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Expression of endothelin type B receptors in uterine artery smooth muscle cells from pregnant Guinea pigs

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A B S T R A C T

Introduction: It is well established that upregulation of endothelin type B (ET_B) receptors in vascular smooth muscle cells plays a role in pathophysiological artery remodeling as response to ischemia and atherosclerosis. This study aimed to investigate the ET_B receptors function and localization under normal physiological remodeling. Specifically, in the guinea pig uterine arteries during pregnancy.

Methods: Uterine artery contractility was assessed with sarafotoxin 6c and endothelin-1 in wire-myography in uterine arteries from non-pregnant and pregnant guinea pigs at gestational day 37 ± 5. Localization of ET_B receptors, proliferation marker Ki-67, and SMC differentiation marker SM22α in uterine arteries were investigated with immunohistochemistry.

Results: Uterine arteries from pregnant guinea pigs showed significantly increased ET_B receptor-mediated vasoconstriction compared to uterine arteries from non-pregnant and to coronary arteries from pregnant guinea pigs ($p < 0.001$), suggesting that ET_B-receptor upregulation in uterine artery SMCs is a normal physiological mechanism taking place during remodeling. Furthermore, uterine arteries from pregnant guinea pigs showed enhanced expression of ET_B receptors, high density of Ki-67 positive SMCs and sparse SM22α staining in SMCs localized in the outer layer of the vessel wall.

Discussion: Our results suggest that ET_B receptors are expressed in dedifferentiated proliferating SMCs of uterine arteries in pregnant guinea pigs. This study provides novel insight into the function and expression of ET_B receptors in uterine vascular remodeling during pregnancy.

1. Introduction

Normal pregnancy involves a progressive rise in uterine blood flow accomplished by extensive remodeling of the uterine vasculature [1]. The process of remodeling comprises dynamic cellular processes that include organization and structural changes of both extracellular and intracellular components, hyperplasia and hypertrophy, eventually leading to reduced uterine artery resistance and outward hypertrophic remodeling [2]. In guinea pigs, the increased uterine blood flow is brought about by increased uterine artery dilation partly mediated by increased nitric oxide (NO) synthesis by extravillous trophoblasts and by dedifferentiation of vascular smooth muscle cells (SMCs), leading to increased SMC proliferation in the uterine and radial arteries [3–7]. The contractile response of the SMCs to various neuronal circulatory factors is known to change in pregnancy i.e. the potency of neuropeptide Y induced contraction is attenuated [8], while α1-adrenoeceptor and vasopressin receptor-mediated contractions are increased in the uterine arteries [9,10]. Furthermore, pregnancy induces transcriptional upregulation and enhanced function of the large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel in uterine SMCs, which has been shown to attenuate the myogenic tone in uterine arteries [11–14].

Endothelins (ET), are powerful vasoconstrictor peptides comprising three distinct isoforms: ET-1, ET-2 and ET-3, coded by three different genes [15]. ET-1 is abundantly expressed in various organs such as heart, lung, ovary and gastrointestinal system and is the most prevalent isotype in the cardiovascular system [15]. ET-1 is synthesized primarily by vascular endothelial cells but during pregnancy, also by the myometrial SMCs [16]. ET-1 is known to be highly involved in uterine blood flow regulation and critical for the development of preeclampsia [17]. ET-2 is mainly expressed in intestines and ovaries [18], while ET-3 is highly expressed in the brain and intestinal tract [19] but also in vagina and endometrium [20]. All three ET isoforms are expressed in the female reproductive organs suggesting that they play a role in the paracrine control of the uterine vasculature [20]. However, the regulation of the endothelin receptor system in the uterine artery remodeling during pregnancy is less well characterized.

ETs acts through their binding to either of two types of G-protein-coupled receptors: the endothelin type A (ET_A) or the endothelin type B (ET_B) receptor. The binding affinity for ET_A and ET_B receptor varies between ET-1, ET-2 and ET-3. Hence, at physiologic concentrations, only ET-3 can distinguish between ET_A and ET_B receptors, because it has the same affinity for ET_B receptors as the two other isoforms, but has much lower affinity for ET_A than ET-1 and ET-2 [21]. ET_A receptors are located in the vascular SMCs and mediate vasoconstriction and

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proliferation [22], while the ET_B receptors are predominantly expressed in endothelial cells mediating vasodilatation (through NO release) and clearance of ET-1 [23]. However, the ET_B receptor can also be expressed in SMCs, where it displays high plasticity in the response to a variety of pathophysiological conditions such as pulmonary arterial hypertension, myocardial ischemia-reperfusion, cerebral ischemia and atherosclerosis [24–27]. ET_B receptors expressed in SMCs have been found to mediate vasoconstriction and proliferation [28], however their exact function has not yet been characterized. The objective of present study was to investigate whether ET_B receptor upregulation is also taking place during normal vascular remodeling of the uterine arteries during pregnancy. Our hypothesis was that pregnancy alters the uterine artery SMC expression and function of ET_B receptors leading to increased ET_B receptor-mediated vasoconstriction and ET_B receptor upregulation in the vessel wall undergoing remodeling.

2. Methods

2.1. Ethical approval

The study was approved by the Danish Animal Experimentation Inspectorate under the Ministry of Justice (permit numbers: 2012-15-2934-00205 and 2016–15 − 0201 − 01011) and approved by the Animal Care and Use Committee (IACUC, University of Copenhagen, Faculty of Health Sciences). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals [29].

