2−ΔΔCT method, and the efficiency of each reaction, as determined by linear regression, was incorporated into the equation.

Western Blot Analysis

Extracts of tissue protein were prepared, and Western blot analysis and polyvinylidene difluoride membrane stripping protocols were followed as reported previously. The primary and secondary antibodies are listed in Table 2. The signal was detected using the SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific, Waltham, MA) and captured using a ChemiDoc MP Imagine System (Bio-Rad, Hercules, CA). UV activation of the Criterion stain-free gel on a ChemiDoc MP Imaging System (Bio-Rad) was used to control for slight variations in protein loading. Band densitometry and quantification were performed using Image Laboratory, version 5.0 (Bio-Rad), and the protein band densities were normalized to the total protein loading control in each sample.

Histologic evaluation of the gravid uterine and placental sections was performed by hematoxylin and eosin (ab245880; Abcam, Cambridge, UK) and Masson trichrome (HT15; Sigma-Aldrich) staining, according to the manufacturers’ instructions. Immunohistochemistry and immunofluorescence were used to evaluate protein localization and intensity, according to previously described methods. The primary and secondary antibodies are listed in Table 2. For immunohistochemistry, sections were imaged on a Nikon E-100 microscope (BergmanLabora AB, Dan- deryd, Sweden) under bright-field optics and photomicrographed using Easy Image 1 (Bergström Instrument AB, Solna, Sweden). For immunofluorescence, sections were examined under an Axiovert 200 confocal fluorescence microscope (Zeiss, Jena, Germany) equipped with a laser-scanning confocal imaging LSM 710 META system (Carl Zeiss, Oberkochen, Germany). The specificity of cellular SM22α and calponin 1 in the non-pregnant rat uterus was demonstrated by dual-immunofluorescence staining (Supplemental Figure S1). The classification of the different stages of uterine SpA remodeling was performed on the basis of immunohistochemistry and immunofluorescence staining with CD31 and α-SMA (Supplemental Figure S2), according to previous reports. The uterus-placenta interfaces from seven different animals per group were randomly selected, stained, and scored. The average number of scored vessels per each uterus-placenta interface was 30. For each sample, the number of blood vessels in each stage was divided by the total number of three or four stages of blood vessels and then calculated as a percentage.
iDISCO Processing and Image Analysis

The sample processing, iDISCO, microscopy, and image analysis were conducted according to the protocol of the previous report with modifications. Tissues were fixed by immersion in 4% paraformaldehyde for 48 hours at 4°C and then at 1 hour at room temperature. They were then treated with a graded series of methanol (20%, 40%, 60%, 80%, and 100% in distilled water; Sigma-Aldrich) for 1 hour each, followed by incubation with a bleaching solution [66% dichloromethane (Sigma-Aldrich)/33% methanol] at room temperature. The samples were washed twice in 100% methanol at room temperature and then chilled in fresh 5% H2O2 in methanol (1 volume of 30% H2O2 to 5 volumes of 100% methanol) overnight at 4°C. The samples were then divided into methanol-containing treatment [100% methanol for 1 hour twice, followed by 80%, 60%, 40%, and 20% methanol for 1 hour, phosphate-buffered saline (PBS; Gibco) for 1 hour twice, and 0.2% Triton X-100 in PBS (PBST) for 1 hour twice] and non–methanol-containing treatment [PBS for 1 hour twice, PBST for 1 hour twice, PBST and 20% dimethyl sulfoxide (DMSO) at 37°C overnight, and then in PBS, 0.1% Tween-20, 0.1% Triton X-100, 0.1% deoxycholate, 0.1% Nonidet P-40, and 20% DMSO for 48 hours at 37°C]. After incubating with PBST, 20% DMSO, and 0.3 mol/L glycine at 37°C overnight, the samples were blocked in PBST, 10% DMSO, and 6% donkey serum at 37°C overnight. The samples were then incubated with primary antibodies (Table 2) dissolved in PBST with 10 μg/mL heparin and 3% donkey serum for 14 days at 37°C, followed by washing with PBST with 10 μg/mL heparin for 5 days at room temperature. Samples were then incubated with secondary antibodies (Table 2) dissolved in PBST with 10 μg/mL heparin for 14 days at 37°C, and then washed with PBST with 10 μg/mL heparin for 5 days at room temperature before clearing and imaging. Next, the samples were treated with a graded series of methanol for 1 hour each, followed by incubation with a bleaching solution at 4°C overnight. Finally, the samples were incubated with 66% dibenzyl ether (Sigma-Aldrich) for 3 hours and then in 100% dibenzyl ether until clear and further stored in 100% dibenzyl ether at room temperature. A light-sheet microscope (LS-18; Nuohai Life Science Co, Ltd, Shanghai, China) equipped with a Hamamatsu camera (Hamamatsu Photonics Co, Ltd, Hamamatsu, Japan) was used to image the intact transparent samples, and IMARIS software, version 9.8 (Oxford Instruments PLC, Abingdon, UK) was used to perform the three-dimensional analysis of the obtained images.

