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Abstract—Rats harboring the human angiotensinogen and human renin genes develop preeclamptic features in pregnancy. The preeclamptic rats exhibit a deeper trophoblast invasion associated with a reduced resistance index by uterine Doppler. Doxycycline inhibits matrix metalloproteinase activity. We tested the hypothesis that matrix metalloproteinase inhibition reduces trophoblast invasion with subsequent changes in placental perfusion. Preeclamptic and pregnant control Sprague-Dawley rats were treated with doxycycline (30 mg/kg of body weight orally) from gestational day 12 until day 18. Placental perfusion was assessed using a micromarker contrast agent. The animals were euthanized on day 18 of pregnancy; biometric data were acquired, and trophoblast invasion was analyzed. Doxycycline resulted in intrauterine growth retardation and lighter placentas in both groups. Maternal body weight was not affected. As shown earlier, preeclamptic rats exhibited a deeper endovascular trophoblast invasion. However, doxycycline treatment reduced trophoblast invasion in the preeclamptic rats. The physiological spiral artery remodeling, as assessed by the deposition of fibrinoid and α-actin in the spiral artery contour, was significantly reduced by doxycycline. The vascularity index, as assessed by perfusion measurement of the placenta, was reduced after doxycycline treatment in preeclamptic rats. Thus, matrix metalloproteinase inhibition with doxycycline leads to reduced trophoblast invasion and associated reduced placental perfusion. These studies are the first to show that reducing trophoblast-induced vascular remodeling decreases subsequent placental perfusion. Our model allows the study of dysregulated trophoblast invasion and vascular remodeling in vivo to gain important insights into preeclampsia-related mechanisms. **(*Hypertension*. 2010;56:304-310.)**

Key Words: pregnancy ■ trophoblast ■ rats ■ animal models ■ doxycycline ■ matrix metalloproteinases ■ preeclampsia

Preeclampsia (PE) affects 3% to 5% of all pregnancies and is a major cause of fetal and maternal morbidity and mortality.[1] Malimplantation of the placenta leads to hypertension, proteinuria, and endothelial dysfunction after 20 weeks' gestation.[2,3] In early onset PE, the endovascular and interstitial trophoblast invasions are reduced, leading to incomplete placental bed spiral artery remodeling, resulting in reduced oxygen and nutrients to the fetus.[4] Uterine blood flow is altered, reflecting the failure of trophoblasts to transform the placenta into low-resistance vessels.[5] Trophoblast invasion and its regulation are poorly understood, and animal models are problematic. Male rats transgenic for human renin mated with dams transgenic for the human angiotensinogen develop hypertension, proteinuria, and fetal loss in the second half of pregnancy.[6,7] However, in contrast to humans, trophoblast invasion in the rat mesometrial triangle (MT) is deeper rather than shallow, as in humans.[8] The uterine resistance index is reduced in the PE-model rats; however, the uterine arteries exhibit endothelial dysfunction.[9] Although human PE differs substantially from this PE rat model, there is homology regarding trophoblast invasion and parallels in important regulatory elements.[10] Studies suggest that trophoblast invasion is regulated by the interaction of autocrine factors from the trophoblasts and paracrine factors from the uterus. Trophoblast invasion, like tumor invasion, is attributed to the active production of proteolytic enzymes to digest extracellular matrices of the host's tissues. The secretion of matrix metalloproteinases (MMP) is a key event. MMPs have been localized in the placental bed,[11] trophoblast,[12] and uterine natural killer (uNK) cells.[13] Although our rat model is imperfect, we elected to investigate MMPs in the process of trophoblast invasion because invasion is aberrant both in human PE and in our animal model, because MMPs could be pivotal to PE.

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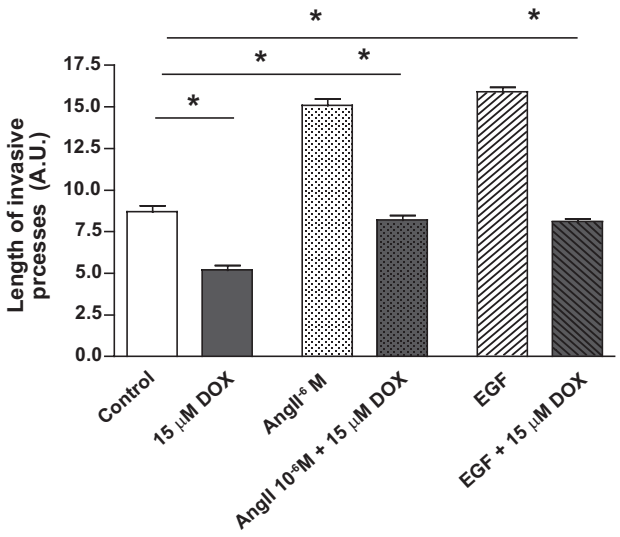

Figure 1. Isolated human trophoblasts (SGHPL-4) were incubated with Ang II, DOX, and EGF. Cells were cultured on microcarrier beads embedded in fibrin gels to assess invasion. Incubation with DOX significantly decreased the motility of the cells in vitro. Ang II and EGF increased invasion of the trophoblasts. This effect was antagonized by DOX (n=4 experiments each with 20 beads per group; *$P<0.05$). The *y* axis represents the length of invasive processes (AU indicates arbitrary units).