2.2. In vivo

Fourteen non-pregnant (weight 395–456 g) and six pregnant (32–43 days gestation; term~ 63 days) Dunkin Hartley guinea pigs (Envigo, Venray, The Netherlands) were included in the study. The guinea pigs received regular chow diet containing 1500 mg vitC/kg diet (Ssniff-Spezialdiäten GmbH, Soest, Germany). Prior to anesthesia animals were sedated with Torbugesic Vet (2 mL/kg) (Butorphanol 10 mg/mL; ScanVet Animal Health, Fredensborg, Denmark) and anesthetized with 3–5% isofluorane (Isoba Vet 100%, Intervet International, Boxmeer, The Netherlands) in oxygen (Conoxia® 100%, AGA A/S, Copenhagen, Denmark) until disappearance of voluntary reflexes. The abdomen was opened and the uterus was exposed. Each of the fetuses were gently removed and immediately euthanized by decapitation. The guinea pig was euthanized by decapitation and the uterus and heart were removed and placed in chilled physiological saline solution.

2.3. Isometric force measurements

The radial (mesometrial) uterine artery [3] and left anterior descending coronary artery [30] were dissected from surrounding tissue, cut into 2 mm long circular segments, and mounted between two 40 μm stainless steel wires in a wire-myograph organ bath (610M, Danish Myo Technology, Aarhus, Denmark). The organ baths contained 6 mL physiological saline solution (PSS) (37 °C, pH 7.4) and were constantly aerated with 5% CO_2 in synthetic air. Vessels were equilibrated for 30 min before normalization to $0.9xL_{13.3kPa}$ for coronary arteries and $0.9xL_{2.7kPa}$ for radial arteries (where L_{xkPa} denotes the circumference of the artery at the specific pressure, x kPa). Artery segments were contracted 3 times with 60 mmol/L KCl-containing PSS before cumulative concentration response curves with the specific ET_B receptor agonist, Sarafotoxin 6c (S6c) (10^{-12} to 10^{-7} M) followed by a concentration response curve with the endogenous ligand, ET-1 (10^{-12} to 10^{-7} M). Contractile responses were measured by calculating the maximal force over a 5-min interval following addition of next concentration of the agonist (LabChart Pro 8, ADInstruments, Oxford, United Kingdom). BQ788 (10^{-7} M) was added 15 min prior to S6c concentration response curve and 0.007% methanol in PSS was used as vehicle control.

2.4. Organ culture

Uterine artery segments from four non-pregnant guinea pigs were dissected out of ligament adipose tissue. One segment was mounted immediately on an organ bath of a wire-myograph, while two segments from each guinea pig were organ cultured (48 h) in Dulbecco's Modified Eagles Medium (DMEM) as described previously [31]. Following organ culture, the segments were mounted on wire-myograph organ baths, and analyzed with isometric force measurements.

2.5. Solutions

PSS had the following composition in mM: 117.8 NaCl, 4.0 KCl, 2.0 $CaCl_2$, 0.9 $MgCl_2$, 1.25 NaH_2PO_4, 20 $NaHCO_3$, and 5.0 glucose. Endothelin-1, human, porcine (Catalogue No. SC324, PolyPeptide Group, Strassbourg, France), and Sarafotoxin S6c (SC457, PolyPeptide Group, Strassbourg, France) were diluted to a 10^{-4} M stock solution in 0.1% bovine serum albumin in milliQ water. BQ788 (B157, Merck, Denmark) was diluted to a 1.5 * 10^{-3} M stock solution in methanol.

2.6. Immunohistochemistry

Markers of cell proliferation (Ki-67) [32], SMC differentiation (SM22α) [33] and the ET_B receptor localization were investigated by immunohistochemical staining. The uterine arteries were fixated in 4% PFA overnight to preserve morphology and switched to 1% PFA. Then the artery segments were processed to paraffin wax and the tissue blocks were sectioned in 1–3 μm transverse sections. Before immunochemical staining, the sections were deparaffinized with distilled water and pre-treated (heated in a microwave) for 2 × 5 min with either citrate buffer (pH 6.0) (for SM22α and ET_B receptor samples) or Tris-EGTA buffer (pH 8.0) (for Ki-67). After 2 × 5 min incubation in Tris (hydroxymethyl) aminomethane/Tris (hydroxymethyl) aminomethane (TBS) (pH 7.6) the sections were blocked for endoperoxidases with hydrogenperoxide (3% for 10 min for SM22α samples and 0.6% for 15 min for Ki-67 and ET_BR) and washed in TBS (2 × 5 min). In order to block nonspecific background staining the sections were incubated for 5 min with Ultra V Block (Lab Vision, Thermo Fisher Scientific, Roskilde, Denmark) (for Ki-67 and ET_BR samples) or in 4% normal rabbit serum (DAKO, Glostrup Denmark) in TBS for 30 min followed by 2 × 15 min Avidin/Biotin blocking (Vector Laboratories, Peterborough, UK) (for SM22α samples). Sections were incubated over night at 4 C with primary antibodies (see Table 1) diluted in 1% bovine serum albumin and TBS. Control incubations were performed with primary ET_BR antibody that had been preincubated with the excess of the respective immunogen peptide (sequence CEMLRKKSGMQIALND), corresponding to amino acid residues 298–314 of rat ET_B receptor (Table 1). The next day the sections were washed in TBS. Binding of primary antibodies to the specific antigens (Ki-67, ET_BR and SM22α) were detected using three different methods (all from (Thermo Fisher Scientific, Roskilde, Denmark): UltraVision LP Detection System (HRP) (for Ki-67), Ultravision one detection system (HRP) (for ET_BR) and Vectastain ABC kit and avidin/biotin blocking (for SM22a) (Table 1). For the UltraVision LP Detection System (HRP) method, the sections were incubated with primary antibody enhancer for 20 min, washed for 2 × 5 min with TBS, incubated for 30 min in HRP polymer, washed 2 × 5 min with TBS, incubated for 10 min in AEC solution and washed 2 × 5 min with distilled water. Then sections were stained for 10 s in Mayers haematoxylin washed for 1 min in tap water and mounted with heated glycerol-gelatine.