Table 2 The Antibodies Used in This Study

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CD31, cluster of differentiation 31 (platelet endothelial cell adhesion molecule 1); CST, Cell Signaling Technology; iDISCO, immunolabeling-enabled three-dimensional imaging of solvent-cleared organs; IF, immunofluorescence; IHC, immunohistochemistry; p-Histone H2AX, phosphorylated histone H2AX; p-p53, phosphorylated p53; RRID, research resource identifier; SM22α, smooth muscle protein 22α; α-SMA, α-smooth muscle actin; WB, Western blot analysis.
Transmission Electron Microscopy

Preparation of the uterine and placental tissues for ultrastructure analysis was performed as described previously. Sections were examined under a transmission electron microscope (H-7650; Hitachi, Tokyo, Japan) equipped with an electron imaging spectrometer.

Statistical Analysis

Data were analyzed using the SPSS statistical software for Windows, version 24.0 (SPSS Inc., Chicago, IL). The Shapiro-Wilk test was used to check whether the data were normally distributed, and for those passing the test, the differences between two groups were analyzed by $t$-test. Data are presented as means ± SEM, and $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ were considered statistically significant. No data, including statistical outliers, were removed from the analyses.

Results

Pregnant Rats Midgestationally Exposed to DHT and INS Display Endocrine and Pregnancy Defects

As shown in Figure 1A, the levels of circulating testosterone, androstenedione, DHT, and dehydroepiandrosterone were higher in DHT + INS–exposed rats than in control rats. However, no significant changes in circulating sex hormone–binding globulin or anti–Müllerian hormone levels were observed in DHT + INS–exposed rats compared with those in control rats. As expected, DHT + INS–exposed rats exhibited reduced glucose tolerance and increased circulating INS levels and homeostatic model assessment for INS resistance (Figure 1B). Furthermore, co-exposure to DHT and INS decreased both total and viable fetus numbers (Figure 1C). These results are consistent with the observation that midgestational exposure to DHT and INS induces hyperandrogenism and INS resistance and increases fetus loss in rats.25,26

Hyperandrogenism and INS Resistance Dysregulate Angiogenic, Vasoactive, and Elastolytic Gene Expression in the Gravid Uterus and Placenta

To assess whether hyperandrogenism and INS resistance affect angiogenesis in the gravid uterus and placenta, the expression of a panel of angiogenesis-related genes was measured by qPCR. As shown in Figure 2A, the expression of vascular endothelial growth factor A (Vegfa), fms-related receptor tyrosine kinase 1 (Flt1), and kinase insert domain receptor (Kdr) was increased, whereas the expression of angiopoietin-1 (Angpt1) and placental growth factor (Pgf) was decreased, in the gravid uterus of DHT + INS–exposed rats compared with that in controls. The expression of Vegfa, Flt1, Angpt1, angiopoietin-2 (Angpt2), TEK receptor tyrosine kinase receptor (Tek), and Pgf was also lower in the placenta of DHT + INS–exposed rats versus that in controls (Figure 2B). There was no significant change in the expression of uterine Angpt2, Tek, or endoglin (Eng) or placental Eng between control and DHT + INS–exposed rats. Furthermore, increased ratios of Flt1/Pgf and Eng/Pgf were observed in the uterus, but not the placenta, of DHT + INS–exposed rats compared with that of control rats (Figure 1, A and B). The present data are largely consistent with previous studies.25,26 Endothelial nitric oxide (NO) synthase (NO synthase 3 (NOS3)) is a key enzyme responsible for the generation of the vasodilator NO in the vascular endothelium, and the gelatinases MMP2, MMP3, and MMP9 are potent elastolytic enzymes that degrade collagens to aid in tissue remodeling. As informed by qPCR analysis, the expression of Nos3, Mmp2, and Mmp9 was decreased in the gravid uterus of DHT + INS–exposed rats compared with that in the control pregnant rats (Figure 2C). There was also a reduction in the expression of Mmp3 in the gravid uterus of DHT + INS rats, but it was not significantly different to that observed in control rats. These results suggest that hyperandrogenism and INS resistance impair uterine tissue remodeling, angiogenesis, and endothelial function during pregnancy.

Hyperandrogenism and INS Resistance Suppress the Expression of Specific Uteroplacental Cell Marker Genes Implicated in SpA Remodeling

The next set of experiments was aimed at understanding the changes underlying altered uterine tissue biology as a result of hyperandrogenism and INS resistance during pregnancy. Towards this end, qPCR analysis was performed for genes that are highly expressed by the trophoblast giant cells that invade the decidua and contribute to SpA remodeling in pregnant rodents (SpA-TGC). The expression of prolactin family 7, subfamily b, member 1
(Prl7b1), regulator of G-protein–signaling 5 (Rgs5), cathepsin 8 (Cts8), atypical chemokine receptor 2 (Ackr2), protein C receptor (Procr), and p21 (RAC1) activated kinase 1 (Pak1), was decreased in the gravid uterus of DHT + INS–exposed rats compared with that in the controls (Figure 2D). In addition, significant decreases in the expression of genes such as platelet-derived growth factor subunit B (Pdgfb; a marker gene for fetal endothelial cells (FECs)), plexin D1 (Plxnd1; a common marker gene for SpA-TGCs and FECs), and hydroxysteroid 17-β dehydrogenase 2 (Hsd17b2; a marker gene for progenitor trophoblasts), were observed in the gravid uterus of DHT + INS–exposed rats compared with that in controls (Figure 2D). There was, however, no significant difference in the expression of the gene encoding CD34 (Cd34; a marker gene of FECs) in response to DHT + INS exposure. The expression of Rgs5, Cts8, Ackr2, Procr, Pak1, Pdgfb, Cd34, and Plxnd1, but not Prl7b1 and Hsd17b2, was also lower in the placenta of DHT + INS–exposed rats versus that in controls (Figure 2E). These results suggest that hyperandrogenism and INS resistance reduced SpA-TGCs and FECs in the gravid uterus and placenta.

