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Citation for published version:

Maybin, JA, Battersby, S, Hirani, N, Nikitenko, LL, Critchley, HOD & Jabbour, HN 2011, 'The expression and regulation of adrenomedullin in the human endometrium: a candidate for endometrial repair', *Endocrinology*, vol. 152, no. 7, pp. 2845-2856. <https://doi.org/10.1210/en.2010-1256>

Digital Object Identifier (DOI):

[10.1210/en.2010-1256](https://doi.org/10.1210/en.2010-1256)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

Endocrinology

Publisher Rights Statement:

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The Expression and Regulation of Adrenomedullin in the Human Endometrium: A Candidate for Endometrial Repair

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After menstruation, the endometrium has a remarkable capacity for repair, but the factors involved remain undefined. We hypothesize adrenomedullin (AM) plays a role in this process. Pre-menstrually progesterone levels decline, stimulating prostaglandin (PG) synthesis, vasoconstriction, and hypoxia. This study aimed to determine 1) AM expression throughout the menstrual (M) cycle and 2) its regulation by PG and hypoxia. Human endometrial biopsies ($n = 51$) were collected with ethical approval and consent. AM mRNA expression was examined by quantitative RT-PCR and was found to be selectively elevated in endometrium from the menstrual (M) phase ($P < 0.001$). AM immunohistochemical staining was maximal in M and proliferative (P) endometrium. Culture of secretory, but not P, explants with 100 nM $\text{PGF}_{2\alpha}$ or hypoxia (0.5% O_2) increased AM mRNA ($P < 0.05$). P explants were induced to increase AM expression using *in vitro* progesterone withdrawal but required the presence of hypoxia ($P < 0.05$). Short hairpin sequences against hypoxia-inducible factor-1 α (HIF-1 α) inhibited AM hypoxic up-regulation but did not alter $\text{PGF}_{2\alpha}$ -induced expression. The AM receptor was immunolocalized to endothelial cells in both lymphatic and blood vessels. Conditioned medium from $\text{PGF}_{2\alpha}$ -treated cells increased endothelial cell proliferation and branching ($P < 0.05$). This was abolished by AM receptor antagonists. In conclusion, AM is elevated at the time of endometrial repair and induces both angiogenesis and lymphangiogenesis by stimulating endothelial cell proliferation and tube formation. In the human endometrium, AM expression is up-regulated by two mechanisms: a HIF-1 α -mediated hypoxic induction and a HIF-1 α -independent $\text{PGF}_{2\alpha}$ pathway. These physiological mechanisms may provide novel therapeutic targets for disorders such as heavy menstrual bleeding. (*Endocrinology* 152: 2845–2856, 2011)

The human endometrium is cyclically subjected to inflammation and tissue destruction during menstruation. Its capacity for scar-free repair is remarkable, but the factors involved remain elusive. Delineation of this efficient repair process may reveal novel therapeutic targets

for complaints such as heavy menstrual bleeding (HMB). In addition, the physiological mechanisms of endometrial repair may be applicable to tissues elsewhere in the body, where inflammation can lead to pathogenic fibrosis and scarring.

ISSN Print 0013-7227 ISSN Online 1945-7170

Printed in U.S.A.

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doi: 10.1210/en.2010-1256 Received October 28, 2010. Accepted April 21, 2011.

First Published Online May 10, 2011

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Abbreviations: AM, Adrenomedullin; CCM, control conditioned medium; CD31, platelet endothelial cell adhesion molecule; CGRP, calcitonin gene-related peptide; CLR, calcitonin receptor-like receptor; COX, cyclo-oxygenase; ES, early secretory; GE, glandular epithelium; HDLEC, human dermal lymphatic endothelial cell; HIF, hypoxia-inducible factor; HMB, heavy menstrual bleeding; HUVEC, human umbilical vein endothelial cell; LS, late secretory; M, menstrual; MPA, medroxyprogesterone acetate; MS, midsecretory; P, proliferative; PCM, $\text{PGF}_{2\alpha}$ -treated conditioned medium; PG, prostaglandin; Q-RT-PCR, quantitative RT-PCR; RAMP, receptor activity-modifying protein; SE, surface epithelium; shRNA, short hairpin RNA; St, stromal compartment.

Premenstrually, the endometrium is exposed to high levels of progesterone, secreted from the corpus luteum. In the absence of pregnancy, the corpus luteum regresses and progesterone levels fall dramatically. It is well established that this progesterone withdrawal initiates a cascade of inflammatory mediators that culminates in tissue destruction and menstruation (1). Hormone levels remain low during menstruation, until the proliferative (P) phase commences and estrogen levels rise. Traditionally, endometrial repair was thought to be regulated by estrogen, but recent studies in a mouse model have suggested it is an estrogen-independent event (2). Therefore, we hypothesized that premenstrual progesterone withdrawal simultaneously triggers endometrial breakdown and initiates repair.

Progesterone withdrawal is known to up-regulate cyclooxygenase (COX)-2, the enzyme stimulating prostaglandin (PG) synthesis (3, 4). PGF_{2α} is a potent vasoconstrictor and, alongside other vasoactive factors, causes constriction of endometrial spiral arterioles. This leads to an episode of local, transient hypoxia in the uppermost endometrial zones during menstruation. This hypoxic episode was first observed in classic experiments in the rhesus monkey (5) and was recently detected in the mouse model of menstruation (6). Use of pimonidazole, a marker of oxygen levels less than 10 mm Hg, demonstrated intense hypoxia in the luminal portion of the endometrium on d 2 of the cycle, with negligible detection by d 5. In addition, markers of tissue hypoxia [carbonic anhydrase IX and hypoxia-inducible factor (HIF)-1α] have been detected immunohistochemically in the human endometrium during menstruation (7, 8).

Adrenomedullin (AM) is a pluripotent peptide that belongs to the calcitonin gene peptide superfamily. It has a wide range of biological actions, including vasodilation (9–11), induction of angiogenesis (12–14), cell growth (15), and inhibition of apoptosis (16, 17). AM has an attractive but previously undefined role in human endometrial repair. AM acts through a G protein-coupled receptor, the calcitonin receptor-like receptor (CLR). Receptor activity-modifying proteins (RAMP) associate with CLR to determine its ligand binding specificity (18). CLR association with RAMP2 or RAMP3 promotes AM binding, whereas heterodimerization with RAMP1 promotes calcitonin gene-related peptide (CGRP) binding. Hypoxia

is known to induce AM gene expression in tumor cells and endothelial cells via the transcription factor, HIF-1 (19, 20). AM is known to be expressed in the human endometrium (12, 21), but its regulation and differential expression across the menstrual cycle remain unknown.

The purpose of this study was to investigate the expression and regulation of AM in human endometrium. We hypothesized that endometrial hypoxia and PG production initiate the up-regulation of factors involved in repair. Here, we have demonstrated that endometrial AM mRNA expression peaked during menstruation, with maximal AM protein observed in M and P phase biopsies. We have identified that progesterone withdrawal, followed by hypoxic conditions and PGF_{2α} synthesis, increased endometrial AM expression. Hypoxia-regulated AM expression was mediated by HIF-1, whereas PGF_{2α} up-regulated AM by a HIF-1-independent pathway. Furthermore, we have demonstrated that endometrial production of AM significantly increased vascular and lymphatic endothelial cell proliferation and branching, suggesting it plays an important role in endometrial repair and regeneration.

