

Cloprostenol, a prostaglandin F_{2α} analog, induces hypoxia in rat placenta: BOLD contrast MRI

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ABSTRACT: Blood oxygen level dependent (BOLD) contrast was used to monitor hypoxia induced by cloprostenol, a prostaglandin F_{2α} (PGF_{2α}) analog, in the rat embryo-placental unit (EPU). It is shown that administration of cloprostenol (0.025 mg/rat) at mid-gestation (day 16) reduced EPU oxygenation, as detected by BOLD contrast MRI, in correlation with induction of vascular endothelial growth factor (VEGF) gene (*Vegfa*) expression in the corresponding placenta ($r = 0.56$, $p = 0.03$). Elevated VEGF mRNA expression in response to cloprostenol treatment was also observed at early gestation (day 9) in the forming placenta ($p = 0.04$) and uterus ($p = 0.03$). Cloprostenol increased the expression levels of endothelin-1 (ET-1) gene (*Edn1*) ($p = 0.03$) and its corresponding peptide ($p = 0.02$) in the forming placenta, as well as the expression of the endothelin receptor type A (ETA) gene (*Ednra*) in both the forming placenta ($p = 0.009$) and the uterus ($p = 0.01$). The levels of the endothelin receptor type B (ETB) gene (*Ednrb*) were not affected in response to cloprostenol, but a significant elevation in the expression level of this receptor was observed in the uterus at mid- and late gestation (day 22) ($p = 0.04$ and 0.01 respectively), suggesting a role for ETB in the vasodilatory status of the pregnant uterus. It is suggested that PGF_{2α} induces uteroplacental vasoconstriction in the rat, and that ET-1 may take part in mediating this effect, probably via activation of ETA receptor. The uteroplacental vasoconstriction induces hypoxia, as manifested by significant changes in BOLD MRI and by upregulation of VEGF. Copyright © 2006 John Wiley & Sons, Ltd.

KEYWORDS: BOLD MRI; PGF_{2α} analog; cloprostenol; endothelin; VEGF; hypoxia; placenta; uterus

INTRODUCTION

The placenta is a complex transient endocrine organ that serves as a circulatory interface between the fetus and the mother. Increasing evidence indicates that most embryonic losses that occur during early human pregnancy may be due to placental vasospasm generated by an impaired balance between vasodilatory and vasoconstrictory agents (1,2). Indeed, reduced placental vascular development and increased vascular resistance have been associated with early embryonic mortality (3,4). Several substances have been suggested to be involved in such pathological events, among which prostaglandin F_{2α} (PGF_{2α}) is included. In support of this idea, elevated PGF_{2α} concentrations have been demonstrated in human decidual tissue at preeclampsia (5). Along this line, it

has been suggested that the elevated levels of progesterone during pregnancy inhibit the detrimental release of uterine PGF_{2α}(6).

It has been reported that endothelin-1 (ET-1), which is a potent vasoconstrictor (7), mediates the action of PGF_{2α} in bovine, ovine and rat corpora lutea (8–11). ET-1 can act through either of the two receptor types, endothelin receptor type A (ETA) or endothelin receptor type B (ETB). It has been shown that ETs activate the ETA receptors on the arterial smooth muscle to cause contraction, whereas activation of ETB receptors on the endothelium releases substances such as nitric oxide and prostacyclin, which relax the artery (12,13). The ratio between vasoconstriction and vasodilatation may determine the vascular tonus in a specific organ. The expression of ET-1 and its receptors in placental vasculature (14) suggests that ETs may play a role in the regulation of the fetoplacental vascular tonus. Indeed, a potential role of ET-1 in hypertensive pregnancy and its possible involvement in preeclampsia have been suggested (15,16). Furthermore, since ET-1 is expressed by the umbilical cord (17), its role in the fetoplacental unit has been of major interest.

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Abbreviations used: PGF_{2α}, prostaglandin F_{2α}; EPU, embryo-placental unit; ET-1, endothelin-1; VEGF, vascular endothelial growth factor; ETA, endothelin receptor type A; ETB, endothelin receptor type B; P4, progesterone; HIF-1, hypoxia-inducible factor-1; BOLD, blood oxygen level dependent.

It has been previously shown that ET-1 mediates the action of PGF_{2α} in rat corpora lutea (11). Thus, it was hypothesized that ET-1 may play a mediatory role in the placental response to PGF_{2α}, and this was examined by analysis of the expression of ET-1 and its receptors in the forming placenta and in the uterus in response to administration of a PGF_{2α} analog at early gestation. It was further hypothesized that ET-1-mediated placental response to PGF_{2α} may induce vasospasm and subsequent ischemic conditions (5,8). To investigate PGF_{2α}-induced hypoxia in the rat embryo-placental unit (EPU), BOLD contrast MRI was applied. As hypoxia triggers the expression of vascular endothelial growth factor VEGF (18), this factor was used as a marker for PGF_{2α}-induced hypoxia at both early and late gestation.

EXPERIMENTAL

Chemicals, drugs and reagents

Highly specific monoclonal antibodies to progesterone (P4) were generously provided by Dr F. Kohen (The Weizmann Institute of Science, Rehovot, Israel). Rabbit antibodies to mouse immunoglobulins were purchased from Dako A/S (Denmark). Sheep antibodies to ETA receptor were acquired from Biogenesis (London, UK). Rabbit antibodies to ETA and to ETB receptors were purchased from Alomone Labs (Jerusalem, Israel). HRP-goat antirabbit IgG (H + L) and HRP-donkey antisheep IgG (H + L) were bought from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Protein assay reagent was acquired from Bio-Rad Laboratories GmbH (Munich, Germany). C18 cartridges were purchased from J.T. Baker Chemical Co. (SPE, octadecyl; J.T. Baker Chemical Co., Phillipsburg, NJ). Enhanced chemiluminescence (ECL) reagents were bought from Amersham Life Science (Buckinghamshire, UK). Estrumate (cloprostenol, an analog of PGF_{2α}) was purchased from Coopers Animal Health Ltd (Berkhamsted, UK). Bovine serum albumin (BSA, fraction V, protease free) and sepharose-protein A conjugated beads were acquired from Sigma Chemical Co. (St Louis, MO). ELISA kit for ET-1 (ET-1 cell culture) was purchased from Biomedica (Vienna, Austria). Primers for mRNA of ET-1, ETA and ETB were acquired from Genosys Biotech. Ltd (London, UK). Acrylamide and bis-acrylamide were from Merck (Darmstadt, Germany). FUJI Medical X-Ray Super RX film was from FUJI Photo Film Co. (Tokyo, Japan). Nitrocellulose (0.2 μm pore size) was obtained from Schleicher and Schuell (Dassel, Germany). All other chemicals were of analytical grade and were acquired from Sigma Chemical Co. (St Louis, MO) or Merck (Darmstadt, Germany).

