Haemoglobin expression in human endometrium

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BACKGROUND: The general concept that haemoglobin is only a carrier protein for oxygen and carbon dioxide is challenged since recent studies have shown haemoglobin expression in non-erythroid cells and the protection of haemoglobin against oxidative and nitrosative stress. Using microarrays, we previously showed expression of haemoglobins α, β, δ and γ and the haeme metabolizing enzyme, haeme oxygenase (HO)-1 in human endometrium.

METHODS: Using real-time quantitative PCR, haemoglobin α, β, δ and γ, and HO-1 mRNA levels were assessed throughout the menstrual cycle (n = 30 women). Haemoglobin and HO-1 protein levels in the human endometrium were assessed with immunohistochemistry. For steroid responsiveness, menstrual and late proliferative-phase endometrial explants were cultured for 24 h in the presence of vehicle (0.1% ethanol), estradiol (17β-E2, 1 nM) or 17β-E2 + Org 2058 (1 nM each).

RESULTS: All haemoglobins and the HO-1 were expressed in normal human endometrium. Haemoglobin mRNA and protein expression did not vary significantly during the menstrual cycle. Explant culture with Org 2058 or 17β-E2 + Org 2058 increased haemoglobin γ mRNA expression (P < 0.05). HO-1 mRNA levels, and not protein levels, were significantly higher during the menstrual (M)-phase of the cycle (P < 0.05), and were down-regulated by Org 2058 in M-phase explants and by 17β-E2 + Org 2058 in LP-phase explants, versus control (P < 0.05).

CONCLUSIONS: The haemoglobin-HO-1 system may be required to ensure adequate regulation of the bioavailability of haeme, iron and oxygen in human endometrium.

Keywords: haemoglobin; haeme oxygenase; human endometrium; menstrual cycle; iron

Introduction

The concept that haemoglobin is merely a protein that carries O2 and CO2 in erythroid cells is now being challenged. It has long been thought that haemoglobin is only produced by erythroid cells, however, recently it was shown that haemoglobins are also produced by activated macrophages, cells of the lens of the eye and alveolar epithelial cells (Liu et al., 1999; Wride et al., 2003; Newton et al., 2006). Evidence is accumulating that haeme-binding proteins, including haemoglobin, protect cells against oxidative and nitrosative stress (Crawford and Goldberg, 1998; Gross and Lane, 1999).

We showed recently using global gene expression analysis that various members of the haemoglobin gene family (haemoglobins α, β, δ and γ) are expressed at the mRNA levels in human endometrium (Dassen et al., 2007). In addition, haemoglobin β expression was reported in the implantation window (Borthwick et al., 2003; Ponnampalam et al., 2004). The haemoglobin molecule is a tetramer composed of a combination of two α-globin chains and two non-α-globin chains (α1 and α2, β, γ or δ-globin). This tetramer is then associated with the iron containing haeme complex (Feng et al., 2001; Poole, 2003).

Haemoglobin, iron and haeme are all oxidative stress-inducing agents and thus potentially toxic for human tissues (Ballal et al., 1993; Abraham et al., 1995). Once haemoglobin is released from the cell, it is oxidized rapidly in the extracellular environment to ferrihaemoglobin, which in turn will readily release the haeme (Ballal et al., 2005). Particularly, the hydrophobic nature of haeme ensures that it can easily cross cell membranes (Ballal et al., 2005). Free haeme levels are controlled in cells by intracellular and extracellular scavenger molecules, such as haeme-binding proteins (haemopexin and albumin) and haeme oxygenases (HOs) (Otterbein and Choi, 2000; Bauer et al., 2003; Szalay et al., 2005), and by anti-oxidative enzymes (Wagen et al., 2003). It is likely that the local production of haemoglobin...
also contributes to the regulation of intracellular haeme bioavailability.

The HOs are the enzymes that neutralize haeme by converting it into iron, carbon monoxide and biliverdin (Lee et al., 1997; Poss and Tonegawa, 1997; Demny et al., 1998; Yachie et al., 1999; Otterbein and Choi, 2000; Appleton et al., 2003; Shibahara, 2003; Wagener et al., 2003). Thus, by clearing the haeme, these enzymes contribute to the release of free iron. The expression of HOs has been reported in the human endometrium (Yoshiki et al., 2001; Casanas-Roux et al., 2002) and placenta (McLaughlin et al., 2000). The protective effect of the HO system was demonstrated in experimental models of various diseases, including acute inflammation, atherosclerosis, degenerative diseases and cancer, in which the induction of HO-1 can prevent or mitigate the symptoms associated with these ailments (Prawan et al., 2005). The aberrant haemoglobin and HO levels in peritoneal fluid and lesions of women with endometriosis suggest their involvement in endometrial pathologies as well (Van Langendonckt et al., 2002).