For the Ultravision one detection system (HRP), the sections were incubated for 30 min with Ultravision One HRP polymer, washed 2 × 5 min in TBS, incubated for 10 min in 3-Amino-9-Ethylcarbazole (AEC), washed 2 times in distilled water, stained 10 s in Mayers haematoxylin, washed 1 min in tap water and 4 min in distilled water and mounted with heated glycerol-gelatine.

Table 1
Antibodies and detections systems used for immunohistochemistry.

Primary antibody	Ig-subtype	Clone	Source	Dilution	Pretreatment	Method
Ki-67	Monoclonal	MIB-1	DAKO, Glostrup, Denmark	1:200	HIER/Tris/EGTA	UltraVision LP Detection System (HRP)
ET_BR	Polyclonal rabbit Anti-Endothelin receptor B Alomone Labs AER-002 1 mg/ml	–	Alomone Labs, Jerusalem, Israel	1:400	Citrate	Ultravision one detection system (HRP)
ET_BR Blocking peptide,	CEMLRKKSGMQIALND, corresponding to amino acid residues 298–314 of rat ET-B 3rd intracellular loop.	–	Alomone Labs, Jerusalem, Israel	1 μg peptide per 1 μg antibody		
SM22α	Anti-SM22 goat polyclonal (ab10135)	–	Abcam®, Cambridge, United Kingdom	1:100	Citrate	Vectastain ABC kit (PK-6101) and avidin/ biotin blocking
	Secondary antibody, biotinylated rabbit anti goat (DAKO E0466)	–	DAKO, Glostrup, Denmark	1:400 in TBS		

In the Vectastain ABC kit and avidin/biotin blocking method, the sections were incubated for 1 h at room temperature with secondary antibody (biotinylated rabbit anti-goat), washed 2 × 5 min in TBS, incubated in ABC complex, washed 2 × 5 min in TBS, incubated 10 in in DAB, washed 2 × 5 min in distilled water, stained for 30 s in Mayers haematoxylin, washed in tap water followed by 4 min in distilled water and mounted with heated glycerol-gelatine. The staining was viewed under light-microscope with 20x and 40x objectives and pictures acquired with an Olympus B × 60 camera and Leica MC170 HD microscope.

2.7. Statistics

Force data (mN) was transformed to wall tension (Nm^{-1}) by dividing by twice the artery segment length and subtracting the baseline tension values. Active tension (T_1) was calculated by subtracting the passive tension from the potassium-induced active tension. In order to correct for different vessel diameters, the transmural pressure (P, kPa) was calculated by dividing the active tension times two with the diameter (d_1, μm) ($P = 2*T_1/d_1$) [34]. Contractions induced by S6c and ET-1 were expressed as a percentage of the maximal potassium-induced contraction and as absolute transmural pressure. Cumulative log(concentration)-response curves were fitted to non-linear regression with variable slope (four parameters). Concentrations of S6c and ET-1 eliciting 50% (EC50) of its own maximal response (Emax) were determined from the fitted non-linear regression equation Y = Bottom + (Top-Bottom)/(1 + 10^((LogEC50-X)*HillSlope)), Y, Bottom and Top were expressed in percentage of maximal potassium-induced contraction, and the X values were the logarithms of concentrations S6c or ET-1. HillSlope describes the steepness of the curves. All statistical analysis and graphs were performed in GraphPad Prism 8.00 (GraphPad Software, La Jolla, CA, USA). Differences between two groups were evaluated by a two-sided Student's t-test. Kruskal-Wallis with Dunn's multiple comparisons tests were used to compare potassium responses and artery segment diameters. For multiple comparisons, repeated-measures two-way ANOVA with Sidak's multiple comparisons test was applied. The results are expressed as mean ± SEM; N = number of animals. P-values of < 0.05 were considered statistically significant.

3. Results

3.1. Uterine artery remodeling

Fig. 1 shows representative pictures of the uterine arteries from the non-pregnant (left panel) and the pregnant (right panel) guinea pig at GD37 ± 5. Along with the uterine growth, the radial arteries undergo profound axial (longitudinal) and circumferential enlargement.

3.2. Effect of S6c and ET-1 on uterine arteries

The specific ET_B receptor agonist, S6c (10^{-10} – 10^{-7} M) induced a negligible contraction in uterine arteries from non-pregnant guinea pigs (Emax = 3.70 ± 0.98%) and no contraction in coronary arteries from pregnant guinea pigs. Pregnancy induced a significantly increased concentration-dependent contractile response for S6c in uterine arteries, with an Emax = 190.7 ± 31.9% and pEC50 = 7.87 ± 0.18 ($p < 0.0001$ compared to contractile responses in both coronary arteries and uterine arteries from non-pregnant guinea pigs; Fig. 2A). The similar significant effect of S6c-induced contraction mediated by pregnancy was found, when the force was normalized to diameter and vessel length (calculated as transmural pressure, kPa) (Fig. 2B).