Figure 1  Pregnant rats midgestationally exposed to 5-dihydrotestosterone (DHT) and insulin (INS) display endocrine and pregnancy outcome defects. A and B: Maternal serum levels of total testosterone (T), androstenedione (A4), DHT, dehydroepiandrosterone (DHEA), sex hormone–binding globulin (SHBG), and anti–Müllerian hormone (AMH); body weight and blood glucose concentration during oral glucose tolerance test (OGTT); area under the curve (AUC) for glucose, INS levels, and homeostatic model assessment for INS resistance (HOMA-IR) in control and DHT + INS–exposed rats. AUC was calculated by the following formula: \[0.5 \times (BG_0 + BG_{30})/2 + 0.5 \times (BG_{30} + BG_{60})/2 + 0.5 \times (BG_{60} + BG_{90})/2 + 0.5 \times (BG_{90} + BG_{120})/2\], where the BG terms are the blood glucose levels at 0, 30, 60, 90, and 120 minutes. HOMA-IR was calculated using the following equation: HOMA-IR = fasting blood glucose (mmol/L) × fasting serum INS (μU/mL)/22.5. C: Pregnancy outcomes. All data were obtained from animals at gestational day (GD) 14.5, aside from the OGTT that was conducted on GD 13.5. In all plots, data are analyzed by statistical tests described in Materials and Methods and are expressed as individual values with means ± SEM (A–C). n = 10 per group (A and B). *P < 0.05, **P < 0.01.
Hyperandrogenism and INS Resistance Impact Vascular EC and VSMC Differentiation in the Gravid Uterus

To assess the impacts of hyperandrogenism and INS resistance on the vasculature of the gravid uterus, various histologic and structural parameters were measured. In the control gravid uterus on GD 14.5, most, if not all, vasculature was completely formed in the mesometrial triangle (Figure 3A) and in the proximal (Figure 3A) and distal (Figure 3A) deciduae (as indicated by morphologic features, including elongated and longitudinally aligned ECs with flattened nuclei in the vessel wall). However, in DHT + INS–exposed rats, although vasculature formation was complete in the mesometrial triangle (Figure 3A) and proximal (Figure 3A) and distal (Figure 3A) decidua, it was incomplete in the proximal and distal (Figure 3A) deciduae.

Figure 2 Exposure to 5-dihydrotestosterone (DHT) and insulin (INS) in midgestation results in dysregulation of circulating pro-angiogenetic and anti-angiogenetic factors and uteroplacental cell, vasoactive, and elastolysis-associated gene expression. Quantification of the expression of angiogenic, vasoactive, and elastolytic gene expression in the rat gravid uterus (A and C) and placenta (B), and spiral artery trophoblast giant cell, fetal endothelial cell, and progenitor trophoblast marker gene expression in the gravid uterus (D) and placenta (E) at gestational day 14.5, as measured by quantitative real-time PCR. Expression was normalized to the average Ct values of the Actb and Gapdh genes as the endogenous controls, and the fold change in expression was evaluated using the $2^{-\Delta\Delta Ct}$ method. In all plots, data are analyzed by statistical tests described in Materials and Methods and are expressed as individual values with means ± SEM (A–E). n = 7 per group, each from different animals (A–E). *P < 0.05, **P < 0.01, and ***P < 0.001.
SpA remodeling during early pregnancy. To understand VSMC phenotype contributes to the completion of uterine SpA remodeling in DHT + INS−exposed rats compared with control pregnant rats.

Using transmission electron microscopy, degenerated ECs and endovascular trophoblasts in the vascular wall were observed in controls (Figure 3B), but not in DHT + INS−exposed rats (Figure 3B). Despite starting with fewer vessels in random tissue samples, collagen deposition was found in DHT + INS−exposed rats (Figure 3B), but not in control rats. This was accompanied by increased fibrillar collagen deposits in the vessel wall of the mesometrial triangle and decidua in DHT + INS−exposed rats (Supplemental Figure S3). Immunohistochemical staining with CD31 demonstrated that in control rats, where CD31-positive ECs were fully present in the vascular wall in the mesometrial triangle (Figure 3C), partial loss of CD31-positive ECs was observed in the vascular walls in the proximal and distal decidua (Figure 3C). However, in the mesometrial triangle and proximal and distal decidua of DHT + INS−exposed rats, CD31-positive ECs completely lined the vascular wall (Figure 3C). Quantitative image analysis showed that the number of unremodeled uterine SpAs (ie, complete CD31-positive vascular structures) was increased, whereas the number of fully remodeled uterine SpAs (ie, little to no CD31-positive vascular structures) was decreased in DHT + INS−exposed rats compared with controls (Figure 3D). Gross examination revealed decreased litter size in rats co-exposed to DHT and INS compared with controls (Figure 4A), indicating fetal loss (Figure 1C). Because the loss of VSMCs is a critical step for the completion of maternal uterine SpA remodeling, whole-tissue imaging using iDISCO combined with dual-immunofluorescence staining for CD31 and α-SMA was performed to get an overview of the distribution of uterine blood vessels at the organ level (Figure 4B and Supplemental Video S1). Qualitative analysis revealed that there was prominent staining of blood vessel walls for CD31 and α-SMA in the proximal and distal decidua in DHT + INS−exposed rats compared with controls (Figure 4B). Furthermore, a gross anatomic distribution of the large volume of CD31-positive vasculature was seen in DHT + INS−exposed rats (Supplemental Video S1). These results are consistent with the hypothesis that hyperandrogenism and INS resistance induce maternal uterine vascular EC abnormalities and reduce SpA remodeling.