The emerging importance of the haemoglobin-HO system in the female genital tract warrants a closer evaluation of the various components in the human endometrium. In this study, we evaluated the expression of various haemoglobin isoforms (α, β, γ and δ) and HO-1 at the mRNA and protein level in the human endometrium throughout the menstrual cycle. In addition, we employed our tissue explant culture system to investigate the steroid regulation of the expression of these proteins in the human endometrium. According to the microarray experiment (Dassen et al., 2007), we predict that the haemoglobin and HO expression will be induced by progesterins and estradiol (17β-E2) combined with a progestin in the explant cultures. We hypothesize that haemoglobin plays an important role during the implantation window and accordingly we predict that the highest haemoglobin expression will occur during the mid secretory phase of the menstrual cycle.

**Materials and Methods**

**Human endometrial tissue**

Endometrium was collected from hysterectomy specimens or by pipelle biopsies during laparoscopy (Pipelle catheter, Unimar Inc., Prodimed, Neuilly-Enthelle, France) in 30 women of 20–45 years of age with regular menstrual cycles, who underwent surgery for in situ endometriosis. Of the 30 biopsies that were collected, 10 were collected in the menstrual (M) phase (cycle day 1–5), 13 were collected in the proliferative phase: early proliferative phase (cycle day 6–11), n = 5; late proliferative (LP) phase (cycle day 12–14), n = 8, and seven were collected in the secretory phase of the cycle: early secretory phase (cycle day 15–18), n = 5; mid-secretory phase (cycle day 19–23), n = 2. A part of each of the collected biopsies was snap frozen in liquid nitrogen.

Human endometrium explant cultures were prepared from M (n = 8) and proliferative phase (n = 8) endometrium as described by Punyadeera et al. (2004). In brief, human endometrium tissue was cut into pieces of 2–3 mm³. Twenty-four explants per well were placed in Millicell-CM culture inserts (pore size of 0.4 μm, 30 mm diameter, Millipore, Billerica, MA, USA) in six-well plates containing 1.2 ml phenol red-free DMEM/Ham’s F12 medium (Life Technologies, Grand Island, NY, USA), supplemented with L-glutamine (1%), penicillin and streptomycin (1%). Cultures were performed for 24 h. Previous experiments have shown that collagenase activity remains very low in proliferative endometria during the first 24 h of culture (Cornet et al., 2002), and that the tissue viability is not affected after 24 h of culture (Marbaix et al., 1992). Treatments included vehicle only (0.1% ethanol), 17β-E2 (1 nM), 17β-E2 + Org 2058 (1 μM each) and Org 2058 alone (1 μM). The steroid hormones were provided by Organon N.V. (Oss, The Netherlands).

**Total RNA extraction and cDNA synthesis**

Total cellular RNA from explants and uncultured samples was extracted using the SV total RNA isolation kit (Promega, Madison, WI, USA) according to the manufacturer’s protocol, with slight modifications. The concentration of DNase-I for DNase treatment of the RNA samples was doubled and the incubation time was extended by 15 min in order to completely remove genomic DNA. Total RNA was eluted from the column in 50 μl RNAse-free water and stored at −70°C until further analysis. The quality of the RNA samples was determined by spectrophotometry (NanoDrop, Wilmington, Delaware, USA). All the samples analysed gave 28S to 18S ratios higher than 1.5. A PCR for a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase, was performed to confirm that the RNA samples were free of genomic DNA.

Total RNA (1 μg) was incubated with random hexamers (1 μg/μl, Promega, USA) at 70°C for 10 min. The samples were chilled on ice for 5 min. To this mixture, a reverse transcriptase mix consisting of 5X reverse transcriptase buffer (4 μl), 10 mM dNTP mix (1 μl) (Pharmacia, Uppsala, Sweden), 0.1 M dithiothreitol (2 μl) (Invitrogen, Breda, The Netherlands) and superscript II reverse transcriptase (200 U/μl) (Invitrogen) was added and the samples were incubated at 42°C for 1 h, after which the reverse transcriptase was inactivated by heating the samples at 95°C for 5 min. The complementary DNA (cDNA) was stored at −20°C until further use.