ET-1 (10^{-10} – 10^{-7} M) induced concentration-dependent contractions in all three arterial segments (uterine arteries from non-pregnant guinea pigs: Emax = 125.1 ± 17.1% of potassium contraction and pEC_{50} = 7.77 ± 0.13; uterine arteries from pregnant guinea pigs:

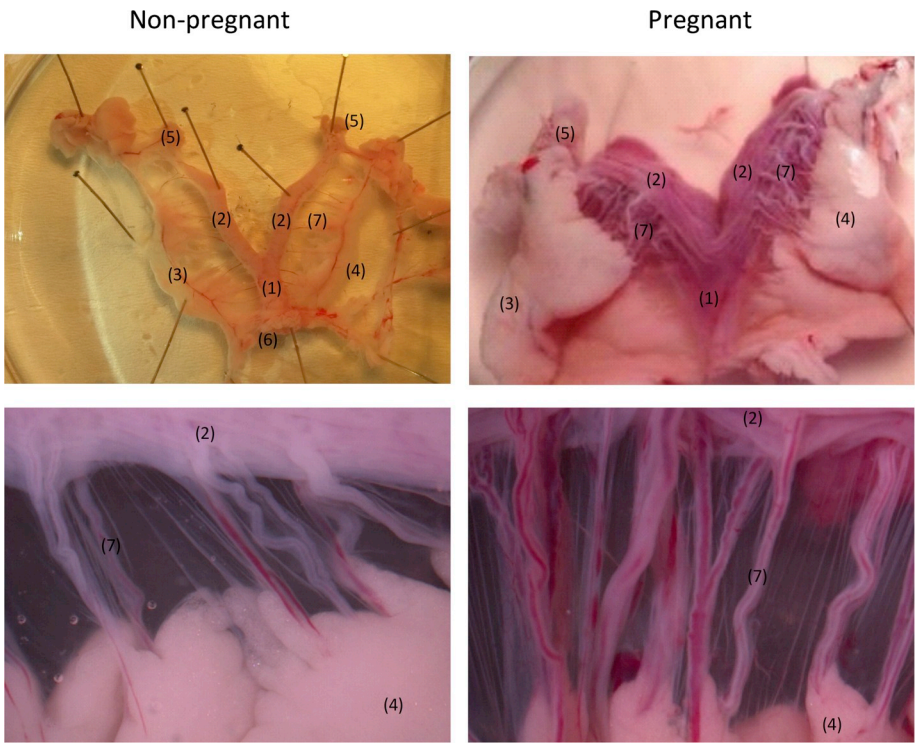

Fig. 1. Representative picture of uteri (upper panels) and (radial) uterine arteries (lower panels) from non-pregnant and pregnant (GD37 ± 5) guinea pigs. Anatomical overview. The Y –shaped guinea pig uterus consists of a joint corpus uterus (1) with and two horns, cornu uteri (2) dexter and sinister (respectively). The uterine artery (3) originates from the internal iliac. The uterine artery runs in the broad uterine ligament (4) (*lig. latum uteri*) along the uterus, lastly anastomosing with the ovarian artery that ensures blood to the ovaries (5). The blood supply to the uterine horns and cervix (6) is ensured by several tortous, vertical vessel branches, the radial arteries (7). As pregnancy advances, the uterine blood supply increases as does the diameter and wall thickness of the uterine vessels. The broad ligament has extensive deposition of adipose tissue, which completely covers the uterine artery of the pregnant uterus.

Emax = 225.6 ± 107.7 and pEC_{50} = 7.23 ± 0.42; and coronary arteries from pregnant guinea pigs: Emax = 161.6 ± 17.4 and pEC_{50} = 7.61 ± 0.08) with no statistical differences between the three groups (Fig. 2C). However, when ET-1-induced tensions were normalized to the diameters, as calculated by the transmural pressure, the uterine arteries from pregnant guinea pigs showed a significantly attenuated response compared to those from non-pregnant guinea pigs ($p < 0.05$) (Fig. 2D).

3.3. Effect of pregnancy on uterine artery diameter and potassium-induced contraction

Diameter of the arteries was calculated from the passive circumference – determined at the myograph normalization procedure. Uterine arteries from pregnant guinea pigs had significantly increased diameters (521 ± 70.5 μm) compared to uterine arteries from non-pregnant (251 ± 33.7 μm) guinea pigs ($p < 0.05$). However, the diameters of the coronary artery segments (437 ± 29 μm) were comparable and not significantly different from the uterine arteries from the pregnant guinea pigs (Fig. 2E). Potassium-induced contractions in isolated uterine arteries from pregnant guinea pigs (3.3 ± 1.1 Nm^{-1}) were not significantly different compared to uterine arteries from non-pregnant (5.3 ± 1.0 Nm^{-1}) or to coronary arteries from pregnant guinea pigs (7.3 ± 1.0 Nm^{-1}), however calculated as absolute transmural pressure revealed significantly lower potassium responses in uterine arteries from pregnant (7.9 ± 1.0 kPa) compared to non-pregnant (25.1 ± 2.2 kPa) guinea pigs ($p < 0.05$). The coronary arteries had a slightly (non-significantly, $p > 0.05$) higher potassium-induced contraction (11.8 ± 1.1 kPa) compared to uterine arteries in pregnant guinea pigs (Fig. 2F).

