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Progesterone-dependent Regulation of Endometrial Cannabinoid Receptor Type 1 (CB1-R) Expression is Disrupted in Women with Endometriosis and in Isolated Stromal Cells Exposed to TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin)

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Abstract

Objective—To examine the differentiation-related expression of CB1-R mRNA and protein in endometrial tissue obtained from women with and without endometriosis and to determine the impact of acute TCDD exposure on CB1-R gene expression in isolated endometrial stromal cells.

Design—Laboratory-based study

Setting—University-affiliated medical center

Patients—Women with and without endometriosis undergoing volunteer endometrial biopsies after informed consent.

Interventions—None

Main Outcome Measures—Analysis of *in vivo* CB1-R mRNA and protein expression in human endometrial tissues and mRNA expression in isolated stromal cells following exposure to TCDD or a progesterone receptor antagonist (Onapristone).

Results—CB1-R mRNA and protein expression was highest during the progesterone-dominated secretory phase in control women, while expression was minimal in endometrial tissues acquired from women with endometriosis, regardless of the cycle phase. Although progesterone was found to induce CB1-R mRNA expression in endometrial stromal cells from control donors, steroid-induced expression of this gene was inhibited by co-treatment with either TCDD or Onapristone.

Conclusions—Our studies reveal a role for the anti-inflammatory actions of progesterone in regulating endometrial cannabinoid signaling, which is disrupted in women with endometriosis.

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The authors have no conflicts of interest to disclose.

Significantly, our studies demonstrate, for the first time, that acute TCDD exposure disrupts cannabinoid signaling in the human endometrium.

Keywords

Cannabinoid receptor CB1-R; progesterone; endometriosis; 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD;dioxin)

INTRODUCTION

Endometriosis, characterized by the presence of endometrial glands and stroma at extrauterine sites, is a common and debilitating reproductive disease of uncertain etiology. Although typically considered an endocrine disorder, women with endometriosis frequently exhibit immune system dysfunctions that not only affect the initial development of the disease but may contribute to the impaired fertility and severe pelvic pain that are frequently noted as co-morbidities [1–2]. Although estrogen has long been considered to represent the most important steroid in the pathogenesis of endometriosis [2–3], research over the past decade suggests that reduced progesterone sensitivity may play an equally relevant role in the initiation and progression of this disease [4–7]. For example, we previously demonstrated an important role for progesterone in suppressing endometrial expression of matrix metalloproteinases (MMPs), enzymes which both regulate extracellular matrix remodeling during the menstrual cycle in healthy women [8–9] and impact the function of the innate immune system [10]. However, in women with endometriosis, an elevated pattern of MMP-3 and MMP-7 expression persisted in the progesterone dominant secretory phase [11]; an alteration which was also associated with the establishment of ectopic disease in a chimeric mouse model of human endometriosis [12–13]. Thus, adequate progesterone response within the stromal compartment of the endometrium is critical for regulating the expression of multiple proteins by various cell types during endometrial maturation in preparation for pregnancy. Equally important, continued progesterone action is critical in mediating the complex interactions that occur between immune and specialized stromal/decidual cells at the maternal-fetal interface throughout pregnancy. Therefore, reduced responsiveness to the anti-inflammatory actions of this steroid within the reproductive tract may contribute to the numerous immunological alterations noted among women with endometriosis-related infertility [10].

During endometrial maturation in healthy women, progesterone principally regulates differentiation-related gene expression following binding to functionally distinct progesterone receptor (PR) isoforms, A and B. These isoforms are selectively produced from a single gene by alternative transcription from two different promoter start sites [14–15]. Importantly, the full-length PR-B is primarily responsible for mediating the anti-inflammatory effects of progesterone [16], while the truncated PR-A isoform can act as a dominant repressor of PR-B activity [17–18]. In women with endometriosis, a predominance of PR-A expression relative to PR-B has been noted in both eutopic and ectopic endometrium, promoting the progesterone resistant endometrial phenotype identified in these patients [4, 7, 19].