Materials and Methods

Local authorities approved the studies that met American Physiological Society guidelines. Details on the transgenic rat model are published.[6] Systolic blood pressure was measured in awake animals by the tail-cuff method before and after the treatment period.[7] Pregnancy was confirmed by inspecting the vaginal plug after overnight breeding, counted as gestational day 1 of pregnancy. Sprague-Dawley females were mated with Sprague-Dawley males as a control group. PE and pregnant control rats were treated with doxycycline hyclate (DOX; 30 mg/kg of body weight per oral gavage per day) for 7 days from gestational day 12 until day 18. This dose inhibits MMP activity.[14,15] DOX was prepared in 4% carboxymethyl cellulose and applied in light-shielded bottles. On day 18 of rat pregnancy, measurement of placental perfusion via ultrasound was performed. Placental perfusion was assessed using contrast agent microbubbles (VisualSonics). After ultrasound examination, all of the animals were euthanized, and organs were harvested, weighed, and analyzed. The MT was separated from the placenta after weighing and either snap frozen in liquid nitrogen or dehydrated in paraformaldehyde or zinc fixative. Analysis of the depth of trophoblast invasion was performed as described earlier.[8] MMP inhibition by DOX was assessed using RT-PCR, Western blotting, gel zymography, ELISA, and activity measurement via fluorogenic peptides. For statistics, we present all of the values as mean±SEM. Student *t* tests or ANOVA was used as appropriate. A value of $P<0.05$ was considered statistically significant. Further methodological details are given in the online Data Supplement (available at http://hyper.ahajournals.org).

Results

The PE rat systolic blood pressure was 165±7 mm Hg, whereas blood pressure was 110±4 mm Hg in the control group. DOX treatment had no effect on blood pressure (PE+DOX: 161±9 mm Hg; control+DOX: 107±4 mm Hg). Treatment of isolated human trophoblasts with 15 μmol/L of DOX caused reduced ($P<0.05$) invasion by 40% (Figure 1). Epidermal growth factor (EGF), a well-known stimulus of trophoblast invasion, increased trophoblast invasion. Adding DOX to EGF resulted in a significant decrease in invasion. Angiotensin (Ang) II (10^{-6} M) also augmented trophoblast invasion, similar to the observed effect with EGF. DOX also reduced the invasion of trophoblasts to untreated controls. We next investigated the in vivo effects of DOX in our transgenic Ang II–dependent PE rat model. DOX 30 mg/kg per day from gestational day 11 to day 18 did not harm the overall ability of the rats to maintain pregnancy. The litter size, as well as the resorption number, did not differ in DOX-treated animals compared with controls. However, DOX resulted in mild intrauterine growth retardation in PE (12% reduced body weight). In control animals, DOX reduced body weight by 8.5% ($P<0.05$). The placentas were also 10.5% lighter in PE and 12.5% in controls ($P<0.05$). Maternal body weight was not affected by the DOX treatment (please see Figure S1 in the online Data Supplement, available at http://hyper.ahajournals.org). DOX treatment did not result in liver and renal dysfunction, measured as liver enzymes, creatinine, and urea (data not shown). DOX also had no influence on the nonpregnant female control and human angiotensinogen rats (data not shown).

DOX treatment inhibited endovascular trophoblast invasion in the MT of the rat placental bed (Figure 2A and 2B). We focused on physiological remodeling of the spiral arteries in response to trophoblast invasion. We analyzed spiral artery segments in 3 depth levels of the MT, the inner compartment 1, the middle compartment 2, and the outer compartment 3 (Figure 2A and 2B). In compartment 2, the spiral artery remodeling was reduced in PE and controls after DOX treatment. There was significantly less fibrinoid deposition, and more α-actin–positive cells were present in the spiral artery wall, indicating less trophoblast-induced remodeling (Figure 2Ci and 2Di). In the outer depth level (compartment 3), DOX reduced spiral artery remodeling as assessed by the deposition of fibrinoid (Figure 2Cii and 2Dii). However, α-actin in the spiral arteries was reduced in the control animals (Figure 2C and 2D). The physiological spiral artery remodeling, as assessed by the deposition of fibrinoid and α-actin in the spiral arteries of the overall MT, was also significantly reduced in PE animals by DOX treatment (data not shown).

The role of apoptosis and uNK cells in DOX-mediated reduction in trophoblast invasion was examined. In the untreated animals, a similar number of perivascular Dolichus biflorus agglutin–positive uNK cells per centimeter squared was found concerning the spiral artery cross-sections from the whole MTs in all of the groups. In the DOX-treated PE rats and in control animals, a significantly enhanced apoptosis was exhibited (Figure 3A; $P<0.05$). We next evaluated the uNK cell content in the MT. There were significantly more uNK cells in the MT of the PE+DOX as compared with the control+DOX (Figure 3B; $P<0.05$).

The vascularity index, as assessed by perfusion measurement of the placenta, was reduced ($P<0.05$) after DOX treatment in PE rats (Figure 4A through 4D). The area under the curve of perfusion of the maternal arterial channel as assessed in the sagittal ultrasound scan was significantly reduced in the DOX-treated animals. However, in controls there was no significant change in perfusion. In the transversal scan of the placenta, DOX treatment significantly reduced the resistance index in the PE rats. In the transversal plane,