Materials and Methods

Tissue collection

Endometrial biopsies were collected with a suction curette (Pipelle; Laboratoire CCD, Paris, France) from women with a subjective complaint of HMB having investigation in the outpatient setting (n = 22) or undergoing hysterectomy (n = 29). At hysterectomy, a full-thickness endometrial biopsy was also collected for immunohistochemical studies. Ethical approval was obtained from the Lothian Research Ethics Committee and written informed consent from all participants. Women were aged 31–52 yr (median, 41; mean, 41) with regular menstrual cycles (21–35 d) and no exogenous hormone or intrauterine device use in the 3 months before biopsy. Women with large (>3 cm) or submucosal fibroids on ultrasound examination were excluded. Women with confirmed endometriosis or with a history of persistent premenstrual dyspareunia or infertility were excluded. Tissue was placed in 1) RNA later, RNA stabilization solution (Ambion Ltd., Warrington, UK); 2) neutral buffered formalin; or 3) PBS for *in vitro* culture and dated according to the criteria of Noyes *et al.* (22). The participant's reported last menstrual period was recorded and serum estradiol and progesterone levels measured at the time of biopsy (Table 1). Biopsies were consis-

TABLE 1. Classification of endometrial samples examined in this study

Histological stage of cycle	Mean age	Day of cycle	Mean E2 pmol/litre (range)	Mean P4 nmol/litre (range)
M (n = 8)	41	2–3	192.25 (55–514)	3.71 (1.24–10.59)
P (n = 16)	42	6–16	441.18 (79–1105)	2.81 (0.97–7.1)
ES (n = 10)	42	17–21	497.50 (289–841)	59.60 (23.2–112.91)
MS (n = 11)	40	21–25	638.00 (242–1949)	64.30 (25.47–114.53)
LS (n = 6)	42	22–29	318.22 (59.09–819)	8.22 (1.06–16.95)

tent for all three criteria and classified as proliferative (P), early secretory (ES), midsecretory (MS), late secretory (LS), or menstrual (M) for analysis.

Immunohistochemistry

AM and CLR localization was studied by immunohistochemistry, using standard methods. Antigen retrieval was performed by pressure cooking in boiling 0.01 M citrate buffer (pH 6.0), and endogenous peroxidase activity was quenched with 10% (vol/vol) H₂O₂ in methanol. After blocking for 30 min in nonimmune goat serum, sections were incubated overnight at 4°C with 1:1000 rabbit antihuman AM (Phoenix Pharmaceuticals, Karlsruhe, Germany) or rabbit antihuman CLR, previously described in Ref. 15. An avidin-biotin peroxidase detection system was used (Dako Ltd., Cambridge, UK) with 3,3'-diaminobenzidine. Controls were incubated with isotype-matched IgG or nonimmune serum instead of primary antibody.

CLR, CD31 (platelet endothelial cell adhesion molecule), and podoplanin expression was studied by triple immunofluorescence immunohistochemistry. Sections were prepared as above, blocked with 20% nonimmune serum, and incubated sequentially with rabbit anti-CLR antibody (1:3000), rabbit anti-CD31 antibody (1:1000) (Abcam, Cambridge, UK), and sheep antihuman podoplanin (1:500) (R&D Systems, Abingdon, UK), for 18 h at 4°C. Control sections were incubated with an equivalent concentration of normal IgG or nonimmune serum. Detection was performed using goat antirabbit or donkey antisheep Fab fragment-peroxidase, as appropriate, at a dilution of 1:500 (Dako Ltd.) followed by incubation with Tyr fluorescence (CLR; green), TyrCy5 (CD31; blue), and TyrCy3 (podoplanin; red) for 10 min at a dilution of 1:50 (PerkinElmer, Wokingham, UK). Between antibodies, sections were microwaved for 2.5 min in 0.01 M citrate buffer (pH 6.0). Sections were counterstained with 4',6'-diamidino-2-phenylindole (Invitrogen, Carlsbad, CA) in PBS and mounted in PermaFluor (Immunotech-Coulter, Brea, CA) and coverslipped. Controls included replacement of the antibody to CLR with an antibody that does not bind to vessels (antihuman CD56 as the first antibody) and preimmune serum from the same rabbit that was used to raise the anti-CLR antibody. Fluorescent images were visualized and photographed using a Carl Zeiss (Jena, Germany) laser scanning microscope Meta LM 510.

Scoring of immunohistochemical staining

Localization and intensity of AM immunostaining was evaluated blindly by two independent observers using a previously validated semiquantitative scoring system (23). Intensity and distribution of staining was rated as described previously (24). Intensity was graded using a three-point scale (0, no staining; 1, mild staining; 2, strong staining). The percentage of cells stained at each of these intensities was assessed in the glandular epithelial cells and stromal cells in both the basal and functional layer of the endometrium. The surface epithelial cells and endothelial cells were also assessed where visible. A value was derived for each of the cellular compartments by using the sum of these percentages after multiplication by the intensity of staining.

Cell culture

Human Ishikawa endometrial adenocarcinoma cells (European Collection of Cell Culture, Centre for Applied Microbiology, Wiltshire, UK) stably expressing the EP2 receptor (EP2S) or

the FP receptor (FPS) were maintained as previously described (25). Approximately 4×10^5 EP2S or FPS cells were seeded in six-well plates. The next day, cells were washed in PBS and incubated in serum-free culture medium containing antibiotics and 8.4 μ M indomethacin for at least 16 h. Cells were treated with vehicle, 100 nM PGE₂ (EP2S cells), or 100 nM PGF_{2 α} (FPS cells) and placed in either normoxia (21% O₂, 5% CO₂, 37°C) or hypoxic conditions (0.5% O₂, 5% CO₂, 37°C) in a sealed chamber (Coy Laboratory Products, Inc., Grass Lake, MI) for 24 h.

To examine the contribution of HIF-1, cells were pretreated with vehicle or 1, 2, 5, or 10 nM echinomycin (an inhibitor of HIF-1 DNA binding activity) for 1 h. Cells were then stimulated with vehicle, 100 nM PGF_{2 α} \pm echinomycin, or hypoxia \pm echinomycin for 8 h.

Tissue culture

Endometrial biopsies (secretory phase, n = 4; P phase, n = 3) were divided into four equal explants and incubated on raised platforms in 24-well plates, with 1 ml of serum-free RPMI 1640 medium and 8.4 μ M indomethacin for 16 h. One explant was then treated with vehicle and one with 100 nM PGF_{2 α} in normoxia (21% O₂, 5% CO₂, 37°C). The remaining two explants were treated in the same way and placed in a sealed hypoxic chamber (Coy Laboratory Products) set at 0.5% O₂, 5% CO₂, 37°C for 24 h.