Animal model

Sexually mature Wistar female rats (Harlan Biotech, Rehovot, Israel; body weight 200–250 g, 12 weeks old), showing three consecutive four-day cycles (examined daily by vaginal cytology) were housed on a 14 h light and 10 h dark cycle schedule at 21 °C and 55% humidity. The rats were allowed free access to food and water. Proestrous females were caged with fertile males overnight, separated on the next morning and analyzed for the presence of a vaginal plug. This day was considered as day 1 of pregnancy. When indicated, a single intraperitoneal injection of cloprostenol (0.025 mg/500 μL), a PGF_{2α} analog, dissolved in saline, was administered on the indicated day of pregnancy. The efficiency of this dose of cloprostenol treatment in the present animal model was previously evaluated by serum progesterone determination (11). Control rats were injected with saline. Untreated pregnant as well as non-pregnant rats at different stages of their estrous cycle were also employed in this study. All protocols were conducted in accordance with the NRC Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy of Science, Bethesda, MD, USA).

MRI studies

Rats on day 16 of pregnancy were anesthetized by one dose of Vetranquil (0.6 mg/50 μL/rat; IP) followed by isoflurane/N₂O/O₂ inhalation and then subjected to MRI analysis. For this purpose, gradient echo (GE) images were acquired using blood oxygen level dependent (BOLD) contrast, showing individual EPUs, before and after administration of cloprostenol or saline. Reduced signal intensity corresponds to increased content of deoxyhemoglobin and thus to reduced blood oxygenation. The amount of deoxyhemoglobin in the tissue can be affected by oxygen extraction in the tissue, oxygen delivery by blood flow, the diameter and density of the vessels and the flux of red blood cells (19). However, in the closed fetal vascular system there are no changes in the total blood volume. In this case, a decreased MR signal intensity represents reduced 'reload' of the fetal red blood cells with fresh oxygen, i.e. hypoxia (20). To allow comparison, the intensity measured for each specific EPU was scaled by calculating the ratio of the averaged MR signal intensity at each time point after the injection of cloprostenol or saline to the average pre-injected signal intensity of that EPU.

GE images were acquired on a horizontal 4.7-T Bruker-Biospec spectrometer (Karlsruhe, Germany) using a whole-body birdcage RF coil. Anesthetized rats were placed supine on a perspex board and immobilized using adhesive tape. Acquisition parameters were: number of slices 15, slice thickness 0.8 mm, no radio frequency

spoiling, *TR* 230 ms, *TE* 10 ms, spectral width 25 000 Hz, FOV 7 cm, 256 × 256 pixels, in-plane resolution 0.27 mm, 117 s/image and no dummy scans. Two averages were acquired for each image, and the total scan time was 1 h per rat. Five images were acquired prior to the injection of cloprostenol, and 18 consecutive images were acquired after the injection of this PGF_{2α} analog. The first image after injection was discarded in the data analysis, to allow time for completion of drug distribution. MRI data were analyzed on an Indigo-2/02 workstation (Silicon Graphics, Mountain View, CA) using Paravision software (Bruker, Germany). Regions of interest (ROIs) including the embryo-placental units (EPU), were manually selected, excluding regions that could include partial volume contribution of the uterus. For each EPU, signal intensity was determined prior to and at each time point after administration of cloprostenol, and the data are presented as the ratio of signal intensity post-injection to the pre-injected intensity. Hypoxia is manifested by signal attenuation and a ratio lower than 1 (26). In a separate experiment, pregnant rats were injected with saline only and analyzed in the same manner.

Tissue preparation

Rats, administered with cloprostenol on either day 9 (early gestation) or day 16 (mid-gestation) of pregnancy, were sacrificed at the indicated time points after treatment and their uteri were recovered. On day 9 of pregnancy, the placenta has not yet fully developed. The mesometrial one-third of the deciduum was therefore isolated (21). This part contains trophoblast giant cells and the ectoplacental cone. It may also include some extraembryonic membranes as the chorion and the allantois. This complex would be referred to as forming placenta. The remaining part of the deciduum, including the fetus, was discarded. By day 16 of pregnancy, the placenta is fully formed and can therefore be easily separated. Uterine tissue was also recovered from untreated rats at the different stages of the estrous cycle, as well as on either day 9 or day 22 of pregnancy (uterine tissue during

pregnancy consists of myometrium). The recovered tissues were immediately frozen in liquid N₂ and stored at -80 °C until use.

Evaluation of VEGF, ET-1, ETA and ETB receptor gene expression

The expression of *Vegfa*, *Edn1*, *Ednra* and *Ednrb* genes encoding for VEGF, ET-1, ETA and ETB respectively was analyzed by reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method (22). The precipitated RNA was washed with 70% ethanol. The concentration of the extracted RNA was calculated by determination of the optical density at 260 nm. The ratio of the optical density of 260 nm to 280 nm was always >1.9.

Aliquots of total RNA (1 μg) were reverse transcribed using random primers. The RT reaction contained 50 units of moloney murine leukemia virus reverse transcriptase (MLV-RT), 200 μM dNTP, 6.5 mM MgCl₂, 20 units of RNasin, 0.5 ng oligo dT and 1.5 × PCR buffer in a total volume of 20 μL. The reaction was performed at 37 °C for 2 h. The vials were stored at -80 °C until PCR was performed. Fragments of the reverse-transcribed ET-1 cDNA were amplified using a radiolabeled nucleotide ([α-³²P]dCTP) and pairs of specific primers. The sequence of the primers used is shown in Table 1. The cDNA amplification products for ET-1 were predicted to contain 382 base pairs (bp). PCR reactions were further performed in the same RT-test vial which finally contained 20 μM of each primer, 200 μM dNTP, 2.5 mM MgCl₂, 2 μCi [α-³²P]dCTP, 1 × PCR buffer and 2.5 units of *Taq* polymerase to amplify a portion of the cDNA. In total, 31 cycles for ET-1 were employed after incubation of 2 min at 94 °C as follows: 94 °C for 1 min (denaturation); 62 °C for 1 min (annealing) and 72 °C for 2 min (elongation), followed by a final extension for 10 min at 72 °C. The radioactive products were size fractionated by 5% non-denaturing polyacrylamide gel electrophoresis in 0.5 × TBE buffer. Gels were dried and radioactivity was visualized by exposure to X-ray film.