While progesterone is the most important endocrine signal for successful initiation and maintenance of pregnancy, emerging evidence suggests a significant role for endometrial endocannabinoid signaling in maintaining fertility [20–23]. Nevertheless, endometrial expression and activation of the endocannabinoid system (ECS) must be carefully orchestrated, since pregnancy failure and other reproductive disorders can result from either the absence or an excess of endocannabinoids and their respective metabolic enzymes [21, 23–31]. The ECS is comprised of the endocannabinoids, anandamide (N-

arachidonoylethanolamide or AEA) and 2-arachidonoyl glycerol (2-AG) as well as their receptors and various regulatory enzymes [32–33]. The membrane bound cannabinoid receptors (CB1-R and CB2-R) are members of the G protein-coupled receptor superfamily [21, 34]. CB1-R is highly expressed in testis, placenta and uterus as well as multiple non-reproductive tissues [29, 35–43]. Within the reproductive tract, the uterus contains the highest concentrations of AEA, the primary endogenous ligand for the CB1-R [36, 44–46]. Although the extent to which the ECS contributes to normal reproductive function has yet to be elucidated, this system has been suggested to direct immune cell migration into the endometrium and homing of the embryo to the site of uterine attachment [29, 44–45]. Not unexpectedly, studies in humans and animals indicate that inappropriate activation of the ECS via exogenous cannabinoids (i.e., *Cannabis*) is associated with reproductive malfunctions including early pregnancy failure and preterm birth [23, 47–49].

Sex steroids have been shown to impact circulating and local endocannabinoid levels as well as the availability of the enzymes required for their synthesis and metabolism [28, 50–52]. Progesterone, in particular, has been shown to play a significant role in regulating activation and suppression of a number of ECS constituents. Specifically, CB1-R mRNA is induced during decidualization of isolated human endometrial stromal cells in response to progesterone treatment [53], and temporal CB1-R mRNA and protein expression has been reported in the human fallopian tube, with the highest expression levels correlating to the progesterone dominant secretory phase [27].

At this juncture, the origin(s) of reduced endometrial responsiveness to progesterone relative to the initiation and progression of endometriosis remain unclear. Based on a review of evidence from multiple laboratories, we recently proposed that exposure to dioxin-like environmental toxicants, including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD or dioxin), may trigger inflammation-related changes within the reproductive tract that promote the progesterone resistant endometriosis phenotype [5, 54–55]. Additionally, using a murine model, we found that an early life exposure to TCDD can lead to infertility and pregnancy loss among female offspring which correlates with a reduced responsiveness to the anti-inflammatory action of progesterone [5, 19, 56]. Although a similar prospective study of early life human exposure to this toxicant is not possible, we have examined the effects of TCDD on primary, adult endometrial stromal cells acquired by biopsy from disease-free women. Within an *in vitro* study, we demonstrated that short-term TCDD treatment of isolated stromal cells led to development of a progesterone resistant cellular phenotype as a consequence of reduced PR-B expression [7]. Furthermore, this same study also revealed that the TCDD mediated loss of PR-B was associated with a failure of progesterone to suppress stromal cell expression of MMP-3 protein. Though several studies indicate that progesterone may play a fundamental role in ECS signaling in the human reproductive tract, whether the progesterone resistant phenotype noted *in vivo* in endometriosis patients or *in vitro* following experimental toxicant exposure affects the ECS has not previously been examined.

In the current study, we evaluated the cyclic expression of CB1-R mRNA and protein in endometrial tissues obtained from women with and without endometriosis. We also utilized an established *in vitro* model of endometrial stromal cell differentiation to examine the relative influence of TCDD, interleukin 1α (IL-1α) and the anti-progestin, Onapristone, on progesterone-regulated expression of CB1-R mRNA. We found that progesterone exposure *in vivo* during the secretory phase was associated with increased CB1-R mRNA and protein expression in endometrial samples obtained from healthy control women while minimal gene and protein expression was observed in similar tissues obtained from women with endometriosis. *In vitro* studies revealed that acute TCDD exposure of isolated primary endometrial stromal cells obtained from healthy control women resulted in a failure of

progesterone to up-regulate CB1-R mRNA expression or reduce MMP-3 mRNA expression. The results of our TCDD treatment studies mirror the results we obtained by treating the cells with the PR antagonist, Onapristone, and further support a role for progesterone in regulating CB1-R expression. Moreover, we demonstrated a synergistic ability of IL-1α and TCDD treatments to synergistically reduce expression levels of PR-B and progesterone mediated CB1-R mRNA expression in isolated stromal cells. Taken together, our current studies identify a previously unrecognized link between the loss of progesterone's anti-inflammatory action in the endometrium and dysregulation of the ECS through a loss of CB1-R expression.