Hyperandrogenism and INS Resistance Impact the Expression of VSMC Differentiation Mediators in the Gravid Uterus

In humans, VSMC dedifferentiation (meaning the loss of the VSMC phenotype) contributes to the completion of uterine SpA remodeling during early pregnancy. To understand the effects of hyperandrogenism and INS resistance on VSMC dedifferentiation, the Western blot analyses and immunohistochemical and immunofluorescence analyses were performed to determine the localization and regulation of differentiation markers (α-SMA, SM22α, and calponin 1) for VSMCs in the uterus of pregnant rats exposed to DHT and INS. As shown in Figure 5A, although there was no difference in uterine α-SMA or SM22α protein abundance between control and DHT + INS−exposed rats, uterine calponin 1 protein abundance was increased in DHT + INS−exposed rats compared with that in controls. Dual-immunofluorescence analysis using confocal microscopy showed that SM22α immunoreactivity was present in ECs and VSMCs, whereas calponin 1 immunoreactivity was only seen in VSMCs (Figure 5B). Immunohistochemical (Figure 5C) and immunofluorescence analyses (Supplemental Figure S4) showed strong calponin 1 immunoreactivity in the mesometrial triangle and the proximal and distal decidua in DHT + INS−exposed rats compared with control rats. Specifically, calponin 1 immunoreactivity was higher in the uterine blood vessels of pregnant rats exposed to DHT and INS when compared with the uterine vessels from control rats. There are different forms of SpA remodeling in the maternal decidua in humans and rats during early pregnancy (Supplemental Figure S2). Although SM22α immunoreactivity was detected in all forms of remodeling, including unremodeled, slightly remodeled, moderately remodeled, and highly remodeled uterine SpAs, highly remodeled uterine SpAs were observed in both groups (Figure 5D). Quantitative image analysis showed that the number of unremodeled uterine SpAs (ie, calponin 1/α-SMA/SM22α-positive vascular structures) was increased, whereas the number of highly remodeled uterine SpAs (ie, calponin 1/α-SMA-negative and SM22α-positive vascular structures) was decreased in DHT + INS−exposed rats compared with controls (Figure 5E). These results indicate a positive correlation between the promotion of VSMC differentiation and compromised uterine SpA remodeling under the conditions of hyperandrogenism and INS resistance.

Hyperandrogenism and INS Resistance Alter Placental Structure and the Expression of Vascular Molecular Indexes

Whether placental structure and molecular indexes of the vasculature might also be influenced by hyperandrogenism and INS resistance was further evaluated. Histologic analysis of placental sections at GD 14.5 showed that both the basal zone and labyrinth zone were present in control and DHT + INS−exposed rats (Figure 6A). Despite their overall normal appearance, the areas of the basal zone, labyrinth zone, and total placenta were decreased in DHT + INS−exposed rats compared with controls (Figure 6A), which is similar to pregnant women with
Figure 3  Exposure to 5-dihydrotestosterone (DHT) and insulin (INS) in midgestation induces maternal uterine spiral artery (SpA) remodeling and vascular endothelial cell (EC) defects. A: Representative bright-field photomicrographs of sections that were hematoxylin and eosin stained to show uterine blood vessels in control and DHT + INS–exposed rats at gestational day (GD) 14.5. The images shown are representative of seven gravid uterine samples from each group that were examined. Arrows specify endothelialized perivillous/extravillous trophoblasts, arrowheads specify vascular smooth muscle cells (VSMCs), dotted lines indicate the swollen ECs, and asterisks indicate the presence of a thick band of acid-staining amorphous fibrinoid material around the uterine SpA lumen (L). B: Representative transmission electron microscopy photomicrographs showing uterine blood vessels in control and DHT + INS–exposed rats at GD 14.5. The images shown are representative of three gravid uterine samples from each group that were examined. White arrowheads indicate ECs, red arrowheads indicate degenerated ECs, red stars indicate the subendothelial space, yellow double arrows indicate the internal elastic membrane, and white arrows indicate VSMCs. Dotted blue and red lines depict the thickness of elastic lamellae. C: Representative photomicrographs of immunohistochemical staining with anti-CD31 in control and DHT + INS–exposed rat uteri at GD 14.5. The images shown are representative of seven gravid uterine samples from each group that were examined. Tissue sections were counterstained with methyl green. Arrows indicate the swollen ECs, and dotted lines indicate CD31-negative ECs. D: A graph comparing the different stages of remodeled blood vessels in the gravid uterus of each group. I to III are unremodeled (100% CD31-positive cells), active-remodeled (including two categories: 5% to 50% and >50% CD31-positive cells), and fully remodeled (<5% CD31-positive cells) uterine SpAs, respectively. In all plots, data are analyzed by statistical tests described in Materials and Methods and are expressed as individual values with means ± SEM. n = 7 rats per group. *P < 0.05, **P < 0.01. Scale bars: 100 μm (A and C); 10 μm (B). C, collagen.
Hyperandrogenism and INS Resistance Induce Cellular Senescence in the Placenta, but Not the Gravid Uterus