3.4. Expression of ET_B receptors, Ki-67 and $SM_{22\alpha}$ in uterine arteries

Pregnancy induced a number of cytological changes in the uterine artery vessel wall that were not seen in uterine arteries from non-pregnant guinea pigs. In uterine arteries from non-pregnant guinea pigs, the ET_B receptors were mainly expressed in the endothelial layer, whereas pregnancy resulted in ET_B receptor expression in tunica intima, media and adventitia of the uterine artery (Fig. 3A, D, G and J). The density of Ki-67 positive cells was exclusively high in the adventitia of uterine arteries from pregnant guinea pigs (Fig. 3B, E, H and K), in addition with high ET_B receptor expression. In contrast, the density of $SM_{22\alpha}$ was remarkably lower in the adventitia compared to the media in uterine arteries from pregnant guinea pigs (Fig. 3C, F, I, L). The observed changes are indicative of dedifferentiation and proliferation of SMCs particularly in the adventitia of the uterine arteries from pregnant guinea pigs.

3.5. Validation of S6c and ET_B receptor immunoreactivity

In order to confirm that S6c (10^{-10} – 10^{-7} M) binds to ET_B receptors, S6c induced responses were tested in presence of the specific competitive ET_B receptor antagonist, BQ788 (0.1 μM). Organ culture of uterine arteries from non-pregnant guinea pigs induced ET_B receptor expression (Fig. 4C–F) and significantly increased S6c contractile responses (Fig. 4A and B) comparable to the pregnant state (Fig. 2A and B). The specific ET_B receptor antagonist BQ788 fully blocked the S6c induced contractile response, confirming a high selectivity of the agonist to ET_B receptors (Fig. 4A and B). In uterine arteries from pregnant guinea pigs, ET_B receptor immunoreactivity was completely abolished after sections were preadsorped with the corresponding blocking peptide (Fig. 4E–H).

4. Discussion

The present study shows for the first time that ET_B receptors are highly expressed in the vessel wall of uterine arteries from pregnant guinea pigs. The ET_B receptor agonist, S6c, induced a net vasoconstriction in uterine arteries from pregnant guinea pigs, whereas uterine arteries from non-pregnant guinea pigs revealed negligible contraction. Uterine arteries from pregnant guinea pigs expressed ET_B receptors in the outermost layer of the vessel wall with high expression of Ki-67 positive cells, whereas the smooth muscle cell differentiation marker, $SM_{22\alpha}$ [35], was less densely expressed in areas with high ET_B receptor