MATERIAL AND METHODS

Acquisition of Human Endometrial Tissue

The use of human endometrial tissues was approved by the Vanderbilt University Institutional Review Board for the Protection of Human Subjects. After obtaining informed consent, tissues were collected from donors (ages 18–45) with predictable menstrual cycles. Exclusion criteria for all donors included recent (<3 months) hormonal therapy (i.e., oral contraceptives) and other medications that could impact the results. Additional exclusion criteria for the disease-free, control population (N=20) included history of adhesions, polycystic ovarian disease or endometrial disorders, including fibroid uterus and endometriosis. Finally, inclusion criteria of patients with endometriosis (N=15) required a previous surgical confirmation with histopathological diagnosis of endometriosis and no hormonal treatment for the disease. Tissues were obtained by Pipelle biopsy (Unimar, Inc, Wilton, CT) at Vanderbilt University Medical Center. Proliferative phase samples (days 9–12; control N=9; endometriosis N=7) were confirmed by a serum progesterone level of ≤1.5 ng/mL. Secretory phase samples (days 14–35; control N=11; endometriosis N=8) were timed from the LH surge, using ovulation predictor kits. Following biopsy, endometrial tissues were washed immediately in pre-warmed, phenol red-free DMEM/F-12 (Sigma, St. Louis, MO) medium. A portion of each sample was formalin-fixed for histological confirmation of cycle phase and an additional portion flash frozen and stored at −80°C for further analysis.

Endometrial Stromal Cell Isolation and Culture

Isolated endometrial stromal cells were obtained by enzymatic digestion and filter separation as previously described [57]. Two hundred thousand stromal cells were seeded onto 1.9-cm^2 wells coated with type I collagen and maintained until approximately 75% confluent (18–24 hours) in phenol red-free DMEM/F-12 with 5% charcoal-stripped calf serum, 1nM 17-β estradiol (E) and 1× antibiotic-antimycotic solution at 37°C and 5% CO_2. Subconfluent cells were removed to serum-free medium with 1nM E with and without 500nM progesterone (P). Some cultures also received 10nM TCDD (in nonane; Cerilliant Corp, Round Rock, TX), Interleukin-1α (IL-1α; 10ng/mL; R&D Systems, Minneapolis, MN) or 5μM Onapristone (Ona; a gift from Schering-AG, Berlin, Germany). Triplicate groups were maintained for either 48h (Ona) or 5 days with media changes every 48h. Terminated cultures were flash-frozen and stored at −80°C until analysis. Steroids and remaining chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Quantitative Real-Time PCR (qRT-PCR)

RNA was extracted from cultured endometrial stromal cells using Qiagen RNeasy kit (Qiagen, CA) while RNA from endometrial biopsies was extracted with TRIzol (Invitrogen, Carlsbad, CA). Reverse transcription was performed using BioRad iScript cDNA Synthesis Kit (BioRad, CA) in duplicate reactions of: 1μl cDNA, 0.5μl (10pmol/μl) each of forward and reverse primers, 5 μl of BioRad SsoFast™ EvaGreen® Supermix (BioRad, CA) and 3μl

nuclease free water (Ambion, Austin TX). Human ribosomal protein, large P0 (RPLP0), was used as the internal reference gene [58]. Primer sequences:

hRPLP0-F 5'-TCGACAATGGCAGCATCTAC-3'

hRPLP0-R 5'-GCCTTGACCTTTTCAGCAAG-3'

hCB1-F 5'-TCCTAGATGGCCTTGCAGAT-3'

hCB1-R 5'-CCCACACTGGATGTCTCCT-3'

hPR-B-F 5'-ACACCTTGCCTGAAGTTTCG-3

hPR-B-R 5'-TCCAAGACACTGTCCAGCAG-3

hMMP-3-F 5'-GCAGTTTGCTCAGCCTATCC-3'

hMMP-3-R 5'-GAGTGTCGGAGTC CAGCTTC-3'

Negative controls included duplicate wells in which cDNA was omitted (volume adjusted with nuclease-free water). qRT-PCR was performed using a BioRad CFX96 Real-Time System™, followed by melt curve to ensure single amplicon. Data was analyzed by ΔΔct method [59].

Immunohistochemical Localization of CB1-R protein

Immunohistochemical staining of human endometrial tissues obtained by biopsy was performed by standard methodology for formalin-fixed, paraffin-embedded tissues. Antigen retrieval was performed on sections using 1× Antigen Retrieval Citra Solution (BioGenex, Fremont, CA) and endogenous peroxidase activity blocked by incubating sections in 3% hydrogen peroxide. To prevent non-specific binding of the antibody, sections were blocked in 3% Bovine Serum Albumin (BSA; Sigma-Aldrich, St. Louis, MO) in PBS-Tween (PBST) for 1 hr at room temperature (RT). The primary antibody (rabbit anti-human CB1-R; Santa Cruz, Santa Cruz, CA), diluted 1:200 in BSA/PBST, was added to the sections which were incubated overnight at 4°C. After washing with PBST, a biotinylated universal secondary antibody (Dako LSAB kit, Dako, Carpinteria, CA) was added to the sections for 30 min at RT, followed by incubation with Streptavidin-HRP (DakoCytomation LSAB+ System-HRP). Color detection was performed using 3,3'-diaminobenzidine (DAB; DAB Substrate kit, Vector Laboratories, Burlingame, CA). The sections were then lightly counterstained with Gill's hematoxylin, dehydrated and coverslipped. Slides were viewed with an Olympus BX51 microscope system and images captured at 1000× using an Olympus DP71 digital camera.