Finally, because cellular senescence may play a regulatory role in uterine and placental physiology and pathology, further experiments were performed to determine whether hyperandrogenism and INS resistance regulate the expression of senescence-associated cell-cycle arrest proteins, including phosphorylated p53 (p-p53), p16, p21, and phosphorylated histone H2AX (p-histone H2AX; a protein marker of DNA damage and repair), in the rat gravid uterus and placenta. Western blot analysis revealed that DHT and INS exposure did not change p-p53, p16, or p21 protein levels in the gravid uterus (Figure 7A), whereas the same exposure increased p-p53 and p16, but not p21, protein levels in the placenta (Figure 7B). Furthermore, exposure to DHT and INS decreased p-histone H2AX protein levels in the gravid uterus (Figure 7A), whereas there was a tendency for elevated p-histone H2AX protein levels in the placenta ($P = 0.053$) (Figure 7B) when compared with the control group. These results demonstrate that uteroplacental cellular senescence is regulated by hyperandrogenism and INS resistance in a tissue-dependent manner.

Discussion

The exact pathobiology of PCOS-associated pregnancy loss remains elusive. Several in vivo studies in PCOS-like pregnant rodent models have been put forward to explain which biological processes and molecular mechanisms are involved, including elevated uterine androgen receptor sensitivity (androgen receptor mRNA and protein expression), disturbed endometrial receptivity, and decidualization, enhanced oxidative stress, augmented proinflammatory cytokine signaling, and increased activation of ferroptosis at the fetal-maternal interface. Hyperandrogenism and INS resistance are the key pathophysiological drivers of PCOS, and this study sought to provide a comprehensive picture of hyperandrogenism and INS resistance—regulated uterine SpA remodeling during pregnancy. The active and complete uterine SpA remodeling by various uteroplacental cells is required for increasing the amounts of oxygen and nutrients needed to support fetal growth and survival. The present data confirmed reports of exposure to DHT and INS during midgestation resulting in aberrant expression of marker genes for angiogenesis, decidual stromal cells, uterine natural killer cells, and progenitor trophoblasts in rats. In this pregnant PCOS-like rat model, the robust down-regulation of the expression of marker genes for SpA TGCSs and ECs in the gravid uterus and placenta was most likely a result of hyperandrogenism and INS resistance. All of the data support the hypothesis that ECs, VSMCs, and TGCSs all contribute to hyperandrogenism and INS resistance—induced impairments of uterine SpA remodeling.

Although the literature regarding the mechanisms underlying PCOS-associated uterine SpA remodeling is limited, numerous observations have suggested that endothelial dysfunction is associated with or is triggered by elevated androgen levels in women with PCOS and in DHT-induced PCOS-like rats. The present data showed that defects in ECs are associated with the abnormal regulation of VSMC-related molecular mediators in the uterus of DHT + INS—exposed rats. These alterations paralleled an increase in the number of unremodeled SpAs and a decrease in highly remodeled SpAs in the gravid uterus. Hyperandrogenism is both a defining sign of PCOS and is assumed to be an etiological factor for the progression of pregnancy-related problems in women with PCOS. The question arises as to how PCOS affects uterine vascular remodeling during pregnancy. There is substantial evidence that, in humans and rodents, uterine vascular ECs and VSMCs express high levels of androgen receptors. In vitro studies also demonstrate testosterone-induced elevation of androgen receptor mRNA and protein expression with enhanced cell growth and survival in human and rat vascular ECs and VSMCs that is blocked by the androgen receptor antagonist flutamide. Thus, it is
Figure 4  Exposure to 5-dihydrotestosterone (DHT) and insulin (INS) in midgestation enhances colocalization of CD31 and α-smooth muscle actin (α-SMA) in the uterine spiral artery. A: Representative gross morphologic images of conceptuses within the uterus (Ut) at gestational day (GD) 14.5 collected from pregnant rats exposed to DHT and INS. Non-specific immunoreactivities of CD31 and α-SMA were found in the residual blood clots in the uterine artery. B: Whole-mount immunolabeling with anti-CD31 (red) and anti-α-SMA (green) of the intact uteroplacental unit from control and DHT + INS—exposed rats at GD 14.5. The images shown are representative of two samples from each group, which yielded similar results. The numbers represent enlarged views of the boxed areas in each image. Scale bars = 100 μm (B). F, fetus; Pl, placenta.
conceivable that in the experimental context of DHT + INS—exposed rats, hyperandrogenism impaired uterine vascular remodeling through the direct activation of androgen receptors in uterine ECs and VSMCs, but this hypothesis needs to be substantiated in future studies.