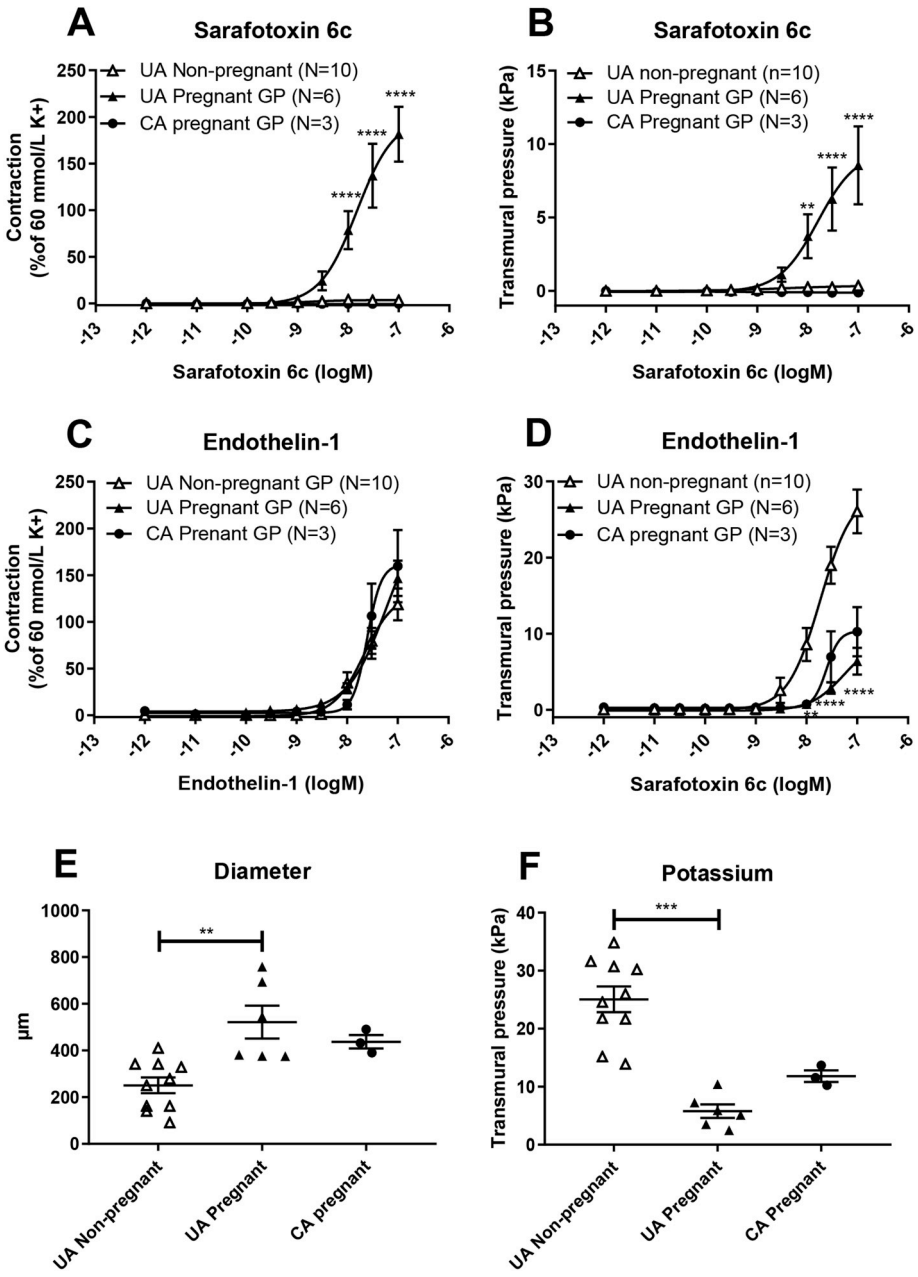

Fig. 2. Contractile responses to cumulative concentrations of S6c (A, B) and ET-1 (C, D); passive diameter calculated from internal diameter (E); contractile responses to high potassium (F); obtained at normalization in coronary arteries (CA) from pregnant guinea pigs (filled circles) and uterine arteries (UA) from non-pregnant (open triangle) and pregnant guinea pigs (filled triangle). Means ± SEM (N = 3–10 animals). *p < 0.05 and ****p < 0.0001.

expression. Collectively, these results suggest that ET_B receptors are expressed in proliferating dedifferentiated SMCs in uterine arteries undergoing outward hypertropic remodeling during pregnancy, whereas differentiated SMCs do not express ET_B receptors.

During normal pregnancy, the uteroplacental vasculature undergoes extensive structural and functional modifications to meet the metabolic needs of the fetus. These changes lead to increased fetoplacental blood flow, which in the guinea pig is accomplished by an increased passive diameter of the uterine and radial arteries along with increased SMC proliferation, which has been found to be most pronounced in the adventitia [36,37]. This remodeling has been shown to induce a 3.5-fold radial artery elongation and a 13-fold increase in mean cross-sectional area of the vessel wall [2,7]. In our study, we observed that uterine arteries from midterm (GD37 ± 5) pregnant guinea pigs had significantly increased passive diameter and decreased potassium-induced transmural pressure responses compared to uterine arteries from non-pregnant guinea pigs.

Our results show that the ET_B receptor agonist, S6c, mediated a net vasoconstriction in uterine arteries from pregnant guinea pigs, whereas uterine arteries from non-pregnant only revealed negligible S6c-induced contraction. During pregnancy, the SMCs of the uteroplacental arteries undergo phenotypic modulation and dedifferentiate as a part of the remodeling process [3,6]. The phenotypic modulation from a contractile differentiated to a dedifferentiated proliferative phenotype is associated with altered expression and reorganization of contractile and cytoskeletal proteins, leading to altered contractile capacity. Furthermore, dedifferentiated SMCs have increased proliferative and migratory capacity along with increased protein synthesis [38]. Our results show that SMCs located in the outermost layer of the uterine artery of pregnant guinea pigs shifted to a dedifferentiated phenotype with increased expression of the proliferation marker Ki-67 and decreased expression of the differentiation marker SM22α.

