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Effects of endocannabinoid 1 and 2 (CB1; CB2) receptor agonists on luteal weight, circulating progesterone, luteal mRNA for luteinizing hormone (LH) receptors, and luteal unoccupied and occupied receptors for LH *in vivo* in ewes

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ABSTRACT

Thirty to forty percent of ruminant pregnancies are lost during the first third of gestation due to inadequate progesterone secretion. During the estrous cycle, luteinizing hormone (LH) regulates progesterone secretion by small luteal cells (SLC). Loss of luteal progesterone secretion during the estrous cycle is increased via uterine secretion of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) starting on days 12–13 post-estrus in ewes with up to 4–6 pulses per day. Prostaglandin $F_{2\alpha}$ is synthesized from arachidonic acid, which is released from phospholipids by phospholipase A2. Endocannabinoids are also derived from phospholipids and are associated with infertility. Endocannabinoid-induced infertility has been postulated to occur primarily via negative effects on implantation. Cannabinoid (CB) type 1 (CB1) or type 2 (CB2) receptor agonists and an inhibitor of the enzyme fatty acid amide hydrolase, which catabolizes endocannabinoids, decreased luteal progesterone, prostaglandin E (PGE), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) secretion by the bovine corpus luteum *in vitro* by 30 percent. The objective of the experiment described herein was to determine whether CB1 or CB2 receptor agonists given *in vivo* affect circulating progesterone, luteal weights, luteal mRNA for LH receptors, and luteal occupied and unoccupied LH receptors during the estrous cycle of ewes. Treatments were: Vehicle, Methanandamide (CB1 agonist; METH), or 1-(4-chlorobenzoyl)-5-methoxy-1H-indole-3-acetic acid morpholineamide (CB2 agonist; IMMA). Ewes received randomized treatments on day 10 post-estrus. A single treatment (500 μg; $N = 5$/treatment group) in a volume of 1 ml was given into the interstitial tissue of the ovarian vascular pedicle adjacent to the luteal-containing ovary. Jugular venous blood was collected at 0 h and every 6–48 h for the analysis of progesterone by radioimmunoassay (RIA). Corpora lutea were collected at 48 h, weighed, bisected, and frozen in liquid nitrogen until analysis of unoccupied and occupied LH receptors and mRNA for LH receptors. Profiles of jugular venous progesterone, luteal weights, luteal mRNA for LH receptors, and luteal occupied and unoccupied LH receptors were decreased ($P \leq 0.05$) by CB1 or CB2 receptor agonists when compared to Vehicle controls. Progesterone in 80 percent of CB1 or CB2 receptor agonist-treated ewes was decreased ($P \leq 0.05$) below 1 ng/ml by 48 h post-treatment. It is concluded that the stimulation of either CB1 or CB2 receptors *in vivo* affected negatively luteal progesterone secretion by decreasing luteal mRNA for LH receptors and also decreasing occupied and unoccupied receptors for LH on luteal membranes. The corpus luteum may be an important site for endocannabinoids to decrease fertility as well as negatively affect implantation, since progesterone is required for implantation.

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1. Introduction

Two cannabinoid receptors are present in mammalian tissues: CB1, originally identified in the central nervous system (CNS) [1–3], while CB2 was identified in macrophages from the spleen, B-cells and natural killer cells [3,4]. Endogenous cannabinoids or endocannabinoids are unsaturated fatty acid derivatives of phospholipids and are ligands for CB1 or CB2 receptors [4–8]. Endocannabinoids bind to receptors and inhibit voltage-gated calcium channels and adenylate cyclase activity, but stimulate mitogen-activated protein kinase C (MAPK) [6,9,10]. Endogenous cannabinoids are also ligands for seven transmembrane receptor G proteins

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(Gi/o, GTP binding proteins) and activate pertussis toxin-coupled cannabinoid receptors [3–5]. Two endocannbinoids produced from phospholipids are arachidonlylethanolamide (anandamide; AEA) and 2 arachidonoyl glycerol (2-AG) [7]. AEA and 2-AG bind to CB1 with slightly greater affinity than CB2 [4]. Likewise inhibition of cAMP by AEA is greater for CB1 than CB2 [11]. In contrast, inhibition of cAMP production by 2-AG is greater for the CB2 receptor [12]. CB1 also inhibits N-type voltage-dependent calcium channels, while CB2 does not appear to couple to ion channels [4]. Both CB1 and CB2 stimulate mitogen-activated protein kinase (MAPK) activity [4,9] and inhibit nitric oxide synthase (NOS) production of NO [13–16].