Table 1. Sequence of the primers used in the study

Gene	Bases	Primers
ET-1	Sense (340–361) Antisense (721–700)	5'-TTG CTC CTG CTC CTC CTT GATG-3' 5'-GGT CTT GAT GCT GTT GCT GATG-3'
ETA	Sense (121–140) Antisense (518–497)	5'-CAG TGC TAA TCT AAG CAG CC-3' 5'-TGC AGA GAA ACA CTC CAA AAT C-3'
ETB	Sense (307–326) Antisense (727–709)	5'-AGG CCA CAC CAT CTC TTC TC-3' 5'-AGC TTG CAC ATC TCA GCT C-3'
VEGF	Sense (324–345) Antisense (528–506)	5'-CAA ACC TCA CCA AAG CCA GCA C-3' 5'-CAA ATG CTT TCT CCG CTC TGA AC-3'
S-16	Sense Antisense	5'-CGT TCA CCT TGA TGA GCC CAT T-3' 5'-TCC AAG GGT CCG CTG CAG TC-3'

PCR for the ET receptors was performed with the specific primers, directed to the non-homologous areas. The cDNA amplification products were predicted to be 398 bp in length for the ETA receptor and 421 bp for the ETB receptor. The following conditions were used for the PCR reaction: 25 cycles both for ETA and for ETB were employed after incubation of 2 min at 94 °C as follows: 94 °C for 30 s (denaturation); 58 °C for 30 s (annealing) and 72 °C for 1 min (extension), followed by a final extension for 5 min at 72 °C.

The following conditions were used for the PCR reaction for VEGF: 26 cycles were employed after incubation of 5 min at 95 °C as follows: 95 °C for 1 min (denaturation), 58 °C for 1 min (annealing) and 72 °C for 2 min (extension), followed by a final extension for 10 min at 72 °C.

The number of PCR cycles for each cDNA product was in the linear range of the amplification curve. Linear correlation between cDNA concentrations and signal intensity has been demonstrated by the analysis of three dilutions of each sample. The identity of the PCR products was confirmed by direct DNA sequencing.

Quantitation was performed by densitometric analysis of the autoradiograms normalized against ribosomal protein S16 mRNA, which served as an internal standard. The RT-PCR reaction of S16 mRNA was performed separately. Densitometric analysis was performed utilizing the Pdi 420oe densitometer supported by Quantity One software (Pdi, Huntington Station, NY, USA).

Western blot analysis

The tissues were homogenized in a lysis buffer (11). Protein concentration was determined by Bradford assay (23), using BSA as the standard protein. Protein fractions of 100 µg were subjected to immunoprecipitation for the ETA receptor that was immunoprecipitated with rabbit-specific polyclonal antibodies, and fractions of 30–80 µg protein were subjected to conventional Western blot analysis for the ETB receptor, all elaborated by Girsh and Dekel (11). Briefly, all samples were subjected to electrophoresis on a 12% polyacrylamide gel, electroblotted onto nitrocellulose membrane and immunodetected with sheep-specific polyclonal antibodies to the ETA receptor (final concentration 12 µg/mL) and with rabbit-specific polyclonal antibodies to the ETB receptor (final concentration 1.5 µg/mL). After subjection to ECL reagents and exposure to X-ray film, the proteins were quantitated by densitometric analysis.

ET-1 extraction and determination

The tissues were extracted as described by Kitamura *et al.* (24). Briefly, tissues were homogenized in 10 vol. of 1 M acetic acid, sonicated and boiled. Homogenates were centrifuged and the supernatants were loaded on C18 cartridges pre-equilibrated with 1 M acetic acid. The cartridges were washed with 1 M acetic acid, and adsorbed

materials were eluted with 3 mL 60% acetonitrile in 0.1% trifluoroacetic acid. The eluates were evaporated under N₂ to dryness, dissolved in a minimum volume of 0.1 M acetic acid and then dissolved in ELISA work solution. ET-1 was determined by a commercial ELISA kit. The standard curve for ET-1 ranged from 2.9 to 94 fmol/mL. The sample was in the linear range of the standard curve. Cross-reactivities of ET-1 antiserum with ET-1, ET-2, ET-3 and big endothelin were 100, 100, <5 and <1% respectively. The intra- and interassay coefficients of variation were 4 and 7% respectively.

Statistical analysis

For each independent experiment, at least three tissues (placenta/uterus) from one individual rat per time point were used. Most of the experiments were repeated 5 times, with a total number of five rats examined for each time point. In MRI experiments, each single EPU in a particular rat was analyzed individually, comparing cloprostenol-injected versus pre-injected control (a total of 47 EPUs). Values were averaged from ROIs of 1–3 slices for each EPU and then used to determine overall signal intensity change measured pre- and post-treatment. The results (*i.e.* *p* values) obtained from EPUs of each individual rat (with a total of six rats) were then combined, and the analysis was performed, per rat. For comparing treated versus pre-injected control, a Wilcoxon signed rank test was used for each EPU. The *p* values obtained from EPUs of each rat (with a total of six rats) were then used as inputs for Fisher's inverse chi-square method. This method combines *Kp* values obtained from *K* independent studies into one *p* value. The analyses were performed by Statistical Analysis System (SAS) for Windows V8 (25). Signal ratios of saline-injected pregnant rats were also calculated, and Student's two-tailed *t*-test analysis was performed, comparing cloprostenol versus saline-injected ratios.

Densitometry data from Western and Southern blot experiments were expressed as arbitrary density units, converted to percentage of control and compared by Friedman's test, followed by Dunn's multiple comparison procedure. Paired results were compared by paired *t*-test. Values of *p* < 0.05 were considered statistically significant.