Imbalances in maternal pro-angiogenic/anti-angiogenic factors and an inadequate modification of uterine SpAs are among the pathophysiological mechanisms underlying the onset of preeclampsia. Given the higher risk of preeclampsia in women with PCOS, the present data also showed that the ratios of Flt1/Pgf mRNA and Eng/Pgf mRNA were increased in the gravid uterus of DHT + INS—exposed rats. Although the circulating soluble FLT1, ENG, and PGF levels, systemic hypertension, and proteinuria as maternal signs and symptoms under the conditions of hyperandrogenism and INS resistance remain to be determined, the present study shed light on the possible roles of hyperandrogenism and INS resistance in preeclampsia. Vascular NO produced by NOS3 is a key modulator for the regulation of uterine blood flow, and the levels of NOS3 mRNA and protein are highly elevated in human uterine arteries during normal, healthy pregnancy. The protein level of apurinic/apyrimidinic endonuclease 1, an enhancer of NOS3 expression and vascular NO levels, is significantly lower in the uterus of DHT + INS—exposed rats compared with that in control rats. The findings in the present study showed that the expression of uterine Nos3 mRNA was decreased in DHT + INS—exposed rats compared with that in controls. Although the uterine NO level was not measured in this study, NOS3 protein levels have been shown to decrease in pregnant rat uterine arteries exposed to testosterone propionate. Furthermore, testosterone propionate—exposed rats from GD 15 to GD 19 both show significant decreases in uterine SpA elongation and blood flow. In addition, uterine VSMC dedifferentiation is disrupted in Nos3-deficient mice. EC-derived NO acting on underlying VSMCs regulates VSMC contraction and relaxation. The functional changes by which PCOS leads to inadequate modification of uterine SpAs remain to be elucidated. However, these observations are consistent with studies showing that rats exposed to androgens (testosterone propionate and DHT) exhibit reduced uterine blood flow and increased maternal blood pressure, as well as suppressed Nos3 activity and depressed endothelial NO production. Furthermore, the present findings of lower levels of uterine Mmp2 and Mmp9 mRNA expression in DHT + INS—exposed rats support the observation that decreased MMP2 and MMP9 activities are linked to impaired uterine vasculization and SpA remodeling. Further studies that specifically isolate uterine SpAs are needed to test the direct role of hyperandrogenism and INS resistance in uterine SpA remodeling, and additional experiments, such as wire myography, are also needed to measure maternal blood flow and blood pressure in DHT + INS—exposed rats.

The VSMC transition from the differentiated to dedifferentiated state is characterized by decreased expression of canonical marker proteins, including α-SMA, SM22α, and calponin 1. In the current study, the tissue distribution pattern of α-SMA indicated that in DHT + INS—exposed rats, α-SMA was expressed at higher levels in uterine SpAs, although no changes in uterine α-SMA protein levels were observed. SM22α contributes to angiogenesis of human umbilical vein ECs. In line with this finding, our analysis indicated that SM22α was also present in uterine ECs in addition to VSMCs. However, exposure to DHT and INS did not affect SM22α immunolocalization or protein levels in the gravid uterus. In contrast to α-SMA and SM22α, the expression of calponin 1 protein was strongly up-regulated in the uterus of DHT + INS—exposed rats. Immunohistochemical and immunofluorescence experiments also showed greater calponin 1 immunoreactivity in uterine SpAs in rats exposed to DHT and INS. Overall, the data indicate that the regulatory patterns of the uterine α-SMA, SM22α, and calponin 1 proteins needed for VSMC dedifferentiation are different in response to hyperandrogenism and INS resistance, at least in pregnant DHT + INS—exposed rats.

Interestingly, although the expression of α-SMA and SM22α proteins was increased in the placenta of DHT + INS—exposed rats compared with control rats, the opposite regulation of calponin 1 protein expression was observed in the uterus from DHT + INS—exposed rats. This highlights the tissue specificity regarding the regulation of α-SMA, SM22α, and calponin 1 protein expression, where they contribute cooperatively to the same VSMC differentiation under conditions of hyperandrogenism and INS resistance.

The placenta integrates signals from the compromised maternal and intrauterine environment, which disturb fetal development and growth. Genes involved in the angiogenesis and trophoblast invasion of uterine SpAs are down-regulated in the placenta of rats exposed to DHT and INS. The current work extends previous findings by showing reductions in the basal and labyrinth zone areas, maternal blood spaces, and labyrinth vasculature coupled to compromised fetal endothelial cell number and integrity in DHT + INS—exposed rats. Pregnant women with PCOS display morphologic, vascular, and pathologic alterations in the placenta. Several placental insufficiency-associated genes, such as Hif1α, Spint1, Spint2, Emp2, and Pparg, were differentially regulated in the placenta by co-exposure to DHT and INS in this study. Hif1α is critical in regulating vascular remodeling and trophoblast cell invasion under hypoxic conditions. In humans, levels of placental Hif1α mRNA and protein are highest in the first trimester of pregnancy and decline thereafter. This study showed that placental Hif1α expression was increased in DHT + INS—exposed rats compared with control rats at GD 14.5. It could be postulated that hypoxia-mediated up-regulation of Hif1α may also contribute to perturbation in uterine SpA remodeling under the conditions of
Figure 5  Exposure to 5-dihydrotestosterone (DHT) and insulin (INS) in midgestation impacts uterine vascular smooth muscle cell (VSMC) differentiation-related protein expression and spiral artery (SpA) remodeling progression.  