Statistical Analysis

Analyses were performed with GraphPad Prism©5 software and presented as mean ±SEM. The statistical difference between samples was determined using unpaired two-tailed t-test (Figures 3 and 4) and one-way analysis of variance (ANOVA) followed by Bonferroni posthoc test (Figures 1A, 2 and Supplemental Figure 1). $P<0.05$ were considered significant.

RESULTS

CB1-R mRNA and Protein Expression in Human Endometrial Biopsies

Following RNA extraction from flash frozen human endometrial biopsies, we examined CB1-R mRNA expression levels in tissues obtained from healthy women and women with surgically-confirmed endometriosis during the late proliferative and midsecretory phases of the menstrual cycle. In disease-free women, CB1-R mRNA expression was significantly elevated in secretory tissues compared to proliferative phase samples (Figure 1A),

suggesting an important role for progesterone in the regulation of this gene. In contrast, CB1-R mRNA expression was minimal in endometrial tissues obtained from women with endometriosis, regardless of the cycle phase (Figure 1A).

Formalin-fixed, paraffin-embedded endometrial tissues from women with and without endometriosis were further examined for immunolocalization of CB1-R protein. These studies revealed that expression of CB1-R protein is minimal in tissues obtained during the proliferative phase from healthy women (Figure 1B), but is abundant in both stromal and epithelial cells in endometrial tissues obtained during the secretory phase (Figure 1B). In contrast, immunolocalization of CB1-R protein, associated with either stromal or epithelial cells, is virtually absent in proliferative and secretory phase samples obtained from women with endometriosis (Figure 1B); an expression pattern which mirrors the mRNA data.

Onapristone Prevents Progesterone-Mediated CB1-R mRNA Expression in Isolated Human Endometrial Stromal Cells

The *in vivo* data described above suggest a role for progesterone in endometrial CB1-R regulation. To more definitively link progesterone treatment to the induction of endometrial CB1-R expression, we isolated stromal cells from the proliferative phase, thereby avoiding *in vivo* exposure of cells to progesterone or downstream mediators of progesterone action. As shown in Figure 2, endometrial stromal cells cultured with estradiol alone (E) exhibited virtually no expression of CB1-R mRNA while a dramatic (>100-fold) increase was observed in the presence of progesterone (EP) ($P<0.001$). Cells exposed to EP and Onapristone, a pure PR antagonist, expressed a significantly lower level of CB1-R mRNA ($P<0.001$) similar to cultures treated only with E.

TCDD Inhibits Progesterone-Dependent Induction of CB1-R mRNA

We have previously demonstrated that acute, *in vitro* exposure of isolated human endometrial cells to the environmental endocrine disruptor, TCDD, reduces the subsequent responsiveness of the cells to progesterone [7, 55]. As shown in Figure 3, the current study demonstrates that TCDD treatment of isolated endometrial stromal cells obtained from control women during the progesterone dominated secretory phase leads to a significant ($P<0.001$) reduction in CB1-R mRNA expression despite continuous exposure to progesterone. Specifically, cells were cultured for 5 days in media supplemented with estradiol and progesterone (EP) in the presence and absence of TCDD. These data further implicate progesterone as a key regulator of the ECS and suggest cannabinoid signaling during endometrial maturation is equally sensitive to disruption by TCDD.

TCDD or Onapristone Prevents Progesterone Suppression of MMP-3 mRNA

Reduced expression of multiple MMPs has been demonstrated during the progesterone dominant secretory phase of healthy women, while persistent expression has been noted in women with endometriosis, regardless of the cycle phase [8, 11, 16]. Using isolated endometrial stromal cells from the proliferative phase of control donors, Supplemental Figure 1 demonstrates that either TCDD treatment (which suppresses endometrial PR-B expression) or treatment with Onapristone (a pure PR antagonist) effectively blocks the ability of progesterone to down-regulate stromal cell MMP-3 mRNA expression. Thus, impeding progesterone action not only leads to a failure to induce CB1-R mRNA expression (Figures 2 and 3), but also prevents regulation of a gene known to be suppressed by this steroid (Supplemental Figure 1) [11, 60–62].