RESULTS

Cloprostenol-induced reduction of oxygenation in the rat embryo-placental unit

The possible effect of PGF_{2α} analog on EPU oxygenation was tested using BOLD contrast MRI. For this purpose, GE images of rats at mid-gestation were acquired, allowing analysis of changes in BOLD contrast in

individual EPUs before and after injection of the PGF_{2α} analog, cloprostenol (Fig. 1). Each bar represents the ratio between the signal obtained before and after cloprostenol or saline injection from one individual EPU. A value lower than 1 represents signal loss due to an elevated level of deoxyhemoglobin, and thus is indicative of hypoxic stress ((26), see details in the experimental section). A significant reduction ($p=0.01$) in this ratio was found at 40 min after injection of cloprostenol in 32 out of the 47 individual EPUs examined (Fig. 1A and data from the following Fig. 3). The general effect of cloprostenol in reduction of the EPU MRI signal was significant ($p=0.02$) even when all 47 EPUs were included, including the non-responsive ones. When testing the response of all EPUs in each dam, a significance of $p=0.008$ was found for rat 1, $p=0.005$ for rat 2, $p=0.05$ for rat 3, $p=0.07$ for rat 4, $p=0.01$ for rat 5 and $p=0.1$ for rat 6. Four out of six rats significantly responded to the treatment, one was close to being significant ($p=0.07$) and one dam did not respond ($p=0.1$). The overall analysis of these six rats revealed a significant ($p=0.02$) mean of 10% signal reduction in rats after cloprostenol treatment. Analysis of images of the same rats acquired 60 min after injection of cloprostenol yielded similar results (data not shown). The duration of continuous anesthesia restricted MRI follow-up to 1 h. In a separate experiment, four pregnant rats were injected with saline only (Fig. 1B) and a mean 1% signal reduction was calculated. The pre- to post-signal ratios of cloprostenol versus saline injection were found to be significantly different ($p=0.025$). Moreover, the MRI data acquired 10 min prior to treatment can serve for internal control, as the main effect of PGF_{2α} analog on the BOLD signal also occurred during the same timescale (Fig. 1C) versus the insignificant effect of saline injection (Fig. 1D).

VEGF gene expression in rat placenta and uterus

Hypoxia induces expression of hypoxia-regulated genes by stabilization of hypoxia-inducible factor-1 (HIF-1) and expression of its downstream targets including VEGF (18). The forming placentae and uteri of rats administered with cloprostenol at early gestation were therefore analyzed for VEGF expression. RT-PCR analysis revealed an elevated expression of *Vegfa* gene encoding for VEGF in the forming placenta and uterus as soon as 1 h after cloprostenol administration ($p=0.04$ and 0.03 respectively) (Fig. 2). The elevated *Vegfa* gene expression was sustained for at least 2 h.

In order to establish the correlation between hypoxic stress and elevated VEGF expression on an individual EPU basis, the following additional experiment was performed at mid-gestation. Rats were anesthetized and administered with cloprostenol. BOLD contrast GE MRI

data were acquired before and at 40 min after treatment. The rats were then sacrificed, and individual placentae from specific EPUs identified by MRI were further analyzed for VEGF expression. A positive correlation was found between the degree of MRI-monitored hypoxic stress (or negative correlation between MRI ratio) for the specific EPUs and PCR-determined expression of *Vegfa* gene in the 14 individually analyzed placentae ($r=-0.56$, $p=0.03$) (Fig. 3).

ET-1 expression in rat forming placenta and uterus

PGF_{2α}-induced reduction of oxygenation may be subsequent to vasoconstriction. It was previously demonstrated that ET-1, a potent vasoconstrictor, mediates the effect of PGF_{2α} in the rat corpus luteum (11). To assess the possible involvement of ET-1 in PGF_{2α} action in rat forming placenta and uterus, the effect of cloprostenol on the expression of *Edn1* gene encoding for ET-1 and its corresponding peptide in these tissues was studied. A substantial elevation of the expression of *Edn1* mRNA in early-gestation forming placenta at 2 h after cloprostenol administration (70%, $p=0.03$) was found (Fig. 4). In the uterus, the expression of *Edn1* mRNA after this treatment was not significantly different from that in the control (Fig. 4).

In accordance with the elevation in mRNA levels, the ET peptide concentration in the forming placenta increased from 96.9 fmol/mg before treatment to 214.7 fmol/mg protein ($p=0.02$) at 2 h after cloprostenol administration (Fig. 5). The elevation of ET in the uterus was not statistically significant (Fig. 5).

ET receptor expression in rat forming placenta and uterus

In addition to modulation of its expression, the effect of ET-1 can be regulated at the level of the corresponding receptor availability. The effect of PGF_{2α} on the expression of the two types of endothelin receptor, ETA and ETB, in rat forming placenta and uterus was therefore studied.

It was found that the forming placenta as well as the uterus of rats on day 9 of pregnancy expresses mRNA of *Ednra* gene encoding for ETA and *Ednrb* gene encoding for ETB. The amount of mRNA for *Ednra* at 1 h after cloprostenol administration was elevated by 25 and 10% in the forming placenta and uterine tissues respectively ($p=0.009$ and 0.01 respectively) (Fig. 6A), with no further alteration at 2 h (data not shown). The expression of the *Ednrb* mRNA in the forming placenta and the uterus was not affected by cloprostenol (Fig. 6B).