A: Representative Western blot analysis of lysates from control and DHT + INS—exposed rats at gestational day (GD) 14.5, showing the analysis of α-smooth muscle actin (α-SMA), smooth muscle protein 22α (SM22α), and calponin 1 in the gravid uterus (Ut). For the quantification of Western blot analyses, total protein was loaded for each sample, and the intensity of each protein band was normalized to the total protein in the individual sample.  

B: Representative photomicrographs of dual immunofluorescence with anti-SM22α (green) and anti-calponin 1 (red) in the uterine decidua of a control rat at GD 14.5. Arrows indicate endothelial cells, arrowheads indicate VSMCs, and brackets indicate the thickness of VSMCs. Small, rounded cells with positive staining in the lumen represent erythrocyte autofluorescence.  

C: Representative photomicrographs of immunohistochemical labeling with anti-calponin 1 in the uterus of control and DHT + INS—exposed rats at GD 14.5. Images shown are representative of seven gravid uterine samples from each group that were examined. The numbers represent enlarged views of the boxed areas in each image. Arrows indicate perivascular/endovascular trophoblasts, red arrowheads indicate calponin 1—positive blood vessels, and pink asterisks indicate calponin 1—negative blood vessels.  

D: Representative photomicrographs of dual-immunofluorescence for anti-calponin 1 (red) and anti-SM22α (green) in the uterine decidua of a control rat at GD 14.5. Dotted lines specify calponin 1—negative blood vessels. The positive signal in small, rounded cells in the lumen was the result of erythrocyte autofluorescence.  

E: A graph comparing the different stages of remodeled blood vessels in the gravid uterus of each group (the average value of two separate sections analyzed from each animal is plotted). I to IV are unremodeled, and slightly, moderately, and highly remodeled uterine SpAs, respectively. In all plots, data are analyzed by statistical tests described in Materials and Methods and are expressed as individual values with means ± SEM (A and E). n = 7 rats per group, each from different animals (A and E); n = 6 (B and D). **P < 0.01, ***P < 0.001. Scale bars: 200 μm (B); 100 μm (C and D). Pl, placenta.
Figure 6  Alterations in placental structure and molecular indexes induced by exposure to 5-dihydrotestosterone (DHT) and insulin (INS) in midgestation. 

A: Representative bright-field hematoxylin and eosin (H&E) staining photomicrographs of the placenta in control and DHT + INS—exposed rats at gestational day (GD) 14.5. The images shown are representative of 10 placental samples from each group that were examined. The graph compares the area of the basal zone (Bz), the labyrinth zone (Lz), and the total area in the placenta of each group (the average value of two separate sections analyzed from each animal is plotted). B: Representative photomicrographs of H&E staining (top panels) and immunohistochemistry with anti-CD31 (bottom panels) in placentas from control and DHT + INS—exposed rats at GD 14.5. The images shown are representative of seven placental samples from each group that were examined. Tissue sections were counterstained with methyl green for CD31 staining. Bottom panels: Insets: Part of a fetal capillary (FC) after CD31 immunohistochemistry at higher magnification. C: Representative transmission electron microscopy photomicrographs showing placental fetal capillaries in control and DHT + INS—exposed rats at GD 14.5. Images shown are representative of three placental samples from each group that were examined. The arrowheads indicate fetal endothelial cells. D: Representative Western blot analyses of lysates from control and DHT + INS—exposed rats at GD 14.5, showing the analysis of α-smooth muscle actin (α-SMA), smooth muscle protein 22α (SM22α), and calponin 1 in the placenta. For the quantification of Western blot analyses, total protein was loaded for each sample, and the intensity of each protein band was normalized to the total protein in the individual sample. E: Quantification of the expression of placental sufficiency-associated gene expression in the rat placenta at GD 14.5 as measured by quantitative real-time PCR. Expression was normalized to the average Ct values of the Actb and Gapdh genes as the endogenous controls, and the fold change in expression was evaluated using the 2−ΔΔCt method. Data are analyzed by statistical tests described in Materials and Methods and are expressed as individual values with means ± SEM (A, D, and E). n = 10 rats per group (A); n = 7 per group, each from different animals (D and E). *P < 0.05, **P < 0.01. Scale bars: 100 μm (A); 10 μm (B and C). MBS, maternal blood space.
hyperandrogenism and INS resistance. Additional studies are needed to fully characterize the molecular pathways and upstream maternal and fetal signals that contribute to the disruption of placental formation and function, and the development of pregnancy loss in response to hyperandrogenism and INS resistance in pregnancy. Further investigations are also needed to assess the impacts of hyperandrogenism and INS resistance on placental oxygen delivery and nutrient transfer in vivo.81