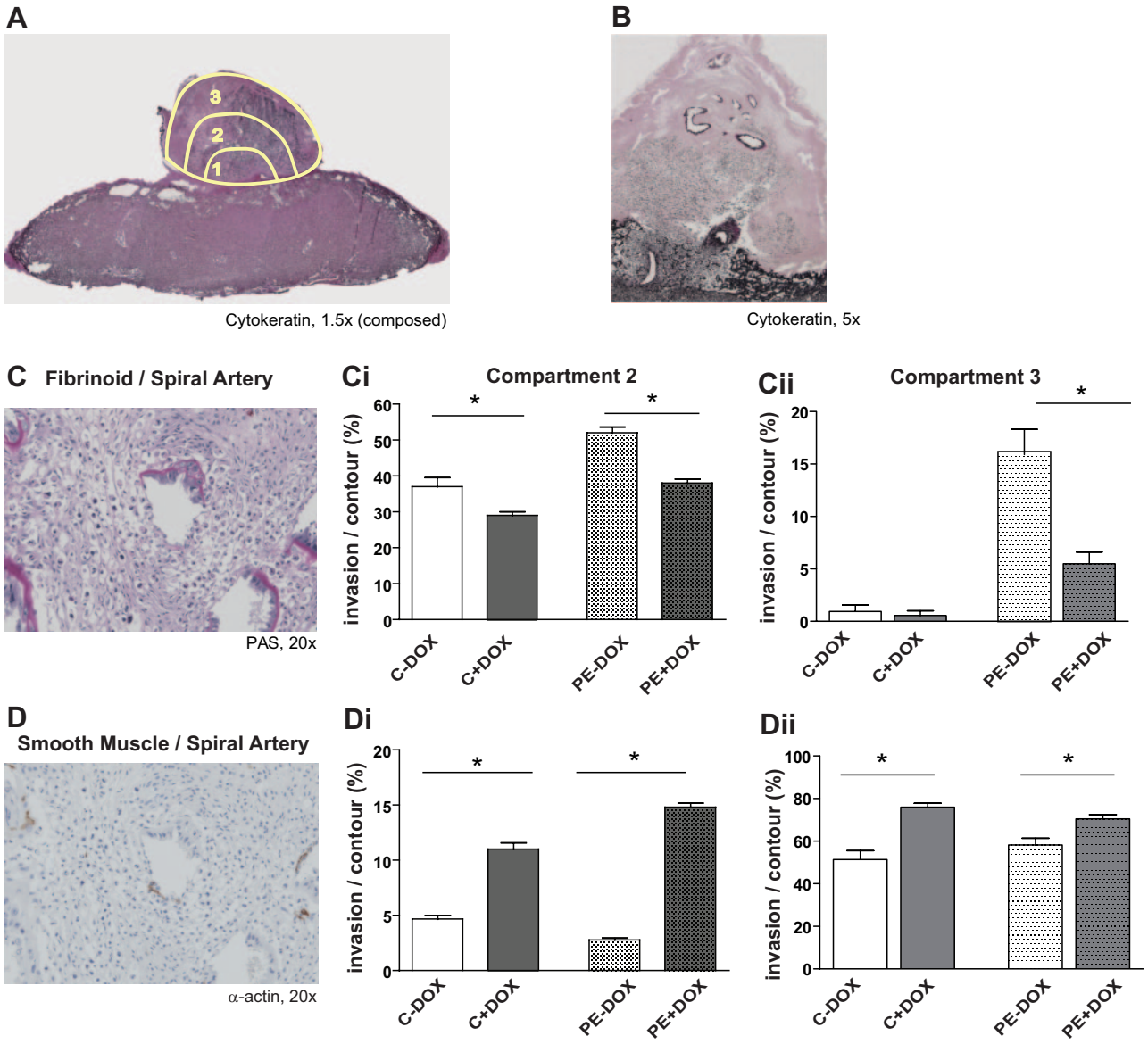

Figure 2. DOX treatment resulted in a decreased trophoblast invasion as reflected by a decreased physiological remodeling of spiral arteries. A representative placental section is shown in A (cytokeratin staining for trophoblasts). The yellow lines represent the depth levels of the MT that were used to analyze the depth of spiral artery invasion by trophoblasts. In B, a representative section of the MT is displayed. C shows a representative Periodic acid-Schiff staining of an MT spiral artery. DOX-treated animals show a significantly lower percentage of fibrinoid staining of the spiral artery contour in compartment 2 (Ci), indicating less physiological and less deep trophoblast invasion (Ci, n=6; *$P<0.05$). Analogous effects are visible in the outer depth level (compartment 3) of the MT. There was almost no fibrinoid deposition as a marker of physiological remodeling visible in the controls, whereas there is a significant reduction of trophoblast invasion in the DOX-treated PE animals (Cii, n=6; *$P<0.05$). D shows a representative α-actin–stained section of an MT spiral artery. DOX-treated animals show a higher percentage of α-actin staining of the spiral artery contour staining in compartment 2 (Di) and compartment 3 (Dii), indicating less physiological change in the MT (n=6; *$P<0.05$).

significant differences in the vascularity index of the untreated controls versus PE animals were observed. The untreated control animals exhibited a significantly reduced area under the curve of micromarker perfusion as compared with the untreated PE animals ($P<0.05$).

Quantitative RT-PCR showed that MMP-2 was increased in the MT of PE+DOX compared with control+DOX (Figure 5A). The results were confirmed by Western blot and ELISA (Figure 5B and 5C), showing a highly preserved MMP2 expression in the PE rats after DOX treatment, which verifies the positive feed back loop after downstream inhibition by DOX. Because DOX is not present in the organ extract after extraction, the preserved MMP-2 expression was enzymatically active (Figure 5D and 5E). Add back of DOX (35 μmol/L) was successful in inhibiting MMP activity (Figure 5F). This proof-of-concept effect was present in the MT, as well as in the placenta (data not shown). Adding back DOX was successful in control tissue and DOX-treated organs (data not shown).