Analysis of uterine samples, recovered at different stages of the estrous cycle as well as throughout

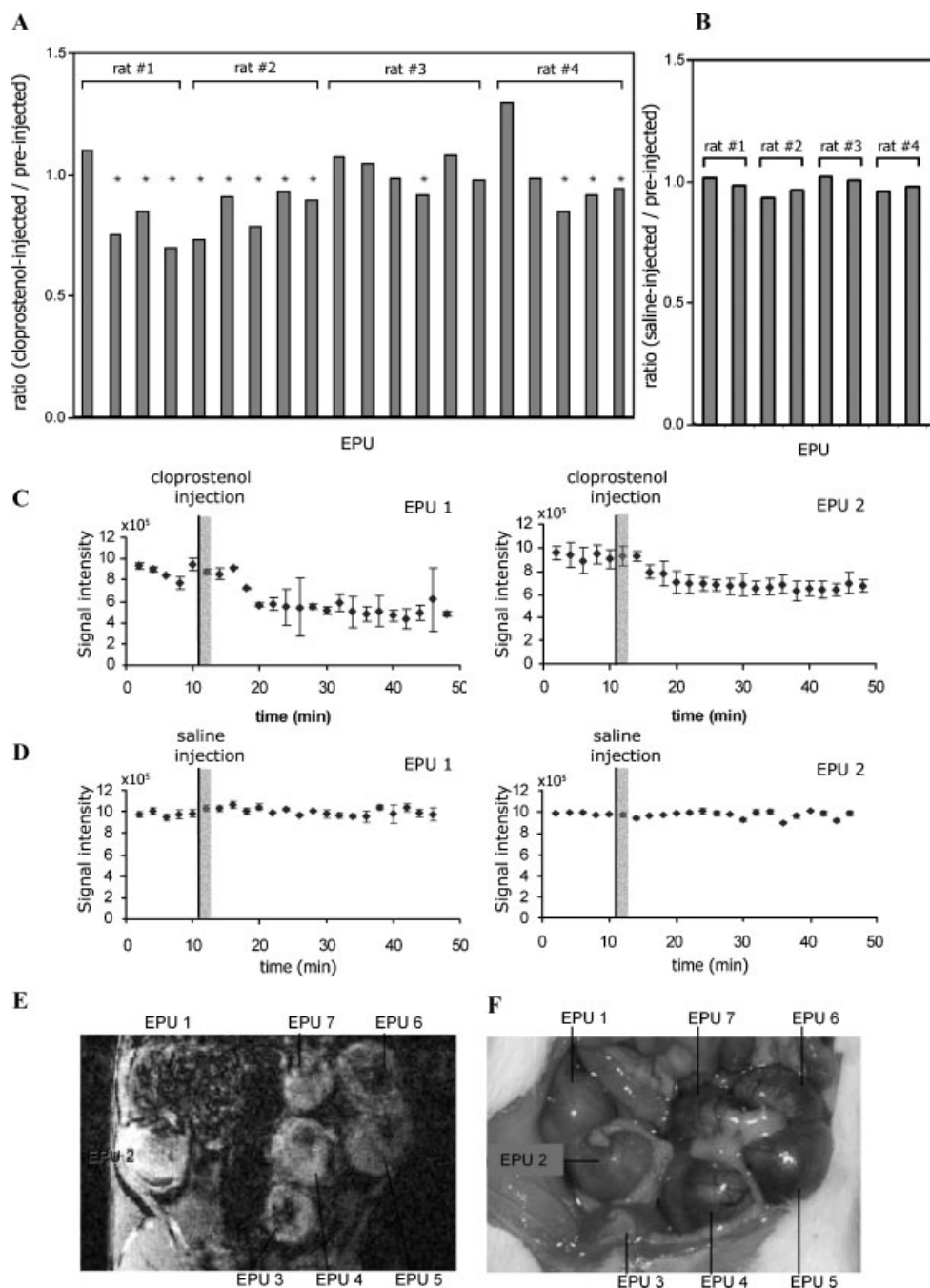


Figure 1. MRI analysis of rat pregnancy after $\text{PGF}_{2\alpha}$ analog treatment. Rats on day 16 of pregnancy were anesthetized. (A) BOLD contrast GE images were used for analysis of the change in signal intensity in specific regions of interest of an individual embryo placenta unit (EPU). The ratio of signal intensity determined 40 min after cloprosteno- administration to the pretreatment signal intensity is presented for each EPU. As described in the materials and methods section, a ratio of 1 represents an MR signal intensity that has not changed after treatment, while values lower than 1 represent reduced oxygenation. Each bar represents one individual EPU, and the asterisks represent significant reduction in oxygenation for that specific EPU ($p = 0.03$). Accordingly, reduction in oxygenation was manifested by a decreased ratio of the averaged MR signal intensity obtained from the images taken at 40 min after cloprosteno- injection and those acquired prior to the injection. Data of 4 dams including a total of 20 EPUs are presented. (B) Ratios of four saline-injected pregnant rats (two EPUs examined per rat). (C) Plots of signal intensity as a function of time for two representative EPUs (black line marks cloprosteno- administration; grey box marks the first image after injection which was discarded in the data analysis to allow time for completion of drug distribution). (D) Plots of signal intensity as a function of time for two representative EPUs of saline injected rats. (E) A representative GE MR image. (F) A macroscopic picture of the rat in (B), taken at the end of the experiment

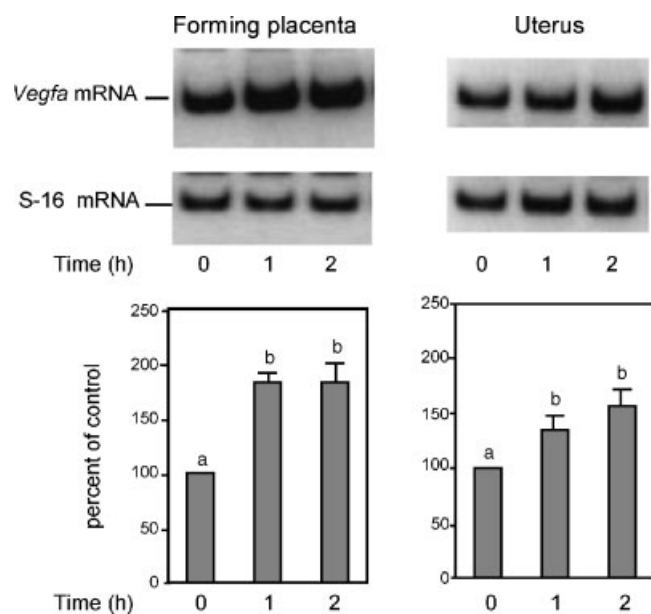


Figure 2. VEGF expression in the forming placenta and the uterus of pregnant rats treated with a $\text{PGF}_{2\alpha}$ analog. Forming placentae and uteri were collected from rats on day 9 of pregnancy at 0, 1 and 2 h after cloprostenol injection. RNA isolated from either the uterus or the forming placenta of individual rats was subjected to RT-PCR analysis, as described in the materials and methods section. Upper panel: the results of RT-PCR analysis of one representative out of five independent experiments with similar results. Lower panel: cumulative results of quantitation of the five independent experiments. Data are presented as mean \pm SE. A total number of 15 rats were examined. Different letters denote significant difference ($p = 0.04$ and 0.03)

pregnancy, revealed that the amount of ETA receptor in the uterus was constant (Fig. 7A). The amount of ETB receptor in the uterus did not change throughout the estrous cycle but was substantially elevated on day 9 and day 22 of pregnancy ($p = 0.04$ and 0.01 respectively, Fig. 7B).

