Mass spectrometry identification of potential mediators of progestin-only contraceptive-induced abnormal uterine bleeding in human endometrial stromal cells☆,☆☆

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Abstract

Objective: Thrombin and hypoxia each target human endometrial stromal cells (HESCs) to mediate long-acting progestin-only contraceptive (LAPC)-induced abnormal uterine bleeding (AUB). Thus, the secretome resulting from treatment of primary cultures of HESCs with thrombin or hypoxia was screened by mass spectrometry (MS) to detect potential protein mediators that lead to AUB.

Study design: Cultured HESCs were primed with estradiol±medroxyprogesterone acetate (MPA) or etonogestrel (ETO), the respective progestins in MPA-injected and ETO-implanted LAPCs, and then treated by incubation with thrombin or under hypoxia. Collected conditioned medium supernatants were used for protein identification and quantitation of potential AUB mediators by liquid chromatography combined with tandem mass spectrometry analysis. Microarray analysis of parallel cultures and immunostaining of endometrial biopsies of LAPC users vs. nonusers corroborated MS results.

Results: MS identified several proteins displaying changes in expression levels from either thrombin or hypoxia treatments that are integral to angiogenesis or extracellular matrix formation. Several MS-identified proteins were confirmed by mRNA microarray analysis. Overexpressed stanniocalcin-1 (STC-1) was observed in endometrium of LAPC users. Unlike controls, all LAPC users displayed endometrial tubal metaplasia (ETM).

Conclusions: MS analysis identified many proteins that can affect angiogenesis or vessel integrity, thereby contributing to AUB. Confirmation of STC-1 overexpression in LAPC users and microarray data supports the validity of the MS data and suggests STC-1 involvement in AUB. The discovery of ETM in LAPC users indicates that LAPC-related side effects extend beyond AUB. The results presented here demonstrate a complex biological response to LAPC use.

Implications: MS identified several HESC secreted proteins deregulated by thrombin and hypoxia that may mediate LAPC-induced AUB. The revelation of overexpressed STC-1 by combined in vivo and in vitro observations identifies a potential target for future studies to prevent or minimize LAPC-induced AUB.

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1. Introduction

Long-acting progestin-only contraceptives (LAPCs) are recommended for use when estrogen-containing formulations are contraindicated (e.g., during lactation and for women at risk for thrombosis). They are generally safe, inexpensive and effective, making them ideal for worldwide
use. However, irregular and prolonged abnormal uterine bleeding (AUB), a source of inconvenience, annoyance and a religious taboo, explains the high discontinuation rate of these inexpensive and effective contraceptives. Such AUB accounts for half of discontinuations during the first year of use and a 30% 5-year discontinuation rate [1].

Unlike menstrual bleeding, which originates primarily from spiral arteries and arterioles in response to circulating progesterone withdrawal, LAPC-induced AUB occurs sporadically and transiently from focally distributed, structurally compromised, dilated and fragile endometrial microvessels [2]. Previously, we demonstrated that LAPC users experience (a) reduced uterine blood flow accompanied by hypoxia/reperfusion and reactive oxygen species generation [3,4] and (b) increased expression by decidualized human endometrial stromal cells (HESCs) of tissue factor, a 47-kDa transmembrane protein that promotes hemostasis via thrombin generation [2,5]. Thrombin also induces gene transcription by binding to protease-activated receptors expressed on the surface of various endometrial cell types [6,7].

In cultured HESCs, hypoxia and thrombin each enhance expression of angiogenic factors such as vascular endothelial cell growth factor (VEGF) and interleukin-8 (IL-8) [8,6]. IL-8 also recruits and activates neutrophils and natural killer (NK) cells [6]. A corresponding increase in VEGF and IL-8 expression as well as excess neutrophil and NK cell infiltration are reported in endometrial sections from LAPC users [8]. The combined effects of this hyperangiogenic state coupled with neutrophil and NK cell-derived proteases and thrombin-enhanced matrix metalloproteinase (MMP) expression in HESCs are expected to damage the microvascular support structure to promote AUB [9].