**Real-time PCR**

In each real-time PCR 50 ng of cDNA template was used. Primers and probes for HO-1 (HS0157965-m1), haemoglobin β (HS00742223-g1) and 6 (HS00426283-m1) and cyclophilin A (HS99999904-m1) were purchased from Perkin-Elmer Applied Biosystems as pre-developed assays. For these PCRs, FAM-TAMRA was the fluorescent tag. The primer and probes for haemoglobin α and γ were designed and purchased from Sigma (Cambridgeshire, UK; sequences are described in Table 1). For these PCRs, SYBR green (SYBR Green PCR Master Mix,
5 min each, the sections were incubated 30 min at room temperature. Tween with diluted antibody. Both antibodies were diluted in PBS phosphate-buffered saline (PBS), the sections were incubated 1 h performed in a microwave at 650 W for 20 min. After washing in Table II. Antigen retrieval with citrate and Tris antibodies, dilutions and antigen retrieval treatments are described in explants. After overnight incubation at 37°C the sections were

cut from uncultured and cultured explants. After overnight incubation at 37°C the sections were dewaxed in xylene and rehydrated. Endogenous peroxidases were blocked by incubating 30 min in 0.3% H2O2 in methanol. The antibodies, dilutions and antigen retrieval treatments are described in Table II. Antigen retrieval with citrate and Tris/EDTA-buffer was performed in a microwave at 650 W for 20 min. After washing in phosphate-buffered saline (PBS), the sections were incubated 1 h with diluted antibody. Both antibodies were diluted in PBS/0.1% Tween/0.1% bovine serum albumin. After three washes in PBS of 5 min each, the sections were incubated 30 min at room temperature with Chemate Envision™ (Dako, Glostrup, Denmark). To visualize the staining, the sections were incubated 10 min in 1:50 diluted diaminobenzidine solution (Dako). The reaction was stopped in water. The sections were briefly counter-stained in haematoxylin, dehydrated and sealed in entalan.

To confirm that the observed haemoglobin staining was specific, increasing amounts of haemoglobin (undiluted and diluted 1:1 to 1:120 from a saturated solution) were added to the antibody mix, 30 min before application to the sections.

The sections were scored according to a previously published method (Pijnenborg et al., 2005). Sections were scored for epithelial and stromal cells separately. A three-point scale was used for both the intensity and percentage (stained cells relative to total number of same cell type). Intensity: negative (score 0), weak (score 1), moderate (score 2), and strong (score 3); Percentage: 0% (score 0), <10% (score 1), between 10 and 50% (score 2), more than 50% (score 3).

Statistical analysis

Statistical tests were carried out using the Statistical Package for the Social Sciences (SPSS 11) (SPSS Inc., Chicago, IL, USA) statistical analysis package. To evaluate whether mRNA and protein expression levels varied significantly throughout the menstrual cycle, the non-parametric unpaired Mann–Whitney U test was used to test for differences versus the M-phase. The non-parametric Wilcoxon signed rank test was used to test for differences between steroid treated explants and controls at a confidence level of 95%.

Results

Haemoglobin and HO-1 mRNA in human endometrium

Transcripts for HO-1 and all haemoglobins were detectable in human endometrium in all the phases of the menstrual cycle investigated. HO-1 mRNA levels in human endometrium were significantly higher during the M-phase of the cycle compared to the other phases of the menstrual cycle (Fig. 1).

The most prominently expressed haemoglobin was haemoglobin α, followed by haemoglobin β (Fig. 2). Haemoglobin δ was expressed at very low levels and was sometimes undetectable in the human endometrium. None of the haemoglobins showed a clear cyclic expression pattern during the phases of the menstrual cycle.

Levels of HO-1 mRNA were significantly reduced by Org 2058 (P < 0.05) in M-phase explants when compared to controls. In LP-phase explants, the expression of HO-1 was reduced by treatment with 17β-E2+Org 2058 (P < 0.05) compared to control (Fig. 3). When M- and LP-phase explants were analysed together, both Org 2058 (P < 0.05) and 17β-E2+Org 2058 (P < 0.05) significantly reduced HO-1 mRNA levels.

Haemoglobin α, β and δ transcript levels were not significantly altered in the presence of 17β-E2, Org 2058 or 17β-E2+Org 2058 when compared to the vehicle treated explants (data not shown). However, there was a tendency for Org 2058 to increase haemoglobin γ mRNA expression in both M- and LP-phase explants. Reanalysis of the data after pooling all experiments (in M- and LP-phase explants) revealed that haemoglobin γ mRNA expression was significantly increased by Org 2058 and 17β-E2+Org 2058 (Fig. 4, P < 0.05).