Discussion

The data indicate a causal relationship among reduced trophoblast invasion, reduced spiral artery remodeling, and

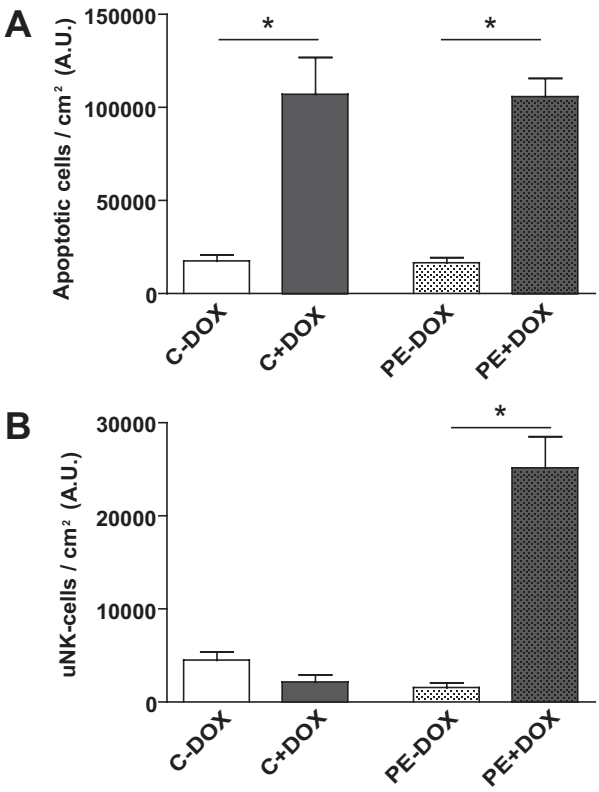

Figure 3. The role of apoptosis and uNK cells in DOX-mediated reduction in trophoblast invasion was examined. A shows a significant proapoptotic effect of DOX in PE and controls (staining for cleaved caspase 3, n=6; *$P<0.05$) In B, uNK cells in the MT are visualized by staining for Dolichus biflorus agglutin lectin (n=6; *$P<0.05$). There were significantly more DBA+ cells in the PE+DOX group. No significant changes were provoked in control (C)+DOX.

subsequent reduction of placental perfusion in a transgenic PE rat model. MMP inhibition with DOX led to reduced trophoblast invasion in the PE group. The physiological remodeling of the spiral arteries in the outer depth level of the MT was reduced by 60%. The novel and accurate method of microbubble-assisted, contrast-enhanced ultrasonography perfusion of the placenta demonstrated that DOX treatment reduced perfusion by 25%. We were able to show inhibition of trophoblast invasion by DOX in vitro by studying trophoblasts from first-trimester human pregnancies. Furthermore, we were able to explain a part of the initially puzzling observation in our PE rats. These rats express a deeper trophoblast invasion compared with controls, which is opposite to the human situation.[8] In the cell culture system, supplementing Ang II with the medium led to an increased invasion of cultured trophoblast cells in vitro. When adding DOX, this effect was blocked. When a known stimulator of trophoblast invasion (EGF) was substituted in vitro, invasion also occurred that could be blocked by DOX.[16]

Invasion of trophoblast into the decidua and myometrium, as well as the remodeling of the spiral arteries, is an important feature of hemochorial placentation.[1] Uterine spiral arteries are vital to supply oxygen and nutrients to the placenta and fetus. The main purpose of trophoblast-induced spiral artery remodeling is to transform the artery into a highly dilated vessel. Although the important role of trophoblasts in the complete physiological change of the spiral arteries is accepted, uncertainties about the molecular pathways and mechanisms of spiral artery remodeling remain. Our knowledge is mainly based on meticulous histological observations. Few data have been generated by ex vivo experimental systems, such as isolated placental bed blood vessels or in vitro models using explanted spiral artery segments.[17,18] Rodent placentation has not been studied in detail. Caluwaerts et al[10] described that changes in spiral arteries of pregnant rats reveal striking similarities with physiological changes seen in human pregnancy. Our study is a follow-up of that pioneering work. We inhibited trophoblast invasion with DOX and demonstrated that placental perfusion is causally connected with trophoblast invasion.

In human and rodent pregnancies, uNK cells control trophoblast invasion.[19,20] Trophoblast invasion follows the demise of uNK cells and is directed toward regions of the MT, which were occupied previously by uNK cells. In our study, apoptosis is increased after DOX treatment in PE and control rats (Figure 3A). We were able to show that the presence of uNK cells is significantly increased after DOX treatment only in PE and not in control rats (Figure 3B). Increased apoptosis of perivascular uNK cells might offer an explanation for the increased endovascular trophoblast invasion. DOX treatment leads to a reduction in endovascular trophoblast invasion by a reduction of uNK-cell apoptosis and a preservation of uNK cells in the MT.

Factors controlling endovascular invasion are still poorly understood. There is clear agreement that trophoblast invasion is not attributed to passive growth pressure but instead is an active biochemical, multistep process involving attachment, degradation, and migration through extracellular matrix proteins.[5,21] MMPs are key proteins in this process.[11,13] MMP levels are elevated in women who subsequently develop PE.[22] This invasive behavior is clearly attributed to the ability of cytotrophoblasts to secrete MMP, because tissue inhibitors of MMP expression inhibit their invasiveness. The expression of MMP-2 and MMP-9 has been shown to mediate cytotrophoblast invasion into Matrigel. Cytotrophoblast production and activation of MMP-9 peak during the first trimester of pregnancy, coinciding with maximal invasive behavior in vivo.[11,13] Our data underscore the importance of MMP. In our model, inhibition of MMP is sufficient to alter the status of associated vascular remodeling and might, thus, contribute to alterations in PE.