Loss of PR-B Correlates with Reduced CB1-R mRNA Induction *in vitro*

As noted above, both *in vivo* and *in vitro* studies suggest that the ability of TCDD exposure to decrease the expression of PR within the reproductive tract correlates with a heightened sensitivity to inflammation [5, 7, 19, 55–56]. Therefore, we explored whether acute TCDD exposure in combination with a pro-inflammatory cytokine might act synergistically to disrupt progesterone-mediated regulation of endometrial CB1-R mRNA expression. Isolated human endometrial stromal cells obtained from endometrial biopsies taken from disease-free women during the secretory phase were cultured for a total of 5 days in media supplemented with estradiol and progesterone (EP) with and without TCDD (10nM), interleukin-1α (10ng/mL) or a combination of these agents. Consistent with our previous studies, TCDD exposure of secretory phase stromal cells leads to a significant decrease in PR-B mRNA expression (Figure 4; left panel). Although only a non-significant decrease in PR-B mRNA expression was observed following stromal cell exposure to IL-1α alone, the levels of this gene were significantly decreased in cells treated with both TCDD and IL-1α (Figure 4; left panel), suggesting a synergistic effect of these compounds. Exposure of cells to TCDD and IL-1α led to a similar pattern of diminished progesterone-mediated expression of CB1-R mRNA (Figure 4; right panel), further linking the loss of progesterone's anti-inflammatory actions to aberrations in cannabinoid signaling within the human endometrium.

DISCUSSION

A woman's exposure to progesterone is recognized as an important negative risk factor for the development of endometriosis [6, 16]; therefore, it is not surprising that recent research suggests that reduced sensitivity to this steroid plays a key role in the pathophysiology of this disease [6–7, 19]. Since the anti-inflammatory action of progesterone is required for normal immune cell migration and function during the menstrual cycle and pregnancy, loss of normal progesterone responsiveness is likely a component of the immunological alterations noted in endometriosis [10]. Importantly, pregnancy establishment requires a controlled inflammatory event, which allows for implantation to occur, yet prevents widespread tissue destruction and spontaneous pregnancy loss. To this end, activation and function of the ECS is known to be required for successful pregnancy establishment and maintenance [20, 63], perhaps due to its ability to mediate a controlled inflammatory response which limits collateral tissue damage [64–65]. Thus, progesterone and local ECS signaling must act in concert to regulate the complex inflammatory microenvironment that allows establishment and maintenance of an appropriate maternal-fetal interface.

In the current study, we explored whether a loss of progesterone action, due to endometriosis [4, 6, 11, 66] or following acute exposure to TCDD [7], would affect stromal cell expression of CB1-R mRNA, the receptor which primarily mediates endometrial response to cannabinoids. Initially, we demonstrated that the expression of CB1-R mRNA and protein in healthy human endometrium is significantly increased during the progesterone-dominant secretory phase (Figure 1A and 1B). These findings suggest that progesterone is involved in regulating endometrial CB1-R expression and agree with the findings of Horne et al, who noted the highest levels of CB1-R mRNA and protein expression in the human fallopian tube during the secretory phase [27]. In contrast, examination of endometrial samples exhibiting the progesterone resistant endometriosis phenotype revealed minimal CB1-R expression, regardless of the cycle phase (Figure 1A and 1B). Thus, our current study indicates that endometriosis patients exhibit alterations in cannabinoid responsiveness, potentially contributing to the infertility and immune dysregulation which is frequently associated with this disease [10].

Additionally, while it is known that acute exposure of women and other primates to TCDD induces pregnancy failure [56, 67], the specific role that this ubiquitous environmental

toxicant may play in the etiology of endometriosis remains uncertain. Using cultures of endometrial stromal cells obtained from disease-free women, we previously found that acute exposure to TCDD leads to a dose-dependent loss of PR-B expression and subsequent failure of progesterone to down-regulate MMP-3 expression [7], mimicking the endometrial phenotype observed in women with endometriosis [4, 6, 19]. In the present study, endometrial stromal cells were similarly obtained from disease-free women during both the proliferative and secretory phases of the menstrual cycle in order to develop a more comprehensive view of TCDD's effects on CB1-R mRNA expression and regulation. As shown in Figure 2, in the absence of TCDD exposure, we found that endometrial stromal cells obtained during the proliferative phase and cultured only with estradiol expressed minimal levels of CB1-R mRNA while progesterone treatment led to a significant increase in expression of this gene, mirroring the cyclic regulation of CB1-R mRNA and protein in endometrial biopsies from control donors (Figure 1A and 1B). In contrast, treatment of proliferative phase stromal cells with the PR antagonist, Onapristone, largely prevented progesterone-mediated induction of CB1-R mRNA (Figure 2) and additionally blocked the suppressive impact of this steroid on MMP-3 mRNA expression (Supplemental Figure 1). The use of proliferative phase samples to obtain stromal cells without a prior exposure to progesterone *in vivo* coupled with the use of Onapristone to block progesterone action *in vitro* confirm progesterone's involvement in regulating CB1-R expression in human endometrial stromal cells.