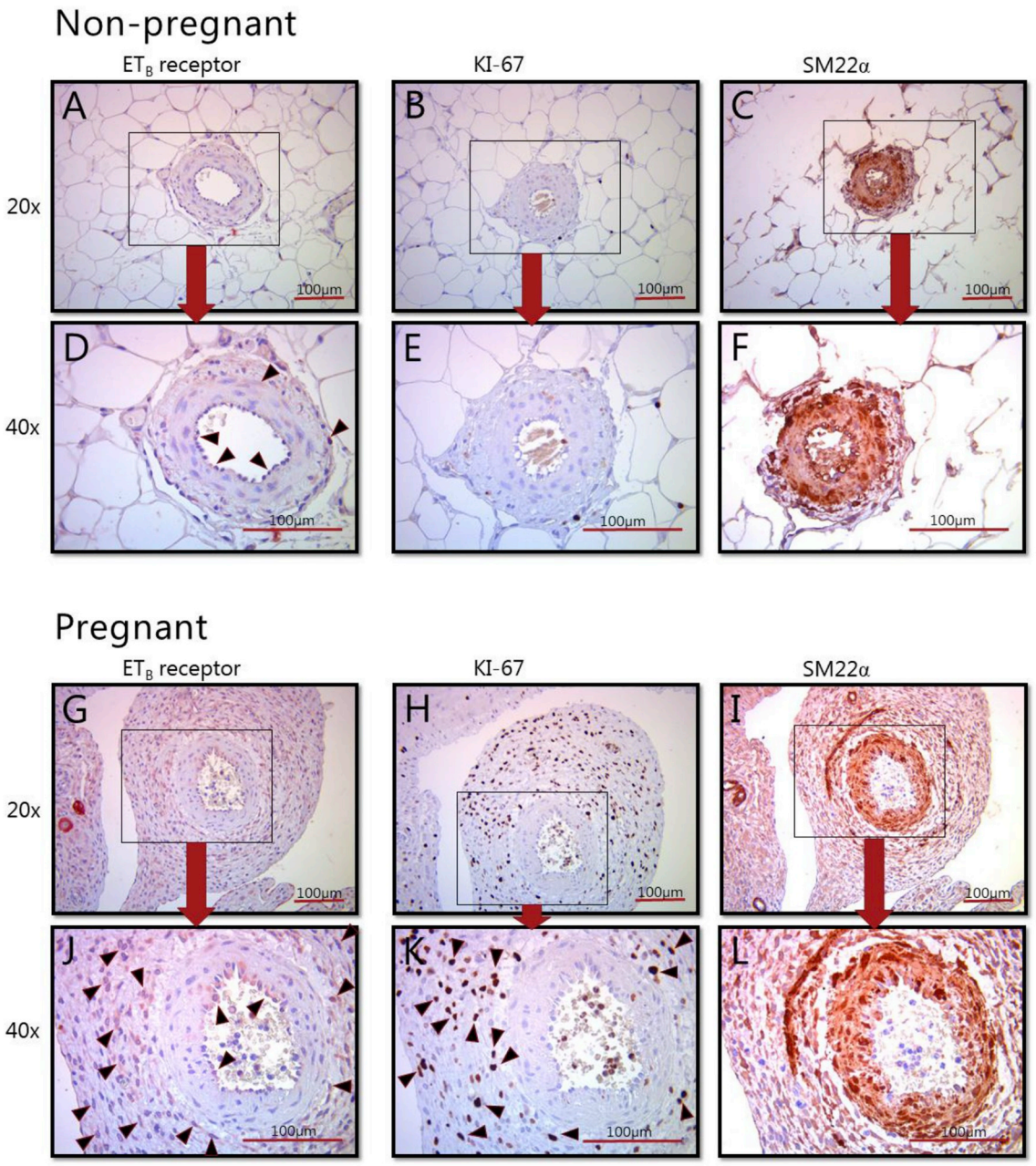

Fig. 3. Representative cross sections of uterine arteries, from non-pregnant and pregnant guinea pigs, immunohistologically stained for the ET_B receptor (panels A, D, G and J), Ki-67 (panels B, E, H and K) and $SM_{22\alpha}$ (panels C, F, I and L) from non-pregnant (A–F) and pregnant guinea pigs (G–L). In uterine arteries from non-pregnant guinea pigs, ET_B receptors are expressed mainly in the endothelial cells (A and D). In the uterine arteries from pregnant guinea pigs, the ET_B receptor expression is however also present in the outermost adventitia layer (G and J). In uterine arteries from pregnant guinea pigs, the expression of ET_B receptors in adventitia along with high density of Ki-67 positive cells (panel H and K), suggesting that ET_B receptors are expressed in proliferating dedifferentiated SMCs. The SMC contractile marker SM22α is weakly expressed in the outermost adventitia layer of uterine arteries from pregnant guinea pigs (panel I and L). In contrast, SM22α positive cells are highly expressed in media (C, F, I and L), where the expression of ET_B receptor and Ki-67 immuno-positive cells are absent. Magnifications 20X and 40X objectives.

Dedifferentiation of SMCs not only takes place under normophysiological conditions but is also important in response to vessel injury and pathophysiological events such as myocardial ischemia-reperfusion and atherosclerogenesis [38,39] — conditions that also show increased SMCs ET_B receptor expression [25,40]. We have recently found that transcriptional regulation of ET_B receptors (EDNRB) in SMCs is controlled by a balance of transcriptional inputs between myocardin-related transcription factors (MRTFs) and ternary complex factors (TCF) [31]. Specifically, we found that several co-activators of serum response factor (SRF) increased the expression of EDNRB, including MRTF, MKL-1, the classical TCF, ELK3, and the atypical SRF co-activator, FLI1. Furthermore, we found that EDNRB expression was highly induced by actin depolymerization [31], a process that is also known to be induced in uterine arteries by pregnancy and after treatment of non-pregnant sheep with 17β-estradiol and progesterone [41]. In the present study, the mechanisms behind ET_B receptor upregulation in pregnant guinea pig uterine arteries were not determined, but future studies will attempt to unravel these.

The endothelin-receptor system is not unique for driving an altered response and expression pattern during uterine artery remodeling.