Our transgenic PE rat model is Ang II driven with high circulating and uteroplacental Ang II levels.[6] Ang II enhances MMP2 release and might, thus, contribute to the observed deeper trophoblast invasion and associated changes of flow in the transgenic rats.[23] Chronic administration of Ang II has been shown to increase MMP expression and subsequently to induce spiral artery remodeling in rats.[24] Inhibition of exaggerated MMP2 release could, therefore, contribute to reduced trophoblast invasion as observed in our intervention. In earlier studies, several authors have shown a different effect of the renin-Ang system on uterine blood flow in comparison with other organs.[25,26] Franklin et al[27] showed that Ang II increased uterine artery blood flow via prostaglandins. Li et al[28] were the first to show that endocrine active decidual cells express both renin and angiotensinogen. They suggested

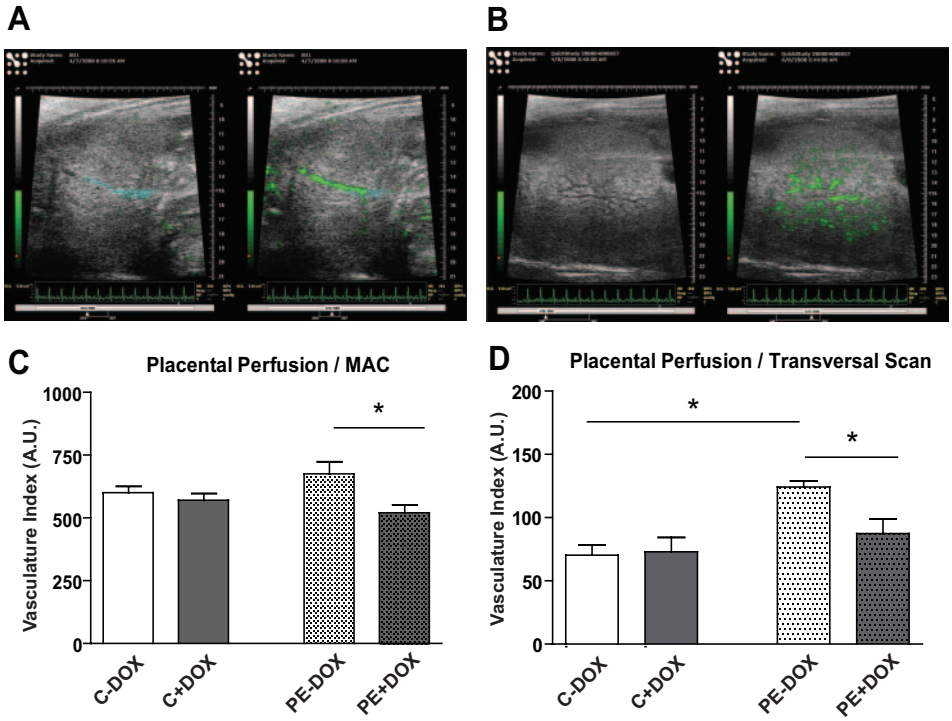

Figure 4. Placental perfusion was measured with microbubbles. The maternal arterial channel of the MT was scanned in a sagittal plane. A shows a representative ultrasound scan before and after the wash-in of the microbubbles into the maternal arterial channel. In a second set of experiments, a transversal scan through the placenta was performed. B shows a representative ultrasound scan before and after wash-in of microbubbles. The vascular index was calculated as a function of wash-in and plateau phase of the microbubble perfusion. The vascular index was reduced in the maternal arterial channel of the MT (C), as well as in the placenta (D) in DOX-treated PE vs PE (n=6; *$P<0.05$). Perfusion was not significantly altered by DOX in C (n=6; $P>0.05$).

that decidual cells regulate an expression of a tissue renin-Ang system in the endometrium. Ferris et al[26] also showed that uterine renin is inducing an increasing uterine blood flow. Renin secretion is increased in response to a reduction in uterine blood flow leading to an increase in circulating or local Ang II. Furthermore, Ang-converting enzyme inhibitors have also been shown to decrease uterine blood flow, which, together with fetopathy, is the reason why Ang-converting enzyme inhibitors should not be used to treat hypertension during pregnancy.[29] DeLano and Schmid-Schonbein[30] have shown that DOX treatment for 24 weeks reduced blood pressure and protease activity in plasma and microvessels in spontaneous hypertensive rats. Castro et al[31] showed a similar reduction in hypertension by DOX in a 2-kidney, 1-clip model for hypertension after 8 weeks of treatment. Thus, we cannot exclude that the differences in placental perfusion between control and PE rats by DOX are at least partially mediated by differences in the uteroplacental or circulating differences in the renin-angiotensin system.

DOX effectively inhibits MMP activity.[32] MMPs are regulated at several levels. Although the expression of most MMPs is transcriptionally regulated, additional modulation exists at the level of activation through the secreted latent proenzyme. The activity of the resultant protease can be inhibited by several endogenous inhibitors, of which tissue MMP inhibitors appear most important.[33] In addition to signaling pathways, DOX is believed to act via both primary direct and secondary indirect mechanisms on MMP activity and cell proliferation.[32] The potential explanations are that DOX nonselectively directly inhibits MMPs by binding to the active zinc sites and also by binding to an inactive calcium site, which causes conformational change and loss of enzymatic activity.[32] Secondary mechanisms of inhibition have also been proposed, which include a reduction in activation,[34] decreased gene expression,[35] and stabilization of specific and nonspecific inhibition.[32] However, the inhibition of the active zinc sites is reversible. After the organs were processed, DOX was no longer present in the cellular extract. Thus, detection MMP inhibition by DOX in the target organ via standard procedures has been difficult. Villarreal et al[36] avoided this problem. They showed that more MMP2 and MMP9 activity was present in the DOX-treated organ in their study. They described a feedback mechanism involving downstream MMP inhibition by DOX. In our study, we could show that MMP2 mRNA and protein expression was increased in the DOX-treated uteroplacental unit. Global MMP activity was higher in PE MTs compared with controls. Because DOX is no longer present in the processed organs, the increased activity, analogous to the protein expression, is an indirect sign that DOX was successful during the treatment period. MMP activity was higher in the formerly DOX-treated organs. We performed an add-back of DOX (35 μmol/L) to the fluorogenic peptide assay. Adding DOX was successful in inhibiting MMP activity, proving the concept of the efficacy of the intervention.