We also examined whether exposure to TCDD disrupts the ability of progesterone to induce CB1-R mRNA expression involved a loss of this steroid's anti-inflammatory action. For this study, secretory phase endometrial stromal cells were maintained in the presence of both estradiol and progesterone and some cultures were further treated with either TCDD (Figure 3), IL-1α or a combination of these agents (Figure 4). These studies were designed to determine whether an inflammatory challenge in cells treated with TCDD further disrupts the ability of progesterone to regulate the differentiation-related expression of CB1-R mRNA. As expected, addition of TCDD to progesterone-treated stromal cells significantly reduced the ability of this steroid to up-regulate CB1-R mRNA ($p<0.001$) compared to sister cultures which were not exposed to TCDD. Interestingly, while exposure of stromal cells to estradiol and progesterone plus IL-1α only modestly affected expression levels of PR-B, exposure to this cytokine significantly ($p<0.01$) inhibited CB1-R mRNA expression (Figure 4), potentially indicating an important intermediary between progesterone and CB1-R gene expression. Regardless, the lowest level of CB1-R mRNA expression was observed following combined treatment with TCDD and IL-1α, an exposure paradigm that also led to a significant reduction of PR-B mRNA expression (Figure 4). Relative to our findings linking loss of CB1-R to inflammation, it is interesting to note that CB1-R/CB2-R knockout mice exhibit an exacerbated inflammatory response following influenza infection [65] and loss of CB1-R in the urinary bladder promotes hypersensitivity to inflammation in humans [68]. These recent studies, taken together with studies presented herein, suggest an important inter-relationship between the anti-inflammatory action of progesterone and the regulation of endometrial endocannabinoid signaling via CB1-R.

To our knowledge, these studies demonstrate for the first time that the TCDD-mediated loss of PR-B expression correlates with the loss of stromal cell expression of CB1-R mRNA. Therefore, these data not only support an important role of progesterone in the regulation of endometrial CB1-R mRNA expression, but demonstrate an ability of a persistent, environmental toxicant to disrupt normal endometrial ECS signaling. However, our studies do not rule out the possibility that progesterone-mediated regulation of CB1-R expression is indirect, via a mediator of progesterone action. Indeed, the promoter of the human CB1-R gene does not contain a known progesterone response element (promoter search 3KB, 5' of transcriptional start site, data not shown), suggesting progesterone regulation of this gene

may be indirect. For example, we and others have previously identified retinoic acid as an important mediator of progesterone-associated endometrial action [66, 69–70], and retinoid signaling has been shown to directly regulate CB1-R mRNA expression in the murine liver [71].

It is important to note that our *in vivo* findings in the endometrium of endometriosis patients are in conflict with two previous studies examining endometrial CB1-R expression. Taylor and colleagues [72] reported that endometrial CB1-R immunoreactivity is “unrelated to the phase of the cycle” while Leconte and coworkers concluded that CB1-R expression is similar in endometrial cells obtained from women with and without endometriosis [73]. However, in contrast to the aforementioned studies, control tissue donors used for this study were carefully screened in an effort to avoid medications or occult gynecologic diseases that may alter endometrial steroid action.

In summary, we demonstrate an important role for the anti-inflammatory actions of progesterone in the regulation of endometrial endocannabinoid response which is dysregulated in women with endometriosis and by *in vitro* exposure to TCDD. Since both progesterone and the ECS are critical to regulating the appropriate inflammatory response during early pregnancy establishment, understanding the mechanisms which lead to a failure of steroid-regulated CB1-R expression may provide a better understanding of endometriosis-associated infertility. Our studies further support the examination of agents which target these systems, potentially providing new and much needed avenues for additional treatment strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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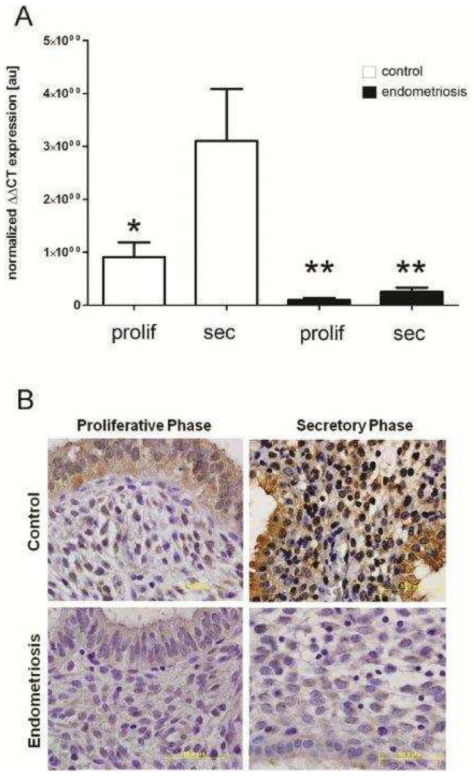