Five further P biopsies were divided into eight equal-sized explants and placed on raised platforms. All explants were treated with 1 μ M medroxyprogesterone acetate (MPA) for 24 h. Explants were then treated with either 1) 1 μ M MPA plus vehicle, 2) 1 μ M MPA plus 8.4 μ M indomethacin (a COX enzyme inhibitor), 3) 1 μ M MPA and 1 μ M RU486 (a progesterone receptor antagonist) plus vehicle, or 4) 1 μ M MPA and RU486 plus 8.4 μ M indomethacin. One plate was placed in normoxic conditions and the other in hypoxic conditions for 48 h.

Quantitative RT-PCR (Q-RT-PCR)

Total RNA from cells and homogenized endometrial tissue was extracted using the RNeasy Mini kit (QIAGEN Ltd., Sussex, UK) according to the manufacturer's instructions. Samples were quantified and assessed for quality as previously described (7, 26). RNA samples were reverse transcribed as described previously (26). A tube with no reverse transcriptase was included to exclude DNA contamination. Real-time quantitative PCR was carried out using an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Warrington, UK) as previously described (26). Primer and probe sequences are listed in Table 2. Data were analyzed and processed using Sequence Detector version 2.3 (PE Biosystems, Foster City, CA). Expression of target mRNA was normalized to RNA loading for each sample using the 18S ribosomal RNA as an internal standard.

TABLE 2. Sequences of primers and probes used for TaqMan RT-PCR analysis

AM	Forward	5'-CCGACGGCCGCGTTGAC-3'
	Reverse	5'-GACACAACCTCTCTCTT-3'
	Probe	5'-FAM-CAAACCTCCCTTGCTCACC-TAMRA-3'
CLR	Forward	5'-GGACTCAATTAGTTGGGAGTTACTAG-3'
	Reverse	5'-GAGCCATCCATCCAGGTT-3'
	Probe	5'-FAM- CCCCATCAACAAGCAGAAGGCGTTT-3'-TAMRA

Short hairpin RNA (shRNA) against HIF-1 α

Two different 19-nucleotide shRNA sequences derived from human HIF-1 α mRNA (U22431; bp 1470–1489 and bp 2192–2211) were used and termed HIF-1 α /shRNA1470 and HIF-1 α /shRNA2192 (27, 28). In addition, control oligonucleotides (TIB MOLBIOL) were used and termed (ShSCR). FPS cells were transiently transfected with lentivirus at multiplicity of infection 10 for 24 h. Cells were incubated in serum-free medium overnight before treatment with 100 nM PGF_{2 α} or placement in the hypoxic chamber for 8 h. Cells were washed with PBS and harvested and RNA or protein extracted for PCR or Western blot analysis.

Adrenomedullin enzyme immunoassay

AM concentration in conditioned media was measured using a human AM (1-52) enzyme immunoassay kit from Phoenix Peptides (Karlsruhe, Germany) according to manufacturer's instructions. All samples were analyzed on the same plate. Minimum detectable concentration, 0.13 ng/ml; interassay error, less than 14%; intraassay error, less than 5%. There was 100% cross-reactivity for rat AM (1-50) and human AM (13-52) and mouse AM (1-50) but no reported cross-reactivity for endothelin-1, amilin, CGRP, CGRP-2, α -atrial natriuretic peptide, or brain natriuretic peptide-32.

Functional assays

Human umbilical vein endothelial cells (HUVEC) (Lonza, Wokingham, UK) and human dermal lymphatic endothelial cells (HDLEC) (TCS CellWorks, Buckingham, UK) were maintained in endothelial cell growth medium, EGM-2 (Lonza) and MV2 (PromoCell, Heidelberg, Germany), respectively, with recommended growth supplements. Flasks for maintenance of HDLEC were precoated with fibronectin (1 mg/ml; Sigma, Poole, UK).

Preparation of conditioned medium

The 1×10^7 FPS cells were seeded in a 162-cm² flask and treated with vehicle or 100 nM PGF_{2 α} in 30 ml of serum-free medium containing indomethacin for 48 h. Vehicle-treated conditioned medium (control conditioned medium, CCM) or PGF_{2 α} -treated conditioned medium (PCM) was then centrifuged and the supernatant frozen at -20 C until use.

Proliferation assay

HUVEC and HDLEC cells were seeded in 96-well plates at a concentration of 3×10^3 cells per well and starved overnight in EBM-2 basal medium supplemented with 1% fetal bovine serum and antibiotic. Cells were treated (six replicates per sample) with vehicle, 1 nM AM, CCM, PCM, or the same in the presence of one of two CLR antagonists, CGRP_{8–37}, or AM_{22–52} (each used at 10 nM) for 96 h, with reagents replenished at 48 and 72 h. Proliferation was then measured with Cell Titer 96 Aqueous One solution reagent (Promega, Southampton, UK) according to the manufacturer's instructions. Results are displayed as PCM-treated cells divided by relative controls, *i.e.* CCM-treated cells.

Network assay

If stimulated, HUVEC and HDLEC cells branch toward each other to forming capillary tubes or networks *in vitro*. The degree of network formation can be assessed by measurement of the number of branch points between the tube-like structures formed, providing a quantification of angiogenic potential. In-

serts of 12-well 0.4 mm transwell plates (Corning Costar, Cambridge, UK) were coated in 80 μ l of growth factor reduced Matrigel (BD Biosciences, Oxford, UK) either alone or in the presence of 10 nM CGRP_{8–37} or 1 nM AM_{22–52}. Matrigel was incubated at 37 C for 30 min; 2.5×10^4 HUVEC or HDLEC cells in suspension were then added to the inserts in a 500 μ l volume of 1% medium; 1 ml of control medium, AM, CCM, or PCM was added to the bottom chamber and incubated for 16 h at 37 C.

Cells were fixed in ice-cold methanol and stained with hematoxylin. Networks were photographed using an Axiovert 200 microscope (Zeiss, Welwyn Garden City, UK), and the number of branch points was counted by an observer blinded to the treatment of the well. As each treatment was carried out in duplicate, the mean branch point count was calculated after unblinding.

Statistics

For mRNA expression in tissue explants, cell culture, or functional assays, results were expressed as fold increase, for which relative expression of mRNA in treated samples was divided by the relative expression in vehicle-treated samples. Data are presented as mean \pm SEM. Significant difference was determined using one-way ANOVA of delta cycle threshold values, with Tukey's *post hoc* test analysis. For endometrial biopsies from across the menstrual cycle, results were expressed as quantity relative to a comparator, a sample of RNA from the liver. Significant difference was determined using Kruskal-Wallis non-parametric test with Dunn's multiple comparison *post hoc* test (Instat, GraphPad Software, Inc., San Diego, CA).