We were faced with the obstacle that sharply reduced trophoblast invasion might have perturbed the pregnancies before they could be completed. The teratogenic risk of DOX is well known. Previous studies by Siddiqui and Janjua[37] showed that DOX administration to pregnant rats is feasible; however, they ob-

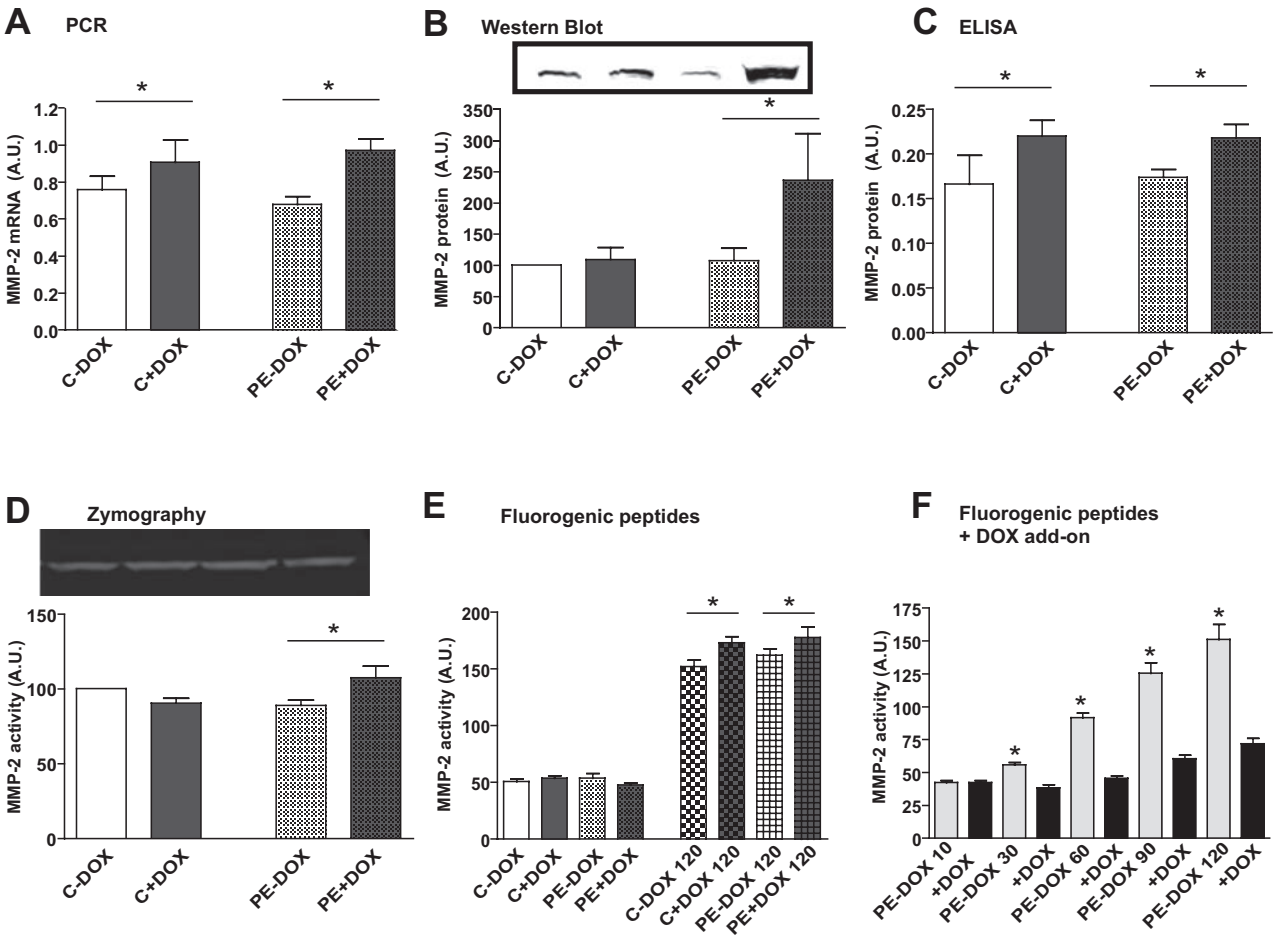

Figure 5. PCR, Western blot, ELISA, zymography, and MMP-2 activity measurements were performed to assess MMP-2 inhibition by DOX in the MT. PCR analysis showed a significant upregulation of MMP expression in DOX-treated controls and PE rats (A, n=4; *$P<0.05$). Western blot analysis showed a highly preserved MMP2 protein expression in the PE rats after DOX treatment, verifying a positive feed back loop after downstream inhibition by DOX (B, n=4; $P>0.05$). Protein content was not significantly different after DOX intervention in the MT of both groups (C, n=4; $P>0.05$). MMP activity was not significantly different as assessed by gel zymography in the MT after DOX intervention in both groups (D, n=4; $P>0.05$). The measurement of MMP2 activity with fluorogenic peptides showed a significantly preserved MMP2 activity after DOX treatment in both groups (E, n=4; *$P<0.05$). To verify the inhibition of MMP2 activity after DOX treatment, add-back of DOX was performed, and activity was measured using the fluorogenic substrates (F, n=5; *$P<0.05$).

served that the skeletal differentiation in the long bones was delayed in the fetuses, when pregnant dams were given DOX. The daily oral DOX dose for effective MMP inhibition in rats ranges between 25 and 100 mg/kg,[34,38] which is substantially higher than the therapeutic doses given to humans (2 to 3 mg/kg). We observed a moderate disturbance of the pregnancies in rats given DOX. DOX reduced fetal weight by 8.5% in control and 11.5% in transgenic PE animals. Placental weight was diminished by 12.5% in nontransgenic and 10.5% in transgenic animals, whereas maternal weight was unaffected. The dose of DOX that we used represents a compromise between a detectable trophoblast inhibition and a pregnancy directly perturbed by DOX.