To gain further insight into the consequences of LAPC use on the endometrium, the effects of mediators of AUB, i.e., hypoxia, thrombin and cytokines, were evaluated on primary cultures of HESCs during coinubcation with medroxyprogesterone acetate (MPA) or etonogestrel (ETO), the progestin components of LAPC formulations. Liquid chromatography was used in combination with tandem mass spectrometry (LC-MS/MS) to screen for the presence of novel potential protein mediators of AUB secreted by HESCs following incubation with thrombin or under hypoxia. Assessment of steady-state levels of specific mRNAs identified by microarray analysis in cultured HESCs and of corresponding protein expression by immunohistochemical analysis of endometrial biopsies from LAPC users corroborated LC-MS/MS results.

2. Materials and methods

2.1. Study participants and sample collection

The endometrial biopsies (n=8) were obtained from women 3 months post-MPA administration after receiving written informed consent at New York University under institutional review board approval. Control endometrium biopsies (n=4), all from the late secretory phase, were obtained from the Yale pathology department with approval by the Yale Human Investigations Committee. HESCs (n=3) were isolated from endometrial biopsy specimens from healthy females who underwent tubal sterilization after receiving written informed consent under approval by the Yale Human Investigations Committee. All the subjects were cycling regularly and had not received hormonal therapy for at least 3 months before surgery. These samples were transported to Ohio State University under a material transfer agreement with Yale University.

2.2. Cell culture

HESCs were isolated as previously described [10]. Aliquots were thawed as needed and decidualized in serum-containing medium with 10^{-8} M estradiol (E2) or E2+10^{-7} M of MPA or ETO in 24-well cell culture plates as previously described [11]. The cell monolayers were washed 4× with phosphate-buffered saline to eliminate traces of serum for LC-MS/MS analysis and incubated in serum-free defined medium [DMEM/F12+5 μM FeSO4, 50 μM ZnSO4, 1 μM CuSO4, 50 μg/ml ascorbic acid (Sigma Chemical Co., St. Louis, MO, USA) and 50 ng/ml EGF (BD Biosciences, Bedford, MA, USA)] containing 10^{-8} M E2 or E2+10^{-7} M MPA or ETO+1 U/ml human alpha-thrombin (Sekisui Diagnostics, Stamford, CT, USA) for 24 h or ±48 h hypoxia (0.2% O2, 5% CO2 and 94.8% N2) as previously described [11]. For hypoxia, a specialized hypoxia system produced by BioSpherix, Ltd (Lacona, NY, USA) was used consisting of a 2-shelf C-Chamber using the BioSpherix ProOx C21 Oxygen and Carbon Dioxide Controller. Regular maintenance and calibration of the oxygen and carbon dioxide sensors were performed routinely using certified, premixed gases. When necessary, the sensors and sampling pump were replaced and the system was recalibrated. For normal operation to maintain a hypoxic environment, 100% nitrogen and 100% carbon dioxide gases were used. Following the hypoxia or thrombin treatments, collected conditioned media supernatant (CMS) was centrifuged for 5 min at 2000g and then for 10 min at 6000g before further processing for LC-MS/MS.

2.3. Mass spectrometry

Following the hormone priming and hypoxia or thrombin treatments of (n=1 patient) HESCs, 80 μl CMS+16 μl of 100 mM NH4HCO3+300 ng trypsin (#V511A; Promega, Madison, WI, USA) was incubated overnight at 37°C and dried, and 20 μl of 2% acetonitrile and 0.1% formic acid was added. The generated peptide samples (4–6 μl) were diluted in 16 μl of 2% acetonitrile in 0.1% formic acid and 18 μl was analyzed by LC-MS/MS with a Thermo Scientific Easy-nLC II (Thermo Scientific, Waltham, MA, USA) coupled to a hybrid Orbitrap Elite ETD (Thermo Scientific, Waltham, MA, USA) mass spectrometer using an instrument configuration as previously described [12]. In-line desalting was accomplished using a reversed-phase trap column.
2.4. Microarray analysis

Total RNA was extracted from cultured HESCs (n=3 patients) using RNeasy Mini and RNeasy MinElute Cleanup Kits (Qiagen, Valencia, CA, USA). RNA quality was initially determined by examining the 260/280 ratio. Subsequently, use of a bioanalyzer confirmed the quality of the RNA by assessing the 20S and 18S RNA peaks. Extracted mRNA was analyzed using Illumina Human HT-12 v4 Expression BeadChip (Illumina Inc., San Diego, CA, USA). Raw data without normalization were analyzed by GeneSpring GX12.5 software (Agilent Technologies Silicon Genetics, Redwood City, CA, USA). Gene readouts were normalized to the 75th percentile of the distribution of all measurements in each chip. Normalization/per-gene across chips used the median value of each gene throughout different chips in the same experimental condition. Normalized data were initially filtered to eliminate genes absent in all experimental treatments then filtered on volcano plots with moderated t test without multiple testing correction. Genes with a fold change of >1.25 and a p value of <.05 were considered differentially expressed.