DISCUSSION

This study suggests a mediatory role for ET-1 in the $\text{PGF}_{2\alpha}$ -induced hypoxia in the rat EPU. The $\text{PGF}_{2\alpha}$ -induced hypoxia is exemplified herein by uniquely utilizing BOLD contrast MRI to monitor hypoxia induced in the rat EPU in response to the $\text{PGF}_{2\alpha}$ analog, cloprostenol. EPU deoxygenation was demonstrated *in vivo* by employment of BOLD contrast GE MRI (Fig. 1). Blood oxygenation can be affected by oxygen extraction, blood flow and the diameter of the vessel, as well as by the density and flux of red blood cells (20). This method has been previously shown to provide a sensitive tool for monitoring changes in vessel density, function and response to therapy in tumor xenografts (26–28). A

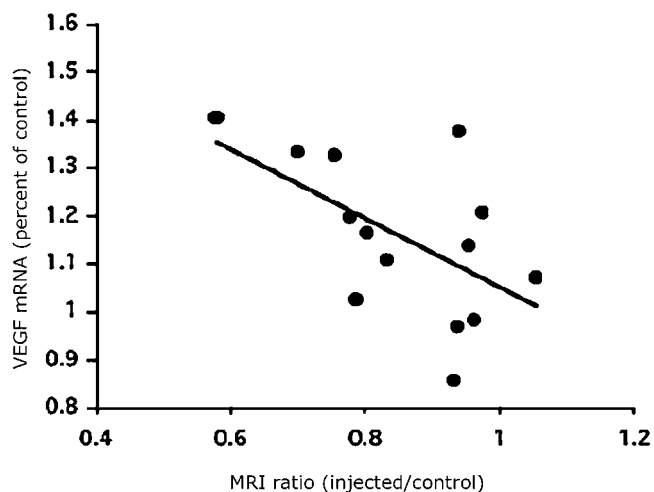


Figure 3. Correlation between MRI analysis of individual EPUs and VEGF expression in individual placentae of pregnant rats treated with a $\text{PGF}_{2\alpha}$ analog on day 16 of pregnancy. GE images before and after treatment were acquired, and the ratio of the MR signal intensity in individual EPUs was calculated as described in Fig. 1 and in the materials and methods section. Individual placentae were recovered from the rats at 1 h after cloprostenol injection and processed for the analysis of VEGF expression by RT-PCR. Results obtained from 14 MRI-EPUs and corresponding placentae-VEGF revealed significant correlation between hypoxia detected by BOLD contrast MRI and placental expression of VEGF ($p = 0.03$)

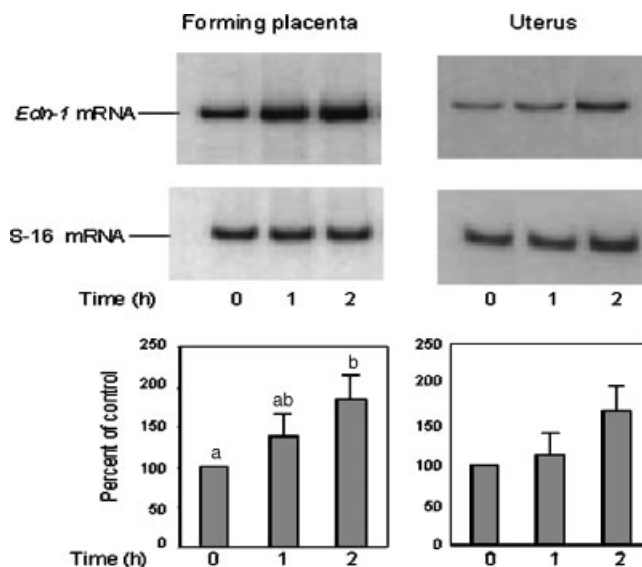


Figure 4. ET-1 expression in the forming placenta and the uterus of pregnant rats treated with a $\text{PGF}_{2\alpha}$ analog. Forming placentae and uteri were collected from rats on day 9 of pregnancy at 0, 1 and 2 h after a single injection of cloprostenol. RNA isolated from either the uterus or the forming placenta of individual rats was subjected to RT-PCR analysis, as described in the materials and methods section. Upper panel: the results of RT-PCR analysis from one representative out of five independent experiments with similar results. Lower panel: quantitation of the results obtained from the five independent experiments. A total number of 15 rats were examined. Data are presented as mean \pm SE. Different letters denote significant difference ($p = 0.03$)

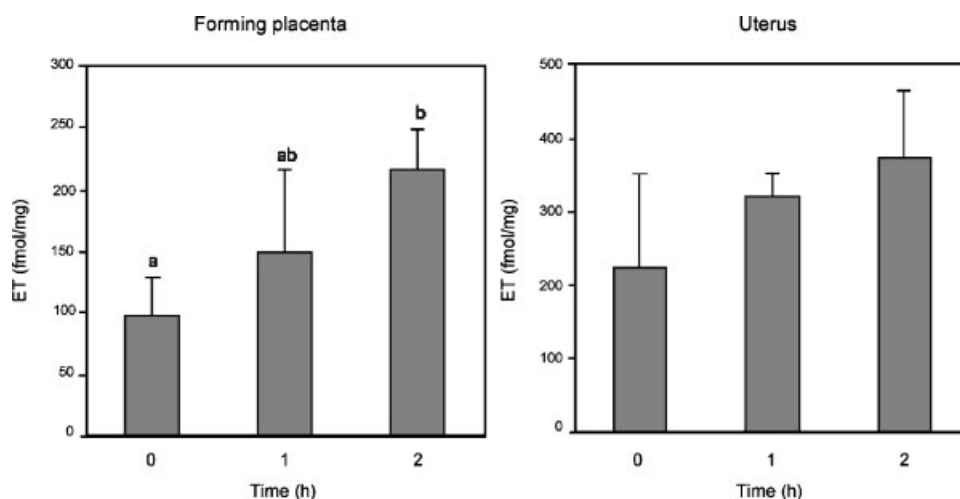


Figure 5. ET content in the forming placenta and the uterus of pregnant rats treated with a $\text{PGF}_{2\alpha}$ analog. Forming placentae and uteri were collected from rats on day 9 of pregnancy at 0, 1 and 2 h after a single injection of cloprostenol. Peptides extracted from either the uterus or the forming placenta of individual rats were subjected to ELISA analysis, as described in the materials and methods section. Cumulative data of three independent experiments including three rats each (a total number of nine rats) are presented as mean \pm SE. Different letters denote significant difference ($p = 0.02$)

novel application of BOLD contrast MRI for monitoring changes in oxygen levels during pregnancy by utilizing the intrinsic contrast of deoxyhemoglobin is reported here. This application takes into account the fact that the fetoplacental vasculature is a closed system. Thus, the reduction in the MR signal intensity detected by this method in the EPU represents a decreased level of blood oxygenation.

The $\text{PGF}_{2\alpha}$ analog-induced expression of ET-1 suggested herein can probably be attributed to the development of the ischemic condition, in agreement with previous findings in rat corpora lutea (11). Moreover, the $\text{PGF}_{2\alpha}$ analog-induced decrease in oxygenation in the EPU that was demonstrated correlated significantly with elevated levels of placental expression of *Vegfa* mRNA, a central hypoxia-induced angiogenic growth factor (18). An increase in vascular tone, frequently associated with a reduced maternal uteroplacental flow, characterizes preeclampsia. The nature of the hypertension in pregnancy suggests that the abnormal increase in blood pressure is dependent on some factor(s) that mediate vasospasm in the vascular system. Tissue 8-iso-prostaglandin $\text{F}_{2\alpha}$ was found to be significantly elevated in decidual tissue of preeclamptic women and may mediate this maternal vascular dysfunction (5). The demonstration that 8-iso- $\text{PGF}_{2\alpha}$ stimulates *Edn-1* mRNA and its protein expression in aortic endothelial cells (29) supports this assumption. The present observation that a $\text{PGF}_{2\alpha}$ analog increased the levels of *Edn-1* mRNA encoding for ET-1 and its peptide in the forming placenta (Figs 4 and 5) further suggests that these two stereoisomers share some similarities in their mechanism of action. Moreover, the present findings demonstrate that the $\text{PGF}_{2\alpha}$ analog administration is consistent with upregulation of *Edn-1*

and *Vegfa* (Fig. 2) in the forming placenta on day 9 of pregnancy, even though vessel formation has not yet been completed. Taken together, these findings support the idea that the reduced level of oxygenation induced by $\text{PGF}_{2\alpha}$ may be mediated by ET-1. The involvement of ET-1 in this pathology was suggested by the demonstration of elevated levels of maternal plasma ET in pregnant women with preeclampsia (15,30–35). It was also suggested that ET-1 has a direct effect on the fetoplacental vasculature, thus contributing to the development of abnormal fetoplacental resistance (36,37).

Endothelial cells are a fundamental compartment of the vascular bed. The vascular density of placental tissues (maternal and fetal) continues to increase slowly throughout gestation (38), and $\text{PGF}_{2\alpha}$ may stimulate this compartment to secrete ET-1. It was previously shown that human placenta at term contains immunoreactive oxytocin which is released from placental cells under $\text{PGF}_{2\alpha}$ stimulation (39). It was shown *in vitro* that oxytocin and $\text{PGF}_{2\alpha}$ stimulate secretion of ET-1 from endometrial cells (40), and further hypothesized that placental blood flow is regulated by compartmentalized eicosanoids (41). It is therefore possible that oxytocin and eicosanoids may contribute to regulation of a uterine vasomotor effect (accompanied with elevation of ET-1), which under pathological conditions may lead to hypoxia. It was further reported that hypoxic conditions augment ET-1 secretion in a positive feedback manner (42).

Previous reports have identified *Edn-1* mRNAs in placental tissue (43–45). Demonstration of prepro-*Edn-1* mRNA in cytotrophoblasts and trophoblastic giant cells of the basal zone, as well as in endothelial cells of the placental vessels, further indicates that the ET-1 in the placenta is derived from both maternal and fetal sources

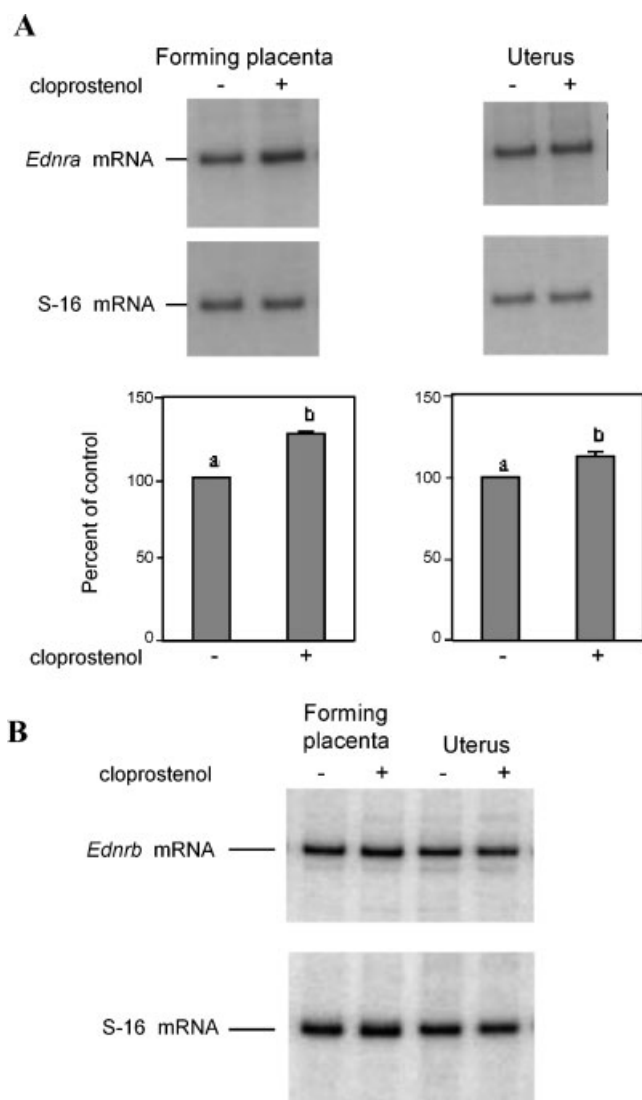


Figure 6. (A) ETA receptor gene expression in the forming placenta and the uterus of pregnant rats. Forming placenta and uteri were collected from rats on day 9 of pregnancy before (cloprostamol -) and at 1 h after (cloprostamol +) a single injection of the $\text{PGF}_{2\alpha}$ analog. The upper panel presents results of RT-PCR analysis from one representative out of five independent experiments with similar results. The histograms represent cumulative data from the five independent experiments (a total number of 15 rats; $p = 0.009$). (B) ETB receptor gene expression in the forming placenta and the uterus of pregnant rats. Data of one representative out of five independent experiments with similar results are presented (a total number of 15 rats were examined; $p = 0.1$).

(14). In accordance with this, ET-like immunoreactivity was shown not only in vascular areas but also in trophoblasts, decidual tissue and the epithelium of the amnion membrane (14,46,47). It is demonstrated in the present paper that the amount of *Edn-1* mRNA is elevated in the forming placenta at 2 h after $\text{PGF}_{2\alpha}$ analog injection and that this elevation is accompanied with an increase in the corresponding ET peptide product (Figs 4 and 5). The present data therefore suggest that $\text{PGF}_{2\alpha}$ may be an

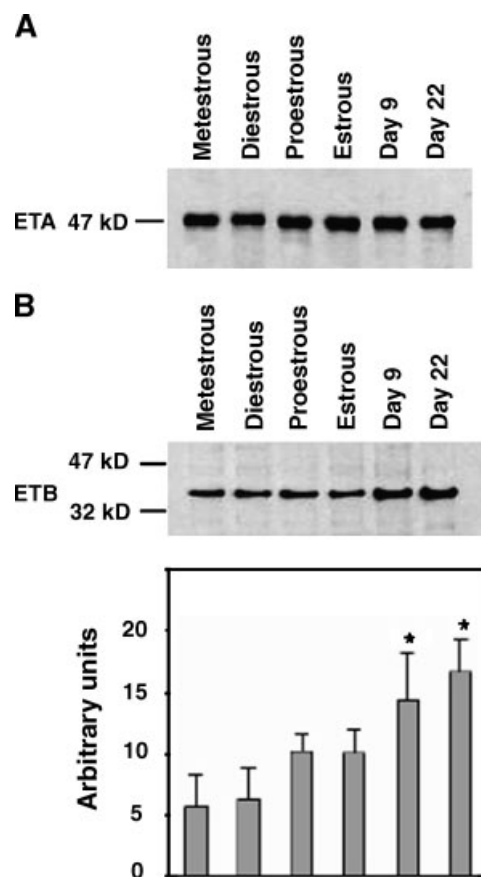


Figure 7. ETA (A) and ETB (B) receptor protein expression in rat uterus. Uteri were collected from rats at different stages of the estrous cycle as well as on day 9 and day 22 of pregnancy. Proteins extracted from uteri recovered from individual rats were subjected to Western blot analysis, as described in the materials and methods section. Data of one representative out of four independent experiments with similar results are presented (a total number of 24 rats were examined). The cumulative quantitated results of the four independent experiments are presented ($p = 0.04$ and 0.01 for day 9 and day 22 respectively).

upstream activator of ET-1 expression in the forming placenta as early as day 9 of gestation.

ET acts through two known types of receptor, type A and type B. Shigematsu *et al.* (14) have shown expression and localization of mRNA for *Ednra* and *Ednrb* encoding for ETA and ETB receptors respectively in the decidual cells and vascular wall of rat placenta at mid- (day 14) and late (days 17 and 19) gestation. Specific ET-1 binding sites have been identified in trophoblasts, decidual tissue and vessels (43,48–50). The present study confirms the presence of mRNA of both types of endothelin receptor in the placenta (Fig. 6). In agreement with the role of ETA in causing vasoconstriction (12,13), it is shown that this gene was upregulated in response to the $\text{PGF}_{2\alpha}$ analog while the levels of ETB were unchanged.

The present study demonstrates that, in addition to the forming placenta, the rat uterus also expresses ET-1 and

its two types of receptor (Fig. 7). Immunoreactivity for big ET-1 and ET-1 is known to be present in the uterine tissue (51–54), and uterotonic effects of these peptides in non-pregnant rats are well documented (55–61). Pregnant as well as non-pregnant rat uteri are highly sensitive to ET-1 (59,62,63) and the contractile effects of ETs on the uterus is mediated by ETA receptors (57,58,61). The ETA receptors are predominantly localized in rat myometrium (64), and ETA-mediated myometrial contractions have indeed been demonstrated (65,66). Furthermore, uterotonic responses were inhibited by the ETA receptor antagonist BQ₁₂₃, but not by the ETB receptor antagonist BQ₇₈₈(67). Utilization of selective antagonists to ETA and ETB receptors recently implied the involvement of endothelin in hypoxia-induced uteroplacental pathophysiology (68,69). The presence of ETB receptors in the rat, rabbit, sheep and human myometrium has previously been demonstrated (53,63–65). The present study demonstrates an elevation of the ETB receptor level during pregnancy, which could be associated with a vasodilatory influence, mediated by this type of receptor.

Taken together, the present results are consistent with the hypothesis that PGF_{2α} induces uteroplacental hypoxia in the rat and that ET-1 may take part in mediating this effect, probably via activation of the ETA receptor. The resulting vasoconstriction associated with the PGF_{2α} effect on uteroplacental vasculature induces hypoxia, detected by BOLD contrast GE MRI and also exemplified by upregulation of VEGF expression. The present study lays the foundation for further studies involving modulation of components of the suggested pathway. The novel use of functional BOLD contrast MRI proposed here to detect and measure uteroplacental hypoxia can be applied for elucidating pathological events such as preeclampsia. As BOLD contrast MRI utilizes the intrinsic contrast of deoxygenation, it not only can be used for basic research of pregnancy but also could potentially be translated for clinical imaging.

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