Figure 1. Progesterone-mediated endometrial CB1-R mRNA and protein expression is disrupted in women with endometriosis

Human endometrial tissues were obtained by biopsy during the proliferative or secretory phase from women with or without endometriosis and a portion flash-frozen for mRNA analysis and a portion formalin-fixed and paraffin embedded for immunohistochemical analysis. Quantitative RT-PCR analysis of CB1-R mRNA expression (**A**) revealed that in samples obtained from disease-free women, CB1-R mRNA expression was dramatically increased in the secretory phase (N=11) compared with samples obtained during the proliferative phase (N=9). Endometrial tissues obtained from women with endometriosis expressed minimal CB1-R mRNA in both the proliferative (N=7) and secretory (N=8) phases of the cycle. qRT-PCR data, normalized to the housekeeping gene (ribosomal protein large P0, RPLP0), was analyzed using the ΔΔCT method [59] and * is indicative of $P<0.05$. Immunohistochemical localization of CB1-R (**B**) was similar to mRNA expression patterns in that protein expression was weak in control samples obtained during the proliferative phase, but robust staining was observed in secretory phase samples. However, CB1-R immunolocalization was minimal in both proliferative and secretory phase samples from women with laparoscopically-confirmed endometriosis. All slides were photographed at 1000× and results representative of at least 5 samples in each group.

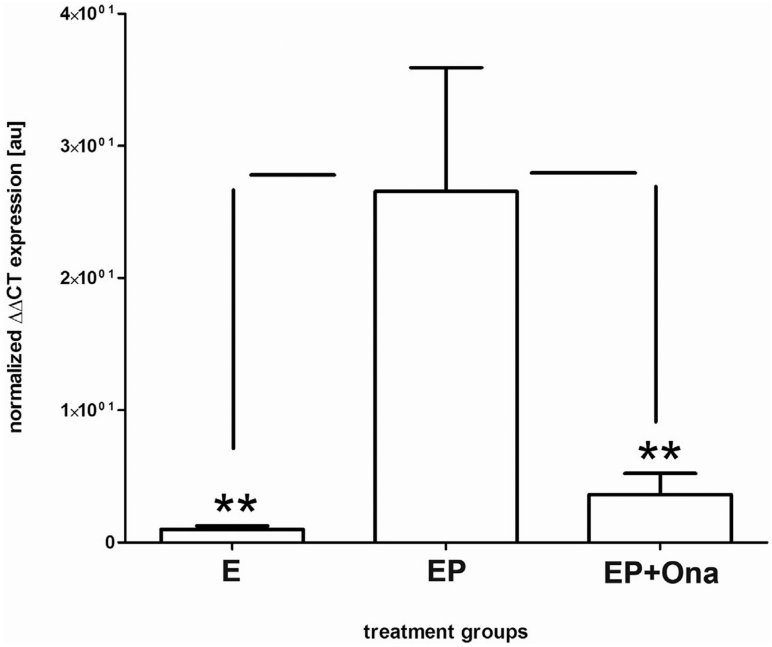

Figure 2. Progesterone-mediated CB1-R mRNA expression in isolated stromal cells is blocked by Onapristone

Stromal cells were isolated from control endometrial biopsies taken during the proliferative phase (N=5) and cultured for 48hr in estradiol (E; 1nM) with or without progesterone (P; 500nM). Some cultures additionally received the progesterone receptor antagonist, Onapristone (Ona; 5μM). CB1-R mRNA expression was low in cells maintained in E alone, but was significantly increased in cells additionally exposed to P ($P<0.01$). Co-treatment with Ona abolished progesterone induction of CB1-R mRNA ($P<0.01$). qRT-PCR data, normalized to the housekeeping gene, was analyzed using the ΔΔCT method [59] and ** is indicative of $P<0.01$.