2.5. Immunohistochemistry

Immunostaining for stanniocalcin-1 (STC-1) on endometrial sections from LAPC users vs. nonusers was performed as previously described [11]. Briefly, following deparaffinization in xylences and incubation in ethanol and water, samples were briefly heated to 100°C in citric acid allowed to cool 5 min and reheated 3 times. After cooling, slides were rinsed in Tris-buffered saline with 0.1% tween-20 (TBS-T) and incubated in 3% H2O2 and 50% MeOH for 12 min at room temperature followed by washing in TBS-T. Sections were blocked for 30 min at room temperature with 5% goat serum in TBS-T and rabbit IgG anti-STC-1 diluted 1:600 (Novus Biologicals, Littleton, CO, USA) or control rabbit IgG at the same concentration in 1% goat serum in TBS-T added for overnight incubation at 4°C. Slides were washed in TBS-T and incubated with biotinylated goat antirabbit IgG (Vector Laboratories, Burlingame, CA, USA) in TBS-T for 30 min at room temperature. Immunoreactivity was developed using biotinylated antirabbit IgG, streptavidin–biotin peroxidase complex (Elite ABC kit PK-6200) and peroxidase substrate kit (sk-4100) (Vector Labs, Burlingame, CA, USA). Slides were then slightly counterstained with hematoxylin. HScores were performed examining combined STC-1 expression in stromal, epithelial and endothelial cells.

3. Results

LC-MS/MS analysis of HESC-derived CMS identified an average of 400 proteins per sample. As previously reported, the number of peptides (peptide counts) detected by LC-MS/MS for a specific protein is proportional to its quantity in an analyzed sample [13,14]. Statistical analysis of peptide counts from the HESC-derived secretome revealed 69 and 42 proteins that demonstrated significant changes (p<.05) following thrombin treatment or incubation under hypoxia, respectively. The volcano plots in Fig. 1 display fold change in peptide counts (represented on the x-axis as log base 2 ratio of the normalized peptide counts) against magnitude of p values (represented on the y-axis as negative log base 10 of p values obtained from the Student’s t test) for individual proteins identified by the LC-MS/MS. Peptides that show significant changes (p<.05) after thrombin or hypoxia treatments are marked in red. Hypoxia and thrombin each induced both up-regulation and down-regulation of several proteins. Compared to the control incubation in which no peptides were detected for STC-1, experimental incubations either under hypoxia or with thrombin show marked increases of 11 (p<.05) and 15 (p=.076) peptide counts on average, respectively, noted as squares on the volcano plots. Finally, the LC-MS/MS peptide counts of the hormone-primed (i.e., E2, E2+MPA or E2+ETO) HESCs following thrombin or hypoxia treatment vs. controls were similar for the proteins demonstrating significant changes and shown in red on the volcano plots.

Tables 1 and 2 display normalized peptide counts and mRNA fold change for proteins shown to be either regulated or not regulated by both analytical methods. “NC” indicates
no change in mRNA levels for the proteins listed in the tables. Table 1 lists peptide counts from proteins that were found by LC-MS/MS to be regulated by thrombin or hypoxia with a p value of <.05, with the exception of thrombin treatment resulting in a p value of .076 for STC-1 expression. The mRNA fold changes for specific proteins found to be either regulated by thrombin or hypoxia corroborate the direction of change in protein expression detected by LC-MS/MS. Following thrombin treatment of HESCs, three proteins — STC-1, chondroitin sulfate proteoglycan 4 (CSPG4) and cysteine-rich angiogenic inducer 61 (Cyr61) — demonstrated significant changes (p<.05) in mRNA expression levels, thus confirming changes in protein levels detected by LC-MS/MS. Following incubation under hypoxia, microarray analysis corroborated the changes detected by LC-MS/MS for the expression of STC-1 as well as insulin-like growth factor-binding protein 3 (IGFBP3). Examination of the unregulated (stable) peptide counts shown in Table 2 demonstrates the capability of LC-MS/MS to discriminate among proteins present at relatively high, medium or low expression levels. No changes in mRNA levels were seen for these proteins in comparisons