DOX had no significant effect on placental perfusion in the control rats. Because the presence of trophoblast invasion in the deep region of the MT is a major determinant, and control rats show only minor deep trophoblast invasion,[8] conceivably DOX was not successful for this purpose. Nevertheless, we could show that DOX had strong effects in the control group, such as intrauterine growth restriction, apoptosis, and vascular remodeling in the more superficial regions of the uteroplacental unit. Another reason for the different potency of DOX in the 2 groups might be the known interaction among hypertension, Ang II, and the MMP system. Hypertension itself and Ang II increase MMP expression and activity in the myocardium and in the vasculature. In PE rats, deeper trophoblast invasion resulted not only in an increased perfusion but also in a dysregulated trophoblast decidua interaction, leading to increased decidual apoptosis and arteriolosclerosis.[7] In this respect, the DOX effect may be regarded as having "normalized" the dysregulated trophoblast invasion.

Perspectives

Trophoblast invasion and spiral artery remodeling in the rat placenta can be investigated in rat models, including PE models. Furthermore, MMP inhibition has a distinct effect on trophoblast invasion in such models. Animal models have thus far been imperfect in terms of duplicating events occurring in PE in our model and in other model systems. However, although rat and human placentas differ substan-

tially, the imaging, pathological, and pharmacological tools that we described here will be applicable to better models that will be developed in the future.

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Disclosures

None.

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Online Supplement

Inhibition of trophoblast-induced spiral artery remodelling reduces placental perfusion in rat pregnancy

Running head: placental perfusion

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Supplementary Method Section

Placental Perfusion Measurements

On day 18 of rat pregnancy, measurement of placental perfusion via ultrasound was performed. The rats were anesthetized with isoflurane. Heart rates and temperatures were monitored (Model THM100, Indus Instruments) and heating was adjusted to maintain a body temperature between 36 and 38°C. All hair was removed from the abdomen by gentle shaving. Pre-warmed gel was applied to the abdomen as an ultrasound-coupling medium (Parker Laboratory). Rat placentas and mesometrial triangles (MT) were imaged transcutaneously using the ultrasound biomicroscope (UBM) and a 30 MHz or 40 MHz transducer operating at 30 frames/s (Model Vevo 660, VisualSonics Inc). Placental perfusion was assessed using micromarker contrast agent (microbubbles, VisualSonics). Analysis of the "wash-in" curves was performed in comparing changes in the slope and plateau and implied differences in blood volume and blood flow in normal pregnant and DOX-treated animals. The microbubbles were administered intravenously as a continuous infusion at a constant rate and concentration. The appearance of the microbubbles in the ultrasound beam represented the wash-in by the placental circulation. After steady state of the plateau phase was achieved, the microbubbles were washed out of placental circulation. This plateau phase reflected the effective microbubble concentration within the placental circulation. The area under the curve (AUC) of the wash-in and plateau of the non-targeted micromarkers were used to describe the vascularity index (VI). Quantification was performed in adapting the concept of quantification of myocardial blood flow with microbubbles as described by Wei et al for the placental bed.[1] Two regions of interest were chosen and contrast agent wash-in and steady state curves were evaluated. In the first set of experiments, the region of interest was the maternal arterial channel (MAC) in the mesometrial triangle (MT). The blood flow inside the MAC was assessed, reflecting the overall remodeling status of the placenta. The MAC was scanned in a sagittal plane to visualize the course of the MAC inside the placenta/mesometrial triangle. In the second examination, a transversal scan was chosen and fetal side of the placenta was assessed.

Blood analysis

Blood was collected in sterile, cold 10-mL tubes and immediately centrifuged at 3000 rpm for 10 minutes at 4°C for the measurement of serum markers of hepatic (alanine aminotransferase [ALAT], aspartate aminotransferase [ASAT], gamma glutamyl transferase [GGT]) and renal function (creatinine and uric acid) in the Charité University Hospital central laboratory facility.

Histology and Immunohistochemistry

Placentas with their associated mesometrial triangle were paraffin fixed and parallel sections were cut step-serially from each implantation site parallel to the mesometrial-fetal axis. PAS staining was performed using standard histological protocols to identify a central maternal arterial channel (MAC) on one set of parallel sections. Parallel sections from the selected set were then stained for cytokeratin as a trophoblast marker, for α-actin as a vascular smooth muscle marker, for cleaved caspase-3 as an apoptosis marker and for DBA (Dolichos Biflorus Agglutinin) to visualize the uNK cells. The depth of trophoblast invasion was assessed using Zeiss KS-400 image analysis system. [2]

MMP-measurements

RT-PCR was performed as described previously.[3] Briefly, two micrograms of total RNA were reverse transcribed and analyzed in triplicate with the ABI 5700 sequence detection system (PE Biosystems). Primer and probes were designed with PrimerExpress 2.0 (Applied Biosystems).