Haemoglobin and HO-1 protein in human endometrium

Haemoglobin staining by the pan-haemoglobin antibody was specific, as evidenced by the disappearance of immunostaining in placenta after pre-incubation with the 1:10 dilution of the

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
<th>Antigen retrieval treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin</td>
<td>rabbit-anti-human haemeoxygenase-1</td>
<td>Stressgen</td>
<td>1:2400</td>
<td>Citrate buffer pH 6.0</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>mouse pan-anti-human haemoglobin</td>
<td>Biodesign</td>
<td>1:2000</td>
<td>TE-buffer pH 8.0</td>
</tr>
</tbody>
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Table II. Antibodies used for immunohistochemistry and incubation conditions used.
saturated haemoglobin solution (Fig. 5). Haemoglobin and HO-1 protein were stained in both epithelial and stromal cells of the human endometrium (Fig. 6). Expression was also observed in endothelium. Haemoglobin expression was higher in the epithelium than in stroma (Table III, Fig. 6). All the epithelial cells were positive for haemoglobin and approximately 75% of the stromal cells (Fig. 6). HO-1 was expressed mostly in epithelial cells (Table III, Fig. 6). In addition, staining was observed in the endothelium, and intensely positive individual cells were present throughout the stromal compartment (Fig. 6).

Treating the explants with 17\(^{b}\)-E\(_2\), 17\(^{b}\)-E\(_2\) + Org 2058 or Org 2058 alone did not significantly alter the intensity of the staining in epithelial and stromal cells or the number of positive stromal cells for either HO-1 or haemoglobin (data not shown). There were also no significant differences in the intensity of the staining or the number of positive stromal cells for either HO-1 or haemoglobin between M- and LP-phase endometrium explants.

**Discussion**

We show in this study for the first time that haemoglobin is present in the human endometrium. The fact that endometrial cells produce gene transcripts for the different haemoglobin isoforms (\(\alpha\), \(\beta\), \(\delta\) and \(\gamma\)) and that haeme is produced by the mitochondria of all nucleated cells (Wagener et al., 2003), suggests that haemoglobin is actively produced by the endometrial cells.

Overall haemoglobin protein production did not vary throughout the menstrual cycle and was not affected by the presence of steroid hormones. The use of a pan-haemoglobin antibody did not allow the distinction between the different haemoglobin isoforms. Only haemoglobin \(\gamma\) mRNA levels responded to steroid hormone treatment, however, the abundant expression of the haemoglobin \(\alpha\) and \(\beta\) mRNA isoforms may have masked changes of the haemoglobin \(\gamma\) at the protein level. Another relevant observation is the fact that the expression of the four studied haemoglobin genes in the M-phase endometrium, at which time hypoxia is most pronounced, is not higher than in the LP-phase endometrium. This suggests that haemoglobin gene expression is not regulated by hypoxia.

Only recently it became clear that haemoglobin is also produced by non-erythroid cells. Why haemoglobin is produced in the human endometrium is not clear, however, it is likely that haemoglobin in the endometrium is involved in the regulation of local iron and haeme levels to prevent overexposure of the cells to these potentially detrimental molecules (Poole, 2003).
Iron overload results in cytotoxicity due to iron-induced formation of reactive oxygen species (Aisen et al., 1990). Yet iron is critical for the proliferation of endometrial cells, and iron levels must therefore be closely monitored. Deprivation of iron was shown to prevent cells to proceed from the G1 to the S-phase of the cell cycle (Le and Richardson, 2002), and Defrere et al. (2006) showed in a mouse model of endometriosis that ip treatment with an iron chelator inhibited proliferative activity in the ectopic endometriotic lesions. Iron may thus be a limiting factor in 17β-E2-controlled endometrial growth during the menstrual cycle.

Haeme is synthesized in all human nucleated cells (Wagener et al., 2003), and most mammalian cells probably maintain a pool of ‘free’ haeme serving both precursor and regulator functions (Ponka, 1999). The function of the haeme molecule is determined by the properties of the polypeptide bound to it. For instance, in haemoglobin, haeme is used for oxygen transport, in cytochromes it is involved in electron transport, energy generation and chemical transformation, whereas in peroxidases it functions in the inactivation of hydrogen peroxide radicals. In addition, haeme is indispensable for the activity of a wide array of enzymes including cyclooxygenase and nitric-oxide synthase, and was shown to influence gene expression at the levels of transcription, protein synthesis and post-translational modifications (Wagener et al., 2003). Haeme therefore can play a determining role in the regulation of endometrial cell function.