Table 1

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<th>Protein Identity</th>
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<th>M</th>
<th>ETO</th>
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<th>M</th>
<th>ETO</th>
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<th>M</th>
<th>ETO</th>
<th>Hypoxia</th>
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Fig. 1. Volcano plots of MS generated peptide count data. Each point on the plots represents the comparison of peptide counts of an individual protein (identified by the MS) of hormone-treated control cells vs. same hormone treatments following incubation with thrombin or hypoxia. Student’s t test was used on normalized peptide counts to calculate statistical significances of pair-wise comparisons of control with E2, E2+MPA and E2+ETO vs. the same treatment with thrombin or hypoxia. The data are from log base 2 of the fold changes in peptide counts vs. negative log base 10 of the p values. Panel A shows proteins that were either up- or down-regulated from thrombin treatment with significant differences (p<.05) indicated in red. Panel B shows proteins either up- or down-regulated following hypoxia with significant differences (p<.05) indicated in red. STC-1 is indicated by square.
between controls vs. treatment groups. Note that, for the peptide counts of calumenin (Table 2) and MMP10 (Table 1), the incubations with E2 vs. E2 plus either MPA or ETO indicate an independent progestin effect on thrombin- or hypoxia-treated HESCs for both proteins. Among several proteins observed to be regulated by incubation with either thrombin or under hypoxia shown in Table 1, STC-1 expression alone is enhanced during incubations with both thrombin and hypoxia. Additionally, no peptides were detected for STC-1 for the controls for both

<table>
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<th>mRNA Fold Change</th>
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<td>Collagen Alpha-2(1)</td>
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**Table 2**  
Peptide counts of proteins unchanged following thrombin or hypoxia

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Fig. 2. (A) STC-1 staining of representative endometrial biopsy sections from an MPA formulation LAPC user. Scale bar in the inset represents 15 μm. Arrowheads (insets) point to cilia on epithelial surface (bottom inset) or intense STC-1 staining of nuclear bodies (top inset). (B) STC-1 staining of MPA formulation LAPC user showing cilia (arrowhead) on epithelium of gland. Inset shows endometrial staining following incubation with control IgG where scale bar represents 15 μm. (C) STC-1 staining of non-LAPC user showing surface epithelium (arrow), stroma (Str) and gland (arrowhead). Scale bars shown represents 50 μm for panels A, B and C. (D) H-scoring of n=8 MPA formulation LAPC users vs. n=4 of nonusers (p<.001).
treatments by LC-MS/MS. This striking observation served as a stimulus to perform STC-1 immunostaining on LAPC-derived endometrial biopsies obtained from women using the LAPC formulation, MPA vs. nonusers. In Fig. 2A, endometrial tissue obtained from a representative MPA LAPC user displays epithelial and stromal cell cytoplasmic and nuclear staining for STC-1 as well as darker-stained nuclear bodies in stromal cells (arrowhead, upper inset). STC-1 nuclear immunostaining staining has been previously observed [15] and the staining of nuclear bodies observed here has been described by others in nuclei of leukemic cell lines [16]. The presence of cilia on the luminal epithelium (Fig. 2A) of the MPA LAPC user indicates endometrial tubal metaplasia (ETM). This observation was confirmed in all of the treated patients examined (n=8), whereas ETM was not detected in four control endometria that were all in the late secretory phase of the menstrual cycle at the time of biopsy collection. The luminal epithelial surface of endometrial glands in MPA LAPC users also displayed cilia (Fig. 2B). Fig. 2C shows light STC-1 staining of non-LAPC users. The HSCOREs (Fig. 2D) demonstrates significant difference in STC-1 staining between MPA LAPC users vs. nonusers (p<.001).

4. Discussion

Downstream LAPC effects were evaluated in primary cultured HESCs. Specifically, the cells were primed with E2 or E2+MPA or E2+ETO±thrombin or under hypoxia, to mimic the endometrial milieu induced by LAPCs. The resulting secretome was interrogated by LC-MS/MS to screen for potential mediators of AUB in the conditioned medium supernatants of HESCs obtained from a single patient. Statistical analysis of LC-MS/MS-generated peptide counts from HESC-derived CMS identified several significantly regulated proteins in response to either hypoxia or thrombin. The presence of numerous proteins with stable peptide counts indicates that peptide count numbers for regulated proteins do not reflect random data acquisition by LC-MS/MS.