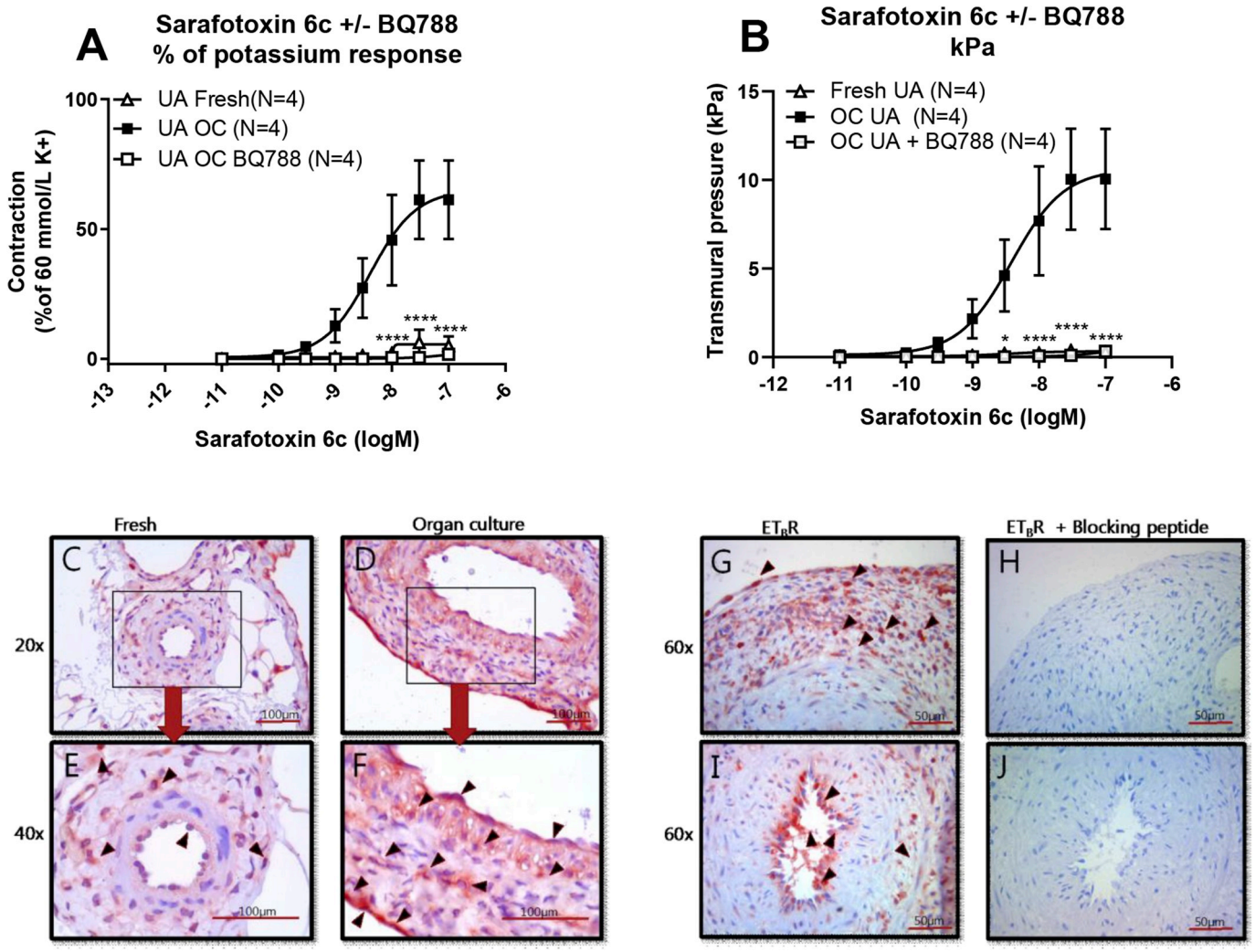

Fig. 4. Contractile responses to cumulative concentrations of S6c (A, B) obtained in uterine arteries (UA) from non-pregnant (open triangle), UA after organ-culture (filled square) and UA after organ cultured and in presence of the ET_B receptor antagonist BQ788 (0.1 μM) (open square). Means ± SEM (N = 4 animals). *p < 0.05 and ****p < 0.0001 comparing OC + BQ788 with OC.

Altered vasoconstrictor responses in uterine arteries induced by pregnancy and sex hormone (17β-estradiol and progesterone) treatment of non-pregnant guinea pigs have been reported for other receptor-ligand systems as noradrenalin/α1-adrenoceptors, vasopressin, neuropeptide Y, oxytocin, serotonin, angiotensin-II/AT1 and AT2 receptors [8–10,42–47].

Our finding that ET_B receptors mediating contraction are upregulated in the pregnancy-induced remodeled uterine artery is a novel and so far unrecognized effect of the uterine artery remodeling process. While our studies focused on the functional (contractile) effects of ET_B receptors it is also possible that ET_B receptors are involved in endothelial cell migration [48] and proliferative mechanisms [49] during remodeling. Future studies are needed to elucidate the mechanisms involved in ET_B receptor upregulation, as well as if the expression is limited to the mid-term of the pregnancy or persist in the entire pregnancy period. Previous studies have shown that ET_A receptors dominate VSMC contraction over ET_B receptor and that a 'crosstalk' mechanism between the two receptors in SMCs amplify their down-stream signaling pathways [50]. Furthermore, ET_B receptors are rapidly desensitized and internalized compared to ET_A receptors [51], which can affect receptor specific vasomotor effects after ligand binding. A limitation of our study is that we primarily focus on the functional regulation and localization of ET_B receptors. Future studies are therefore required to elucidate the involvement of ET_A and ET_B receptors in regulation of uterine blood flow during pregnancy including the effects of the three isoforms of endothelin, ET-1, ET-2 and ET-3, respectively. Furthermore, it would be interesting to correlate the ET_B receptor density with the remodeling index of the uterine arteries.

In conclusion, our results show that ET_B receptors are expressed in the vessel wall of uterine arteries of pregnant guinea pigs. The ET_B receptors mediate a net constriction in uterine arteries from pregnant guinea pigs, whereas uterine arteries from non-pregnant only produce negligible constriction. The ET_B receptors are expressed primarily in the adventitia of the uterine arteries with Ki-67 positive cells and low $SM_{22\alpha}$ expression, suggesting that ET_B receptors are expressed in dedifferentiated proliferating SMCs of the uterine arteries in pregnant guinea pigs. Our study provides novel insights into the function and expression of ET_B receptors in uterine vascular remodeling during pregnancy.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2019.01.015.

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