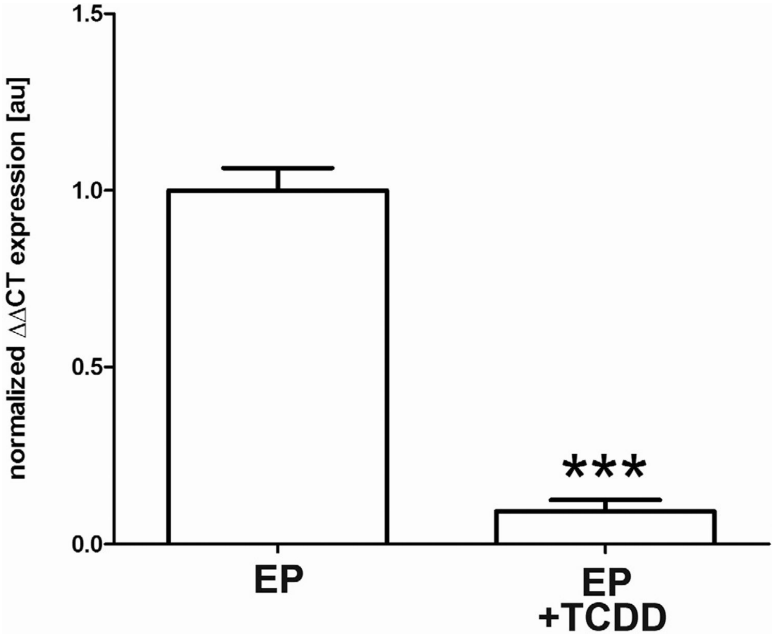

Figure 3. ***In vitro*** **TCDD exposure blocks progesterone induction of CB1-R mRNA expression in endometrial stromal cells obtained from control donors**

Stromal cells were isolated from control endometrial biopsies taken during the secretory phase (N=5) and cultured 4 days with estradiol + progesterone (E; 1nM; P; 500nM) with or without TCDD (10nM). CB1-R mRNA expression was robust in cells maintained in EP, while those cells treated with TCDD showed significantly reduced expression of this transcript ($P < 0.001$). qRT-PCR data, normalized to the housekeeping gene, was analyzed using the ΔΔCT [59] method and *** is indicative of $P < 0.001$.

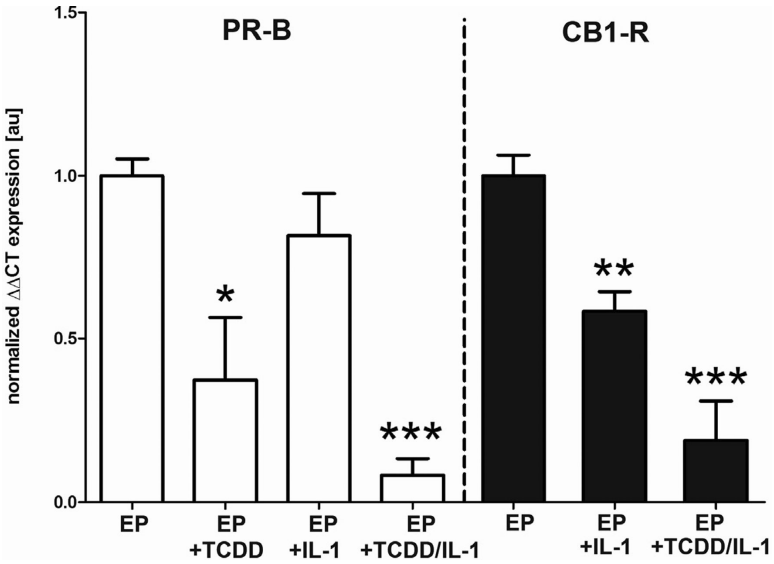

Figure 4. Synergistic effect of TCDD and IL-1α in isolated stromal cells

Control endometrial stromal cells were isolated from the secretory phase and cultured 5 days with estradiol + progesterone (E; 1nM; P; 500nM) in the presence and absence of TCDD (10nM). Some cultures were challenged with IL-1α (10ng/mL) during the last 24 hrs of culture. Expression of PR-B (**left panel**) and CB1-R mRNA (**right panel**) was robust in cells treated with EP, while cells treated with TCDD showed a marked reduction in PR-B mRNA expression levels ($P< 0.05$), similar to CB1-R mRNA levels observed in Figure 3. Treatment with IL-1α alone led to a non-significant reduction in PR-B mRNA expression while CB1-R mRNA levels were significantly reduced ($P< 0.01$). Combinatorial treatment with EP, TCDD and IL-1 led to the lowest expression levels of both genes ($P< 0.001$). qRT-PCR data, normalized to the housekeeping gene, was analyzed using the ΔΔCT method [59]. **P<0.01; *** P< 0.001.