In conjunction with the MS study, HESCs from n=3 patients were (hormone) primed and then treated with thrombin or under hypoxia and mRNA microarray analysis was performed on the isolated RNA. The statistical characterization of the mRNA microarray analysis was used in conjunction with the MS peptide count data to select a target for characterization of endometrial biopsies from LAPC users vs. nonusers using immunohistochemistry (IHC) analysis. Both thrombin and hypoxia induced substantial increases in the number of peptides for STC-1, whereas no peptides were detected for their respective controls by LC-MS/MS analysis. The microarray results corroborated the effect of thrombin and hypoxia on STC-1 expression. Additionally, all other common proteins identified in the microarray data corroborated the analysis of the peptide count MS data.

The current study identified several proteins found to be regulated by hypoxia or thrombin previously reported to influence angiogenesis as well as extracellular matrix (ECM) and vascular integrity. Among thrombin-induced proteins secreted by HESCs are several that are involved in angiogenesis and/or vessel formation. Specifically, these include (1) mediators of angiogenesis or vessel formation such as Cyr61 [17], STC-1 [18] and CSPG4 [19]; (2) mediators of ECM formation/degradation such as MMP10 [20] and MMP2, which can effect vessel formation [21] and, finally, (3) emilin-1, an ECM reported to affect vessel integrity [22]. Two of the HESC secreted proteins affected by hypoxia also are also known to modulate angiogenesis. These include IGFBP3, a potent mediator of tube formation [23] and STC-1. Taken together, the results confirm that LAPC use induces a complex response that may trigger AUB. A recent study using LC-MS/MS to examine the secretome of glioma cells incubated under hypoxia found that both IGFBP3 and STC-1 are significantly up-regulated and that their expression is highly correlated with glioma grade [24].

Microarray analysis demonstrated that thrombin and hypoxia each induce changes in HESC-derived steady-state mRNA levels that parallel the LC-MS/MS observations. Although all proteins showed the same directional change in mRNA levels, only for STC-1 were steady-state peptide counts and mRNA levels up-regulated by both thrombin and hypoxia. STC-1 was originally discovered in bony fish as a secreted product of the corpuscles of Stannius, an endocrine organ involved in Ca^{2+} homeostasis [25]. As described above, STC-1 has been reported to be involved in angiogenesis. Specifically, this includes activity in affecting tube formation [18], promoting angiogenic sprouting of cultured human umbilical vein endothelial cells [26] and decreasing production of vascular endothelial growth factor (VEGF) [27], the primary mediator of angiogenesis [28]. Moreover, STC-1 is highly expressed in small vessels in colon adenocarcinomas [29]. Other studies from our laboratory indicate that LAPC-induced AUB involves endometrial hyperangiogenesis suggesting that local STC-1 expression may be a contributing factor to such AUB. Other cellular effects exerted by STC-1 likely to be involved in the occurrence of AUB include (1) increased mitochondrial electron transport chain activity and calcium transport [30], (2) inhibition of apoptosis [31] and (3) protection against hypoxia-induced superoxide generation [32]. Recently, STC-1 mRNA overexpression was described in placentas associated with small- and large-for-gestational-age deliveries and gestational diabetes as well as preeclampsia with elevated STC-1 plasma levels accompanying preeclampsia [33].

STC-1 immunostaining observed in several endometrial cell types in AUB patients vs. control endometrial sections in the current study strengthens the link between aberrant
endometrial STC-1 expression and the onset of AUB. IHC confirmation of the STC-1 MS and microarray findings support the need for further study of the array of proteins shown here to be dysregulated by thrombin and hypoxia. The expression of STC-1 on ETO LAPC user endometrial biopsies was not examined during this study due to sample unavailability. However, the similarity of the MS and microarray findings for the array of dysregulated proteins described here from the MPA- and ETO-primed HESCs suggests that a similar outcome would be observed on the endometrium of an ETO LAPC user. However, future studies are necessary to confirm this supposition.

These observations stress the importance of integrating in vitro observations in which LC-MS/MS is used to search for novel potential mediators of AUB with immunostaining of endometrium of an ETO LAPC user. However, future routine operation. Rapid Commun Mass Spectrom 2003;17:2093–8.