Results

AM expression across the M cycle

AM mRNA expression in homogenized endometrial biopsies was low in samples from the P and secretory phases of the cycle. Expression peaked at menstruation, when AM mRNA levels were significantly higher than during the P phase ($P < 0.001$) (Fig. 1A). Immunostaining for AM was detected in a cytoplasmic location in the surface epithelium (SE), glandular epithelium (GE), stromal compartment (St), and endothelial cells (Fig. 1B). More positive staining was observed in the functional endometrial layer than in cells located in the basal portion of the endometrium (Figs. 1, B and C, i–iv). Strongest staining was observed in the SE, and this did not vary significantly throughout the cycle (Fig. 1C, v). In contrast, semiquantitative assessment of staining in the GE revealed significantly greater staining in the M phase, compared with MS endometrium, in both the functional and basal layers ($P < 0.05$). There was also increased staining of P phase GE cells, but this did not reach statistical significance (Fig. 1C, i and ii). There was no significant difference in staining score of the stromal cell compartment across the cycle (Fig. 1C, iii and iv). Endothelial cells from all phases stained positively for AM (Fig. 1C, vi).

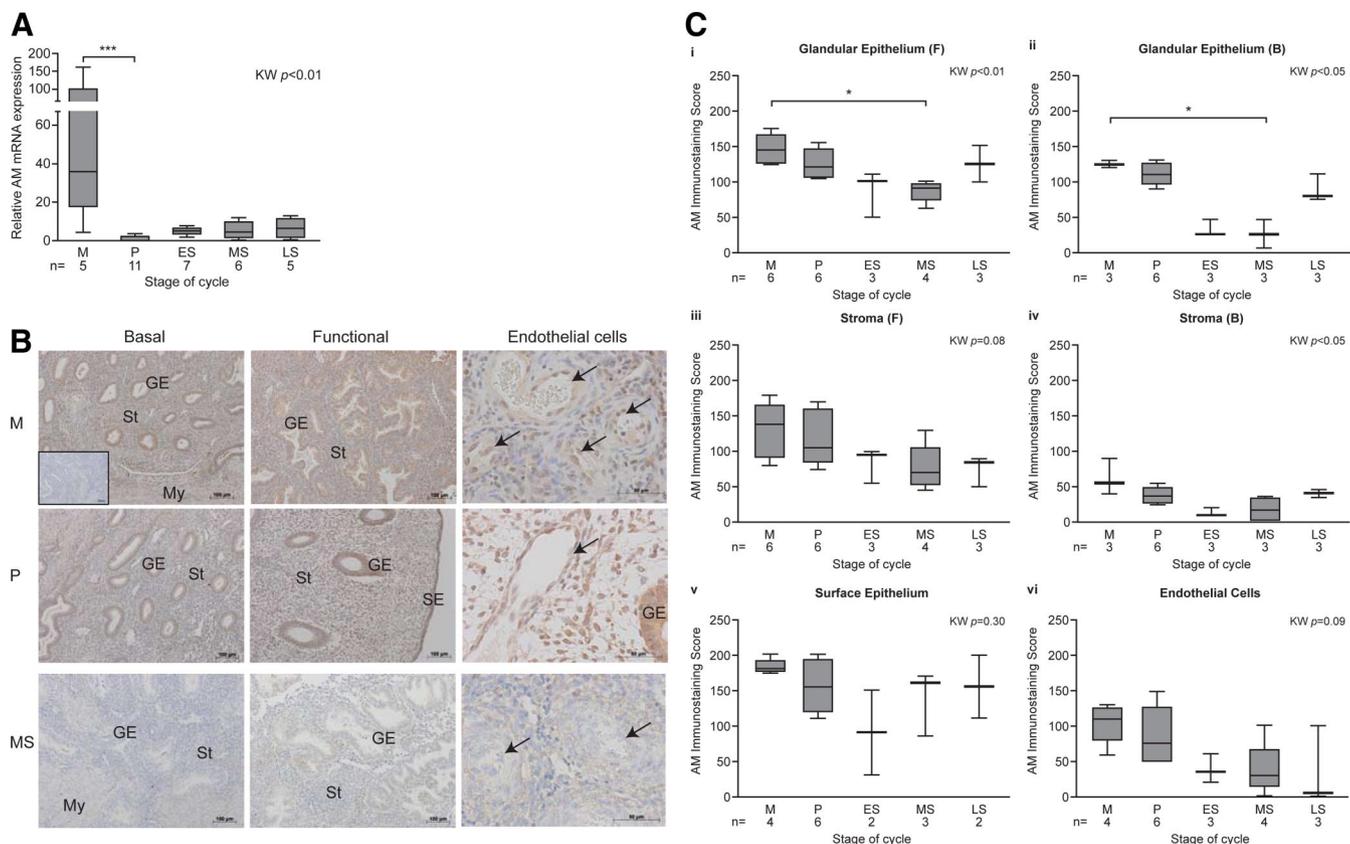


FIG. 1. AM levels in the human endometrium at different stages of the menstrual cycle. **A**, AM mRNA expression was determined by Q-RT-PCR, normalized using 18S rRNA as an internal standard. **B**, AM protein was present in the surface epithelial (SE), glandular epithelial (GE) cells, the stromal compartment (St), and endothelial cells (arrows) during the M and P phase. Staining was markedly decreased during the secretory phase. *Inset*, Negative control. *Scale bar*, 100 or 50 μ m as marked. **C**, Scoring of immunohistochemical staining for AM in GE cells of the functional (F) layer (i), GE cells in the basal (B) layer (ii), the St of the functional endometrial layer (iii), the St in the basal layer (iv), SE cells (v), and endothelial cells in both endometrial layers (vi). My, Myometrium; KW, Kruskal Wallis statistical analysis. *, $P < 0.05$.

Endometrial CLR expression

CLR mRNA was present throughout the menstrual cycle (Fig. 2A). Highest levels were observed in the MS phase, when CLR expression was significantly higher than in the P ($P < 0.01$) and LS stage ($P < 0.05$) (Fig. 2A). To characterize the site of CLR expression, sections were stained sequentially with antibodies to CLR, CD31 (a pan-endothelial marker), and podoplanin (a lymphatic endothelial marker). CLR and CD31 were expressed in all vessels (Fig. 2, B and C). In contrast, podoplanin was expressed in only a proportion of vessels, predominantly in the basal layer of the endometrium (Fig. 2D). This shows CLR was present both in the blood vessels (podoplanin-negative, CD31-, and CLR-positive vessels) and lymphatics (CD31-, CLR-, and podoplanin-positive vessels).

Regulation of endometrial AM expression

PGF_{2α} and hypoxic conditions up-regulate AM expression in endometrial epithelial cells

To determine the impact of PG and hypoxia on AM expression, we first used an endometrial epithelial cell line stably transfected with either the PGE₂ receptor (EP2S) or with

the PGF_{2α} receptor (FPS). Treatment of EP2S cells with 100 nM PGE₂ had no significant impact on AM expression at 8 h (Fig. 3A) or 2, 4, 24, or 48 h (data not shown). EP2S cells placed in hypoxic conditions (0.5% O₂) displayed a significant up-regulation of AM mRNA expression, with a 50-fold increase over cells in normoxic conditions ($P < 0.001$) (Fig. 3A). There was no further increase when cells were cotreated with hypoxia and 100 nM PGE₂. Treatment with 100 nM PGF_{2α} significantly increased AM mRNA expression in FPS cells, resulting in a more than 11-fold increase over vehicle-treated cells after 8 h ($P < 0.001$) (Fig. 3B). Hypoxic conditions significantly increased AM expression in FPS cells more than 6-fold ($P < 0.001$). Interestingly, cotreatment of FPS cells with PGF_{2α} and hypoxia resulted in a synergistic increase in AM mRNA expression (>28-fold increase over normoxic vehicle) that was significantly greater than treatment with PGF_{2α} or hypoxia alone ($P < 0.05$ and $P < 0.001$, respectively). Analysis of culture supernatants by ELISA revealed no significant differences in AM-secreted protein levels after 24 h, but a similar pattern emerged, with increased secreted AM protein from cells treated with both PGF_{2α} and hypoxia compared with vehicle treatment in normoxia (Fig. 3C).