Previous results25,26 and the results presented here positively link hyperandrogenism and INS resistance with uterine and placental cell death. In contrast to the gravid uterus, the expression of pro-apoptotic and necroptotic genes is increased in the placenta of pregnant rats exposed to DHT and INS.63 Moreover, the placenta is less susceptible to ferroptosis than the gravid uterus under the conditions of hyperandrogenism and INS resistance.41,63,64 The present study found that, in DHT + INS−exposed rats, the protein levels of p-p53, p16, and p21 were unaffected, despite decreased p-histone H2AX protein levels in the gravid uterus. However, in the same animals, there were increased levels of p-p53 and p16, and a tendency for elevated p-histone H2AX protein abundance, which indicate senescence-associated cell-cycle arrest, apoptosis, and DNA damage and repair in the placenta. These results suggest that hyperandrogenism and INS resistance have different effects on the control of uterine and placental cell senescence and death pathways. There is time-dependent regulation of apoptosis during placental development,82 and dysregulation of placental senescence is implicated in adverse pregnancy outcomes.83 Thus, apoptosis, necroptosis, and cell senescence are affected by hyperandrogenism and INS resistance and likely contribute to placental dysfunction and pregnancy loss in DHT + INS−exposed rats. Although the current data support the hypothesis that uterine senescence may not be regulated by exposure to DHT and INS, it does not support excluding the notion that placental senescence might contribute to the impact of hyperandrogenism and INS resistance on uterine function.

Besides the uterine and placental defects in women with PCOS,10,11 clinical evidence shows that ovarian dysfunction and impaired oocyte developmental competence may be the potential causative factors for the pathogenesis of PCOS-induced pregnancy complications.84,85 The significantly decreased ovarian weight and corpus lutea number per ovary in DHT + INS−exposed pregnant rats suggest that ovarian dysfunction could contribute to fetal loss.58 However, as the DHT + INS exposure only commenced on GD 7.5, a direct role on the oocyte in our rat model is unlikely. However, it may impact the postimplantation embryo. Consistent with previous work,25,26 induced endocrine and

Figure 7 Exposure to 5-dihydrotestosterone (DHT) and insulin (INS) in midgestation induces tissue-specific dysregulation of select cell senescence-associated proteins. Representative Western blot analyses of lysates from control and DHT + INS−exposed rats at gestational day 14.5, showing the analysis of phosphorylated p53 (p-p53), p16, p21, and phosphorylated histone H2AX (p-Histone H2AX) in the gravid uterus (A) and the placenta (B). For the quantification of Western blot analyses, total protein was loaded for each sample, and the intensity of each protein band was normalized to the total protein in the individual sample. In all plots, data are analyzed by statistical tests described in Materials and Methods and are expressed as individual values with means ± SEM (A and B). n = 7 per group, each from different animals (A and B). *P < 0.05, **P < 0.01.
metabolic disturbances in DHT + INS—exposed pregnant rats were similar to those in pregnant patients with PCOS. However, it would be interesting to identify the individual roles of hyperandrogenism and INS resistance to the defective SpA remodeling, placental senescence, and other uteroplacental changes seen in future work. This could employ the use of anti-androgens or metformin, which may also inform the development of new therapeutic strategies to effectively prevent or treat PCOS-induced uterine and placental dysfunction.

In summary, this is the first report to show that hyperandrogenism and INS resistance, the key features of PCOS, disrupt maternal uterine SpA remodeling and placental senescence, which may account for decreased fetal growth and survival in DHT + INS—exposed rats. This work contributes to a greater understanding of hyperandrogenism and INS resistance—induced uteroplacental dysfunction and may explain, at least in part, why patients with PCOS have increased chances of miscarriage during pregnancy.

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Author Contributions

L.R.S. conceptualized and supervised the study; M.H., Y.Z., and L.R.S. developed methods and validation; M.H., X.Z., X.Y.Z., X.H., Y.Lu., Y.Li., and L.R.S. analyzed the data; M.H., Y.Z., X.Z., X.Y.Z., X.H., Y.Lu., Y.Li., and L.R.S. performed experiments; M.H., Y.Z., X.Y.Z., X.H., Y.Lu., and L.R.S. curated data; M.H., Y.Z., X.Z., X.Y.Z., X.H., Y.Lu., and Y.Li. performed visualization; M.H., Y.Z., and L.R.S. wrote the manuscript; M.B., A.N.S.-P., X.H., Y.Lu., and Y.Li. performed visualization; M.H., Y.Z., X.Z., X.Y.Z., X.H., Y.Lu., Y.Li., and L.R.S. conceptualized and supervised the study; and M.H., Y.Z., X.Z., X.Y.Z., X.H., Y.Lu., Y.Li., and L.R.S. performed visualization; M.H., Y.Z., X.Z., X.Y.Z., X.H., Y.Lu., Y.Li., and L.R.S. wrote the manuscript; and M.B., A.N.S.-P., X.H., Y.Lu., and Y.Li. performed visualization; M.H., Y.Z., X.Z., X.Y.Z., X.H., Y.Lu., Y.Li., and L.R.S. conceptualized and supervised the study; M.H., Y.Z., X.Z., X.Y.Z., X.H., Y.Lu., Y.Li., and L.R.S. analyzed the data; M.H., Y.Z., X.Z., X.Y.Z., X.H., Y.Lu., Y.Li., and L.R.S. performed experiments; M.H., Y.Z., X.Y.Z., X.H., Y.Lu., and Y.Li. performed visualization; M.H., Y.Z., and L.R.S. wrote the manuscript; and M.B., A.N.S.-P., L.R.S., and H.B. reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

Disclosure Statement

None declared.

Supplemental Data

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