For western blotting, protein from DOX-treated and untretaed placenta and MT were processed as described earlier.[4] For gelatin zymography, Placentas MT from DOX-treated and

untreated rats were extracted with 10 mmol/l Tris _HCl (pH 7.5) extraction buffer. Zymography was performed using 7.5% SDS-PAGE with copolymerized gelatin (2 mg/ml) as substrate. At the end of each run, gels were washed with 2.5% Triton X-100 and incubated for 48 h in an enzyme assay buffer (50 mmol/l Tris, pH 7.0, 5 mmol/l CaCl2, 0.15 mol/l NaCl, and 0.05% Na3N) to allow for the development of enzyme activity bands. Gels were stained with 0.05% Coomassie brilliant blue G-250 in a mixture of methanol: acetic acid: water (2.5:1:6.5) and destained in 4% methanol with 8% acetic acid. The gelatinolytic activity was detected as transparent bands against the background of Coomassie brilliant blue-stained gelatin. Gels were scanned using Fluor-S Multi-Imager (Bio-Rad) and analyzed for pro-MMP-2 and activated MMP-2 (72-and 64-kDa bands, respectively).

MMP-2 protein content was measured using commercially available ELISA kits and were performed according to the manufacturer's instructions (R&D Systems). Global MMP-2 activity was measured by the substrate: MCA-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2 (Biomol). Fluorescence was kinetically assessed using a microplate reader (340 excitation and 405 emission).

Cell culture measurements

A trophoblast invasion assay was used in which SGHPL-4 cells were grown on gelatin coated cytodex-3 microcarrier beads embedded in fibrin gels. In brief cells were at a density of 500000 cells/ml with 1500 microcarrier beads/ml and incubated for 1h at 37°C. Fibrin gels were prepared by dissolving bovine fibrinogen (>95% of protein clottable, Sigma) in PBS at a concentration of 2.5mg/ml and 200U/ml aprotinin (Trasylol, Bayer, Germany) was added. Cell-coated microcarrier beads were added at a density of 150 beads/ml and clotting was induced by the addition of thrombin (0.625U/ml). After clotting the gels were equilibrated with culture medium supplemented with 0.5% (v/v) FCS for 1h. The medium was replaced with fresh medium (1:1 volume with the gel) and the cells incubated at 37°C in a humidified atmosphere of 5% CO2. After 24h, the medium was replaced with medium containing the stimulus and/or the inhibitor as indicated. After 48h at least 10 beads from each plate were chosen at random and invasion was observed using an Olympus IX 70 phase contrast microscope. Images were captured and the number and length of the processes formed were determined using Image Pro-Plus software (Media Cybernetics).[5]

Literature:

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Supplementary Figure:

Figure S1

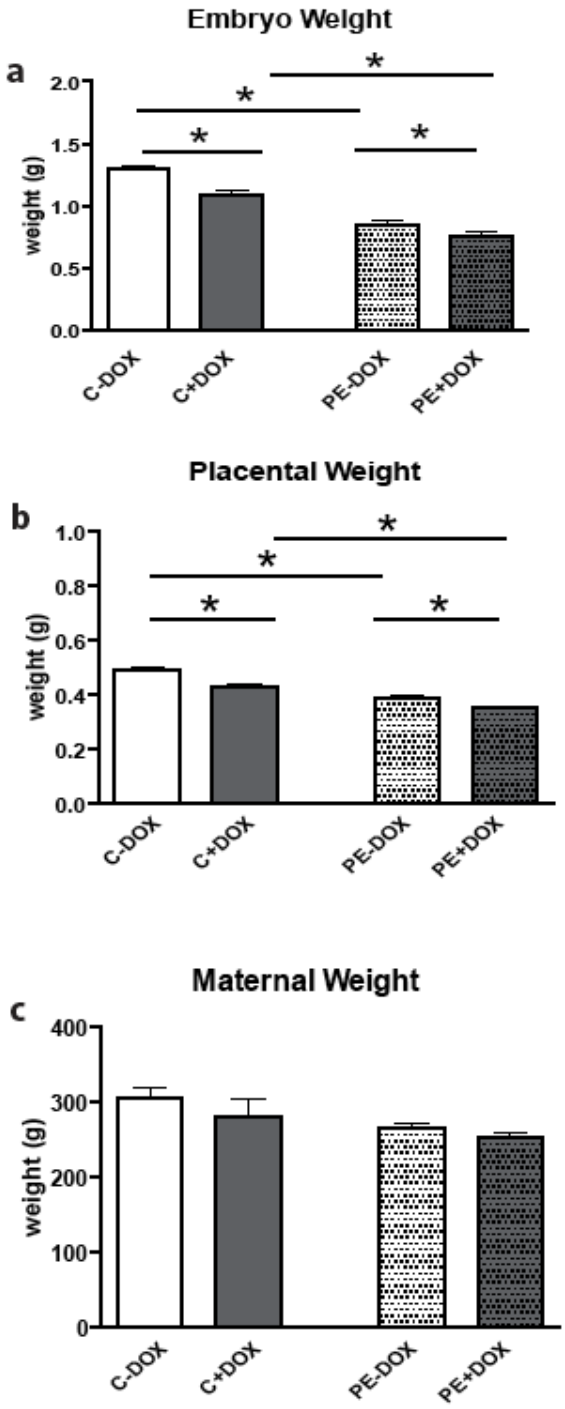

Fig S1. Embryonic weight (a) and placental weight (b) were reduced by treatment with 30 mg/kg bodyweight DOX per day from gestational day E11 - E18. Embryonic growth was reduced by 11.5 % in PE and 8.5 % in C. Placental weight was reduced by 10.5 % in PE and

12.5 % in C (n=45, *p<0.05). Maternal bodyweight (c) was not affected by the DOX treatment (n=6, n.s.).