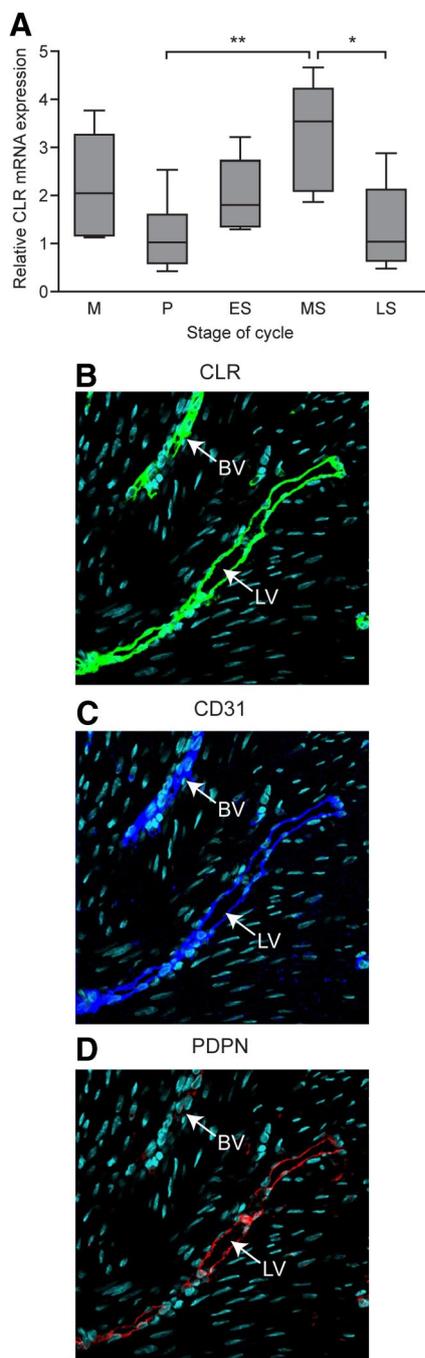


FIG. 2. CLR in human endometrium from across the menstrual cycle. A, CLR mRNA expression. ns, No significant difference; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Immunolocalization of CLR (B) (green) was present in the vasculature of the endometrium and colocalized both with endothelial cell marker CD31 (C) (blue) and lymphatic vessel marker podoplanin (PDPN) (D) (red). Blood vessel (BV) is negative for podoplanin, but lymphatic vessel (LV) is positive for all three antibodies.

PGF_{2α} and hypoxia up-regulate AM expression in secretory endometrial tissue

To further investigate $PGF_{2α}$ and hypoxia-mediated AM expression, we treated endometrial tissue explants with 1) vehicle, 2) 100 nM $PGF_{2α}$, 3) hypoxic conditions, or 4) $PGF_{2α}$ and hypoxia. P biopsies revealed no significant differences in AM mRNA expression with these treat-

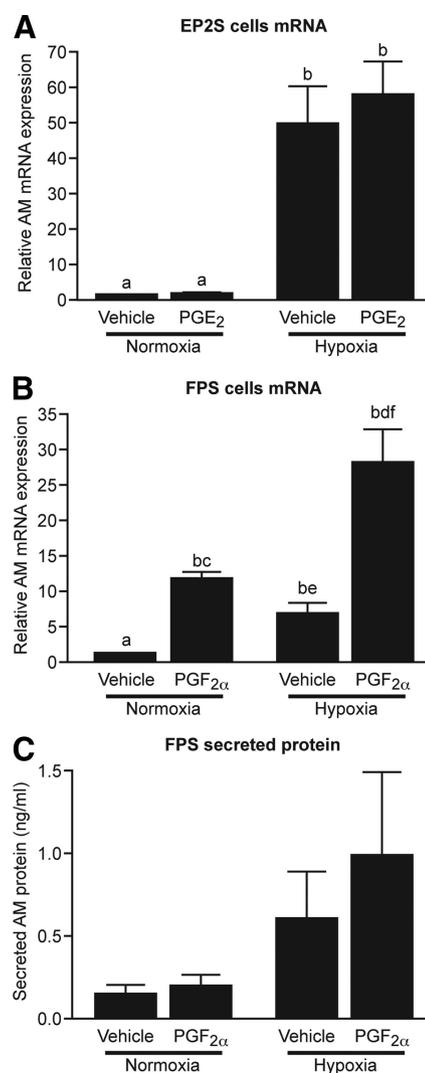


FIG. 3. $PGF_{2α}$ and hypoxia up-regulate AM expression in an endometrial epithelial cell line. A, 100 nM PGE_2 did not up-regulate AM expression in EP2S cells after 8 h, whereas hypoxic conditions (0.5% O_2) significantly increased AM expression. B, 8-h treatment with 100 nM $PGF_{2α}$ or hypoxia significantly increased AM expression in FPS cells. Cotreatment with both factors revealed a synergistic increase in AM expression. C, Secreted AM protein levels in conditioned media from FP cells treated with 100 nM $PGF_{2α}$ and/or hypoxia for 24 h. a and b, $P < 0.001$; c and d, $P < 0.05$; e and f, $P < 0.01$.

ments ($n = 3$) (Fig. 4A). In contrast, endometrial tissue from the secretory phase did show a significant increase in AM expression when placed in hypoxic conditions ($n = 4$) (>4-fold, $P < 0.05$) (Fig. 4B). In addition, there was a nonsignificant 2-fold increase in AM expression after treatment with $PGF_{2α}$ in normoxia. This suggests that previous exposure to progesterone is a prerequisite to enable up-regulation of AM by $PGF_{2α}$ and hypoxia.