Endocannbinoids are catabolized by fatty acid amide hyrolase (FAAH) [7,8]. Endocannabinoids affect an array of physiological responses in the CNS as well as the endocrine and immune systems [17–20] in both the male and female, including the hypothalamus, pituitary, gonads, sex accessory ducts, spermatogenesis, oocytes, and fertilization [21–73]. Among these effects are a negative impact on fertilization, testicular function, sperm production and motility, embryo viability, embryo transport in the oviduct, embryonic development, implantation, establishment and maintenance of pregnancy and an increased occurrence of ectopic pregnancy [23–29,33–73].

CB1, but not CB2, binding sites for AEA are expressed in uterine endometrial luminal epithelium and stroma [63]. Cannabinoids upregulated some estrogen responsive genes in the uterus of ovariectomized mice and tetrathydrocannabinol (THC) increased the expression of some estrogen target genes to a higher level than that induced by estradiol [63]. Messenger RNA for CB1 and CB2 receptors and AEA production have been reported in the human ovary [69]. CB1 and CB2 are expressed in the ovary with expression of CB2 in granulosa cells of primordial, primary, secondary, and tertiary follicles and granulosa cells of the corpus luteum and corpus albicans being greater than CB1 [69]. Concentrations of AEA are also greater in follicular fluid of mature follicles and only oocytes of tertiary follicles express CB2 mRNA [69]. Ooctyes may not respond to AEA until late in follicular development [69]. Cannabinoids also influence reproduction via the hypothalamic–pituitary–ovarian axis [6]. CB1 receptors are present in the arcuate nucleus and preoptic area of the hypothalamus as well as the adenohypophysis [6]. Levels of CB1 in the adenohypophysis change throughout the estrous cycle with expression being higher during diestrus and lower during estrus, which is presumably regulated by steroids [6]. However, AEA expression in the adenohypophysis is lower during diestrus, but peaks at estrus [6]. In humans, smoking marijuana during the luteal phase decreased circulating LH by 30 percent within 1 h compared to controls, but not during the follicular phase [51]. Women who smoked marijuana at least four times per week had shorter menstrual cycles, which was via a dose-dependent decrease in circulating LH during the luteal phase resulting in a shortened luteal phase [51]. Furthermore, THC prevented the LH surge, blocked ovulation, and delayed puberty in female rats, and caused irregular estrous cycles with decreased concentrations of follicle stimulating hormone (FSH) and LH. Similar results have been described in the Rhesus monkey [6]. Inhibitory effects of cannabinoids on ovulation are reversed by exogenous gonadotropin releasing hormone (GnRH), indicating that cannabinoids negative effect on reproductive function could occur at the level of the hypothalamus [6,50].

Chronic administration of AEA prolonged pregnancy, increased stillbirths, and decreased serum LH, progesterone, $PGF_{2\alpha}$, and prolactin in pregnant rats, indicating both positive and negative effects on reproduction [6]. Endocannabinoids and their receptors as well as corticosteroids, corticotrophin releasing hormone (CRH), and $PGF_{2\alpha}$ increased in uterine tissue at the time there was a decrease in progesterone just prior to parturition, indicating endocannabinoids may play a role in parturition [66]. A decreased progesterone/estradiol ratio is associated with pre-term labor [67–71]. Moreover, CRH increased earlier in CB1 deficient mice, around day-14, and remained high through day-20 [67–71]. This rise in CRH was accompanied by an increase in corticosterone on days 14–16 [71]. CRH regulates length of pregnancy in women and pre-term birth occurs in about 10 percent of human pregnancies [66–71,74].

Concentrations of FAAH, the enzyme that catabolizes endocannabinoids, were lower in women who miscarried versus those with a normal pregnancy [6]. However, FAAH was down-regulated during pregnancy and pseudopregnancy in the mouse and estrogen supplementation decreased FAAH expression [19]. These data suggest that changes in FAAH expression in the mouse uterus are not dependent on the presence of embryos in the uterus, but are regulated by steroids [19]. However, embryos expressed FAAH up through the blastocyst stage of development and endocannabinoids decreased mitotic rate and arrested development and hatching of embryos [6]. Knockout of CB1, CB2, and CB1/CB2 knockout mouse embryos resulted in asynchrony of embryonic and uterine development by day-4, which is when progesterone, estradiol, NO, and PGE_2 are obligatory for implantation [71–73]. By day-4, 98 percent of normal wild type embryos were at the blastocyst stage, while only 62, 71, and 61 percent of embryos deficient in CB1, CB2, and both CB1/CB2, respectively, reached the blastocyst stage [70,90]. Cannabinoids also affect the movement of embryos through the oviduct. Knockout of the CB1 receptor in mice was associated with oviductal retention of embryos [66] and endocannabinoids appear to be associated with increased occurrence of ectopic pregnancies [66–68].

Low doses of AEA stimulated mouse blastocyst attachment and outgrowth on a monolayer of uterine