One of the enzymes that control intracellular haeme levels is HO. The HO system was shown to prevent or mitigate the symptoms associated with various diseases, including acute inflammation, atherosclerosis, degenerative diseases and cancer (Prawan et al., 2005). HO-1 was expressed in the human endometrium and was predominantly present in the epithelium. HO-1 expression can be induced by oxidative stress (Keyse and Tyrrell, 1989; Bauer et al., 2003), hypoxia (Lee et al., 1997; Panchenko et al., 2000) and haeme (Alam et al., 1989), which could explain why the HO-1 mRNA expression was higher in M-phase endometrium compared to the rest of the menstrual cycle. However, this difference was not reflected at the protein level. There are various reports suggesting that the expression of HO-1 is regulated by steroid hormones (Acevedo and Ahmed, 1998; Toth et al., 2003; Szalay et al., 2005; Cella et al., 2006). In this study using our steroid-responsive explant cultures of human endometrium, we clearly showed that even though HO-1 mRNA levels were suppressed by Org 2058, no effects were noted at the protein level, nor was there a difference between proliferative and secretory endometrium, which agrees with the findings of Yoshiki et al. (2001) and Casanas-Roux et al. (2002).

Despite rapid cyclical changes in leukocyte infiltration, angiogenic activity, hypoxia, proliferation, hormone concentrations and metabolic activity, expression levels of haemoglobin (and HO-1 particularly) in epithelial cells remained stable. This suggests that the haemoglobin-HO-1 system functions as a buffer in the supply of haeme and iron under these rapidly changing conditions, thereby contributing to the regulation of endometrial function.

Jauniaux et al. described that the earliest stages of embryo development take place under a low O2 concentration of approximately 1–9% (Jauniaux et al., 2004). They also found that the first trimester placenta limits rather than facilitates O2 supply to the fetus, reducing the oxygen stress of the fetus and protecting it against free radicals (Jauniaux et al.,
regulation of the O2 and subsequent free radical concentration. So haemoglobin and HO-1 could be involved in the early villous tissue and causes miscarriage (Jauniaux et al., 2006). A high or rapidly fluctuating O2 concentration damages the early villous tissue and causes miscarriage (Jauniaux et al., 2006). So haemoglobin and HO-1 could be involved in the regulation of the O2 and subsequent free radical concentration in the uterus during implantation.

In summary, we have shown that haemoglobin is present in, and probably synthesized by, endometrial cells. The expression of the various haemoglobin isoforms suggests that haemoglobin is indeed produced by the endometrial cells. The α- and β-globins, the globins needed for haemoglobin A, were the most abundantly expressed haemoglobins in the human endometrium. The δ-globin, required for haemoglobin A2, and γ-globin, required for haemoglobin F, were also expressed. Only γ-globin expression was significantly enhanced by Org 2058. Steroid regulation of the total haemoglobin pool could not be demonstrated. HO-1 mRNA levels, which degrades haeme, were slightly higher in M-phase endometrium and inhibited by Org 2058. However, no changes were observed at the protein level. The presence of the haemoglobin-HO-1 system may be required to ensure adequate regulation of the bioavailability of haeme, iron and oxygen in the different compartments of the endometrium.

### References


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**Table III.** The average immunoscores for haemoglobin and haem oxyg enase-1 (HO-1) as assessed in all explant cultures (*n* = 19).

| Haemoglobin | M-phase | | | LP-phase | | |
|---|---|---|---|---|---|
| (Mean ± SD) | Intensity | Percentage | Intensity | Percentage |
| Epithelium | 2.2 ± 0.6 | 3.0 | 1.8 ± 0.7 | 3.0 |
| Stromata | 2.1 ± 0.6 | 2.9 ± 0.2 | 1.6 ± 0.8 | 2.9 ± 0.2 |
| HO-1 (Mean ± SD) | | | | |
| Epithelium | 1.6 ± 0.6 | 3.0 | 1.4 ± 0.4 | 3.0 |
| Stromata | 2.8 ± 0.4 | 1.3 ± 0.6 | 3.0 | 1.1 ± 0.3 |

M-phase, menstrual phase of cycle; LP, late proliferative phase.


Submitted on August 8, 2007; resubmitted on November 24, 2007; accepted on December 17, 2007