Progesterone withdrawal, in the presence of hypoxia and PG, up-regulates AM expression in P endometrial tissue

To examine the contribution of progesterone exposure and withdrawal, we used an *in vitro* model of progester-

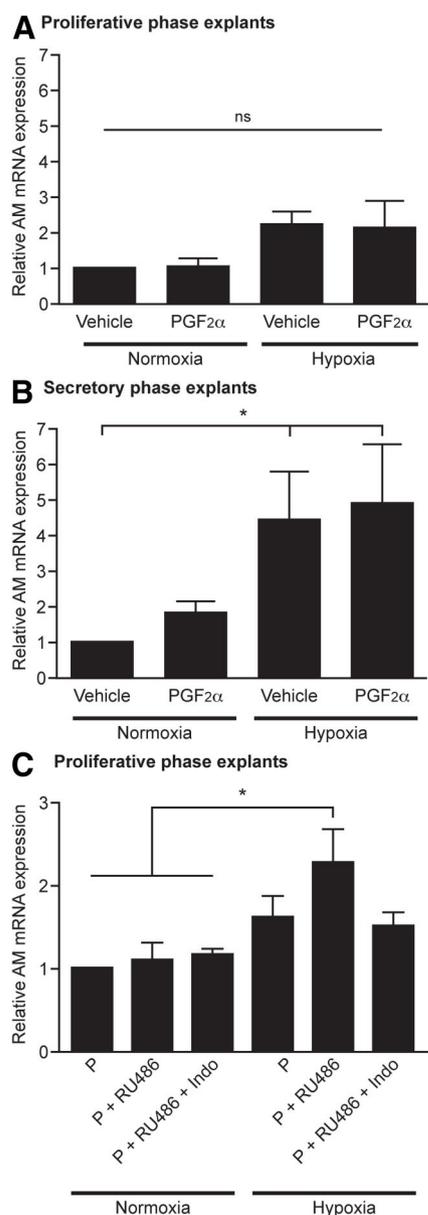


FIG. 4. Hypoxia and PGF_{2α} significantly increase AM expression in endometrial tissue previously exposed to progesterone. A, P phase explants of endometrium exposed to vehicle, 100 nM PGF_{2α}, 0.5% O₂ or both for 24 h (n = 3). B, Identical treatment of secretory phase biopsies (n = 4). C, P phase explants (n = 5) were pretreated with 1 μM MPA and then 1) maintained in 1 μM MPA (P), 2) cotreated with 1 μM MPA and 1 μM RU486, a progesterone receptor antagonist (P + RU486), or 3) cotreated with 1 μM MPA, 1 μM RU486, and 8.4 μM indomethacin, a COX enzyme inhibitor (P + RU486 + Indo). Identical treatments were incubated in either normoxia (21% O₂) or hypoxia (0.5% O₂). ns, Nonsignificant. *, P < 0.05.

one antagonism. P explants were treated with progesterone followed by progesterone and mifepristone (RU486, a progesterone receptor antagonist), to mimic progesterone withdrawal. There was no significant difference in endometrial AM mRNA expression in explants subjected to progesterone withdrawal when compared with those maintained in progesterone. However, when progesterone withdrawal was carried out in hypoxic conditions, a sig-

nificant increase in AM mRNA expression was observed (P < 0.05) (Fig. 4C). The contribution of PGs, which are up-regulated downstream of progesterone withdrawal, was assessed by coculture of explants with indomethacin, a COX enzyme inhibitor. Addition of indomethacin resulted in abrogation of the AM increase observed after progesterone withdrawal in hypoxic conditions (Fig. 4C).

AM induction by hypoxia, but not by PGF_{2α}, is dependent on HIF-1α

To assess the contribution of the transcription factor HIF-1α, we treated endometrial epithelial cells with echinomycin. This small molecule inhibits HIF-1 binding to hypoxic response elements on target genes, thereby preventing HIF-1-induced transcription (29). Cotreatment of FPS cells with 100 nM PGF_{2α} and 1–10 nM echinomycin had no significant effect on AM mRNA expression (Fig. 5A). In contrast, echinomycin prevented AM up-regulation by hypoxia in a dose-dependent manner (Fig. 5B). AM expression was significantly reduced in cells treated with hypoxia and 5–10 nM echinomycin when compared with hypoxia treatment in the absence of echinomycin.

To confirm these findings, we used two different shRNA sequences against HIF-1α (HIF-1α/Sh1470 and HIF-1α/Sh2192). HIF-1α silencing was confirmed by Western blot analysis and TaqMan Q-RT-PCR (Supplemental Fig. 1, A and B, published on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>). Specificity of the shRNA sequences was confirmed with measurement of Lamin A/C mRNA levels (Supplemental Fig. 1C). HIF-1α silencing had no significant impact on PGF_{2α}-induced AM mRNA expression (Fig. 5C). However, hypoxic induction of AM was significantly lower in those cells in which HIF-1α was silenced, when compared with untransfected cells or cells transfected with HIF-1α/ShSCR (P < 0.05) (Fig. 5D).

Endometrial AM regulates endothelial cell function

To investigate downstream effects of endometrial AM production, we treated endothelial cells (HUVEC/HDLEC) for 96 h with 1 nM AM, PCM, and the same reagents in the presence of two AM receptor antagonists CGRP_{8–37} (10 nM) and AM_{22–52} (10 nM). Endothelial cell proliferation and network formation were assessed in both cell types and displayed in Fig. 6 as fold increase over controls (vehicle treatment or cells treated with control conditioned medium).

Treatment of HUVEC cells with AM or PCM induced 1.69 (±0.28)- and 2.1 (±0.1)-fold increases in HUVEC proliferation, respectively. These effects were inhibited by the AM antagonists (Fig. 6A). Similarly, treatment of lymphatic endothelial cells (HDLEC) with AM and

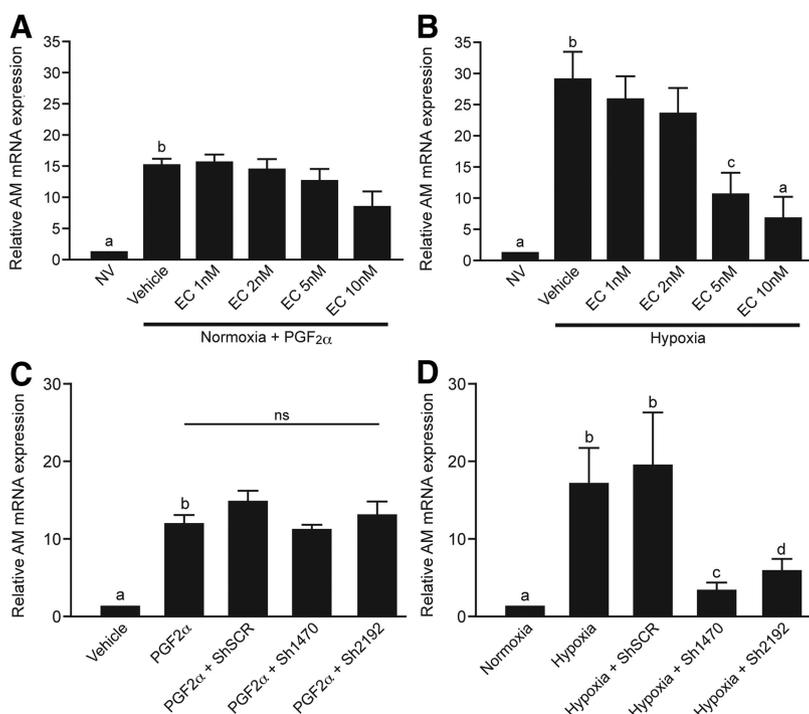


FIG. 5. Inhibition of HIF-1 prevents hypoxia-mediated AM expression but not $\text{PGF}_{2\alpha}$ -induced expression. A, Endometrial epithelial cells (FPS) treated with 100 nM $\text{PGF}_{2\alpha}$ and echinomycin (EC) (a pharmacological inhibitor of HIF-1 binding) showed no significant difference in AM expression ($n = 3$). B, Cells treated with 5–10 nM echinomycin in hypoxic conditions significantly abrogated the hypoxic induction of AM expression ($n = 3$). C, Silencing of HIF-1 α with two short hairpin sequences against HIF-1 α (Sh1470 and Sh2192) had no significant effect on $\text{PGF}_{2\alpha}$ -mediated AM expression. D, Cells transfected with shRNA against HIF-1 α had a significantly abrogated response to hypoxic stimulation, with low levels of AM expression compared with untransfected cells or those transfected with a scrambled sequence (ShSCR). a and b, $P < 0.001$; b-c, $P < 0.01$; b-d, $P < 0.05$. ns, Nonsignificant; NV, normoxia and vehicle.

PCM induced small but significant increases in HDLEC proliferation, which were inhibited by chemical antagonists (Fig. 6B).

To further investigate the effect of $\text{PGF}_{2\alpha}$ -mediated endometrial AM expression on endothelial cell function, network formation of endothelial cells was studied in the presence of AM and PCM. AM induced a $1.56 (\pm 0.19)$ -fold increase in the number of networks formed by HUVEC, whereas PCM caused a $1.84 (\pm 0.05)$ -fold increase. These effects were significantly inhibited by the AM receptor antagonists ($P < 0.05$) (Fig. 6C). A similar effect of AM and conditioned media on network formation was observed in HDLEC. In this case AM and PCM induced $1.25 (\pm 0.05)$ - and $1.75 (\pm 0.23)$ -fold increases in network formation, respectively, which were again significantly inhibited by the antagonists ($P < 0.05$) (Fig. 6D).

Discussion

This study demonstrates significant, novel advances in our understanding of human endometrial repair. Firstly, we

show significant menstrual up-regulation of AM mRNA expression. Protein levels are also highest during the M and P stages, a temporal pattern consistent with a role in repair and regeneration. Secondly, we demonstrate that endometrial AM is regulated by two distinct mechanisms: a HIF-1-mediated hypoxic induction and a novel, HIF-1 independent, $\text{PGF}_{2\alpha}$ pathway. Both these pathways are induced by progesterone withdrawal during the LS phase. Finally, we show that AM produced by endometrial cells has a significant impact on both vascular and lymphatic endothelial cell function *in vitro*. These data suggest that AM may have an active role in endometrial repair.

The expression of AM in the human endometrium has been studied previously (12, 21, 30, 31), but we present the first detailed analysis of endometrial AM expression at well-defined stages of the menstrual cycle. We show, for the first time, that AM mRNA and protein are high during menstruation, when repair is initiated. Immunolocalization revealed AM in the SE, GE, St, and endothelial cells, in agreement with previous *in situ* hybridization studies (30). We acknowledge that our study population had a subjective complaint of HMB. Previous studies have shown that 64% of women with subjective heavy loss actually had a normal objective measurement (32). However, further studies are required to determine whether blood loss has an impact on endometrial AM expression.

Maximal AM localization was in the functional endometrial layer. A recent study comparing the functional and basal endometrial layers at menstruation found that genes associated with extracellular matrix biosynthesis were greatest in the lysed functional layer (33), suggesting fragments of this layer have an active role in endometrial repair. Our finding of high levels of AM protein in P endometrium is consistent with findings in the mouse and rat uterus (34, 35). However, our study also revealed high levels of AM protein during the M phase, when estradiol levels are low. This suggests an additional estradiol independent mechanism of AM up-regulation. This is consistent with observations in the mouse model of simulated menstruation (2). The absence of estradiol support in ovariectomized mice, with additional removal of extraovarian estrogenic influence using an aromatase inhibitor, had no significant impact on the rate of endometrial repair. Notwithstanding the limitations of this mouse model, these data

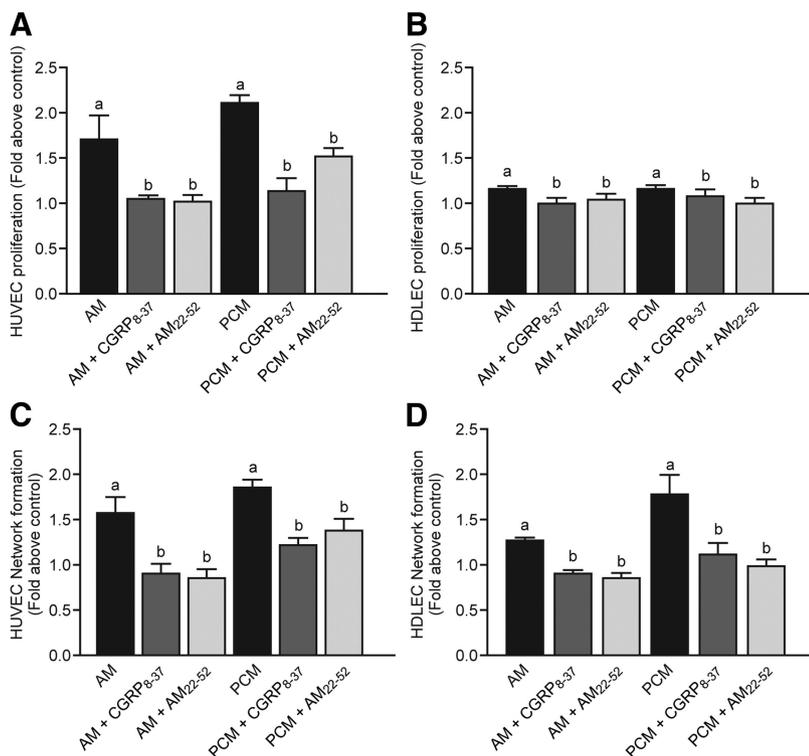


FIG. 6. $\text{PGF}_{2\alpha}$ -induced AM secretion up-regulates proliferation and network formation in human vascular endothelial cells and in HDLEC. Proliferation in HUVEC cells (A) and HDLEC cells (B) in response to AM, AM in the presence of CLR antagonists (CGRP₈₋₃₇ and AM₂₂₋₅₂), $\text{PGF}_{2\alpha}$ treated conditioned medium (PCM), or PCM in the presence of CLR antagonists ($n = 9-10$). Network formation in HUVEC cells (C) and HDLEC cells (D) in response to AM, PCM, and AM or PCM in the presence of CLR antagonists (CGRP₈₋₃₇ and AM₂₂₋₅₂) ($n = 4-5$). a and b, $P < 0.05$.

support our findings in the human endometrium, suggesting factors with a role in initial repair are not reliant upon estrogen for their expression.

Nikitenko *et al.* (30) previously identified the endometrial expression of CLR by RT-PCR but did not assess its level of expression across the cycle. We observed high CLR expression during the ES phase, with low levels during the M and P phases. This inversely correlates to the pattern of endometrial AM expression. Internalization and desensitization of the endogenous CLR in endothelial cells occurs after exposure to AM (15), suggesting the function of CLR is tightly regulated by its ligand. A similar pattern of reduced CLR expression was seen in the mouse model of sepsis, where AM expression is markedly up-regulated (36). Further studies of this receptor, in particular investigation of the endometrial expression of the receptor activating binding proteins, are required to confirm its functional role and affect on AM responsiveness.

Our finding that CLR protein is localized to endothelial cells of blood vessels in the human endometrium is consistent with previous studies (30, 37, 38). In addition, we have demonstrated CLR localization in the lymphatic vasculature. Nikitenko *et al.* (15) have previously shown that treatment of primary endometrial endothelial cells with

a synthetic AM peptide increased their growth *in vitro*. Our study supports and extends this work. We have demonstrated significantly increased proliferation and network formation of macrovascular and lymphatic endothelial cells treated with AM produced endogenously by endometrial cells. Confirmation of these findings in microvascular endothelial cells, as found in the human endometrium, is necessary. These processes are essential for the repair of damaged blood vessels at menstruation (39, 40). Endometrial lymphangiogenesis has recently emerged as an important process in the normal menstrual cycle (40). The lymphatics play a vital role in tissue fluid balance and immune surveillance. A study of endometrial lymphatic vessels identified small sparsely distributed vessels in the functional layer, with larger lymphatic vessels in the basal layer, often closely associated with spiral arterioles (41). This association may indicate that the lymphatics have a regulatory role in spiral arteriole function. Importantly, AM has been shown to promote the proliferation, migration, and network formation of cultured lymphatic microvascular endothelial cells and reduces secondary lymphoedema in mice with tail injury (42). Our results suggest that endometrial epithelial cell AM production increases blood and lymphatic endothelial cell proliferation and network formation. An alternative explanation is that $\text{PGF}_{2\alpha}$ has an autocrine effect on endothelial cell AM production to increase their proliferation (15). Either way, these data provide further support for a functional role for AM in human endometrial repair.

The regulation of endometrial AM expression has not previously been defined. Here, we have demonstrated that endometrial cell AM expression is induced by hypoxia and by a novel $\text{PGF}_{2\alpha}$ -mediated pathway. As our immunohistochemistry data showed strong staining in epithelial cells, we used an endometrial epithelial cell line stably transfected with either the PGE_2 receptor (EP2) or the $\text{PGF}_{2\alpha}$ receptor (FP). These cell lines were used because primary endometrial epithelial cells have a limited capacity for proliferation *in vitro* but do express PG receptors *in vivo* (43, 44). We have shown that $\text{PGF}_{2\alpha}$ significantly up-regulated endometrial AM expression, whereas PGE_2 had no such effect. Inflammatory cytokines, such as $\text{TNF-}\alpha$ and $\text{IL-1}\beta$, have previously been shown to regulate AM expression (45), but our report is the first on AM regulation by $\text{PGF}_{2\alpha}$.

PGF_{2α} is emerging as a potential key player in the initiation of endometrial repair factor expression. We have previously demonstrated PGF_{2α} induction of a host of endometrial angiogenic factors, including vascular endothelial growth factor, fibroblast growth factor-2, and IL-8 (46–48). In addition to this novel pathway of AM regulation, we also confirmed hypoxic induction of endometrial AM expression, an effect previously demonstrated in other cell types (49, 50).

To further delineate the mechanisms controlling endometrial AM expression, we assessed the contribution of HIF-1α. HIF-1 is a transcription factor composed of two subunits: the oxygen-destructible HIF-1α subunit and the constitutively expressed HIF-1β. As a heterodimer, it binds to hypoxic response elements on target genes, which include angiogenic and mitogenic factors, such as AM, vascular endothelial growth factor, connective tissue growth factor, and IL-8 (51–54). In addition to its stabilization in hypoxic conditions, there is evidence that inflammatory mediators, *e.g.* PG, can induce HIF-1 in normoxia (7, 55). Therefore, we examined the contribution of HIF-1 to both hypoxia and PGF_{2α}-mediated up-regulation of AM in our endometrial cell line. Both pharmacological inhibition of HIF-1 binding and silencing of HIF-1α with shRNA elicited a significant abrogation of hypoxia-induced AM up-regulation. These results concur with findings in cardiomyocytes and human tumor cell lines (53, 56). In contrast, we found that inhibition of HIF-1 had no impact on the ability of PGF_{2α} to increase endometrial cell AM expression. Therefore, we propose that two independent pathways are active in the endometrium to regulate AM expression: a HIF-1-dependent hypoxic pathway and a novel PGF_{2α} pathway that is not reliant on HIF-1 activity.

Interestingly, when examining the effect of PGF_{2α} and hypoxic treatment of primary endometrial explants, increases in AM expression were only observed in explants taken during the secretory phase of the cycle. This suggested that previous progesterone exposure is necessary for endometrial AM up-regulation. To test this hypothesis, we subjected P endometrium to a previously described *ex vivo* model of progesterone antagonism (57), to mimic progesterone withdrawal in the LS phase. When explants were cotreated with progesterone and a progesterone receptor antagonist, there was no significant increase in AM expression over explants maintained in progesterone alone. However, when progesterone is withdrawn *in vivo* the spiral arterioles constrict, resulting in a hypoxic episode that was not accounted for in our model. Therefore, we treated explants from the same biopsies in an identical manner but placed them in a hypoxic chamber. Progesterone withdrawal in hypoxic conditions significantly in-

creased endometrial AM expression when compared with explants treated in normoxic conditions. Addition of the COX inhibitor indomethacin abrogated this AM up-regulation. These data suggest that progesterone withdrawal up-regulates endometrial AM expression but only in the presence of hypoxia and PGs. Although the contribution of hypoxia to the initiation of menstruation is currently controversial (58), our results suggest that endometrial hypoxia has an important role in the initiation of repair.

In summary, our work describes the expression of AM and its receptor in human endometrium across the menstrual cycle. Our study identified a peak in AM mRNA and protein expression at menstruation, coinciding with the initiation of endometrial repair. We provide evidence that AM produced endogenously by endometrial cells is functionally active, in that it can stimulate proliferation and capillary branching of vascular and lymphatic endothelial cells. In addition, we demonstrate regulation of endometrial AM expression via two separate pathways: a novel PGF_{2α} pathway and hypoxic induction via HIF-1. We propose that these pathways are activated in the endometrium after progesterone withdrawal in the LS phase, to up-regulate AM for endometrial repair. Aberrant AM expression and regulation in the endometrium may contribute to defective repair and subsequent clinical presentations of prolonged bleeding or HMB. AM expression in women with objective measurement of their menstrual loss remains to be determined. Further work in this area may aid development of effective medical treatments for this common complaint.

Acknowledgments

We thank Sharon McPherson and Catherine Murray for their help with recruitment, Sarah McDonald and Omaira Idris for technical assistance, Ronnie Grant for his help with illustrations, and Sheila Milne (all affiliated with The University of Edinburgh, Edinburgh, United Kingdom), for secretarial support. shRNA constructs were a kind donation from Professor T. Cramer (Charité-Universitätsmedizin, Berlin, Germany), and we thank Dr. Pamela Brown for her help using them.

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This work was supported by Medical Research Council Grants U.1276.00.004.00002.01 and G0600048 and, in part, by the Wellcome Trust (L.L.N.).

Disclosure Summary: The authors have nothing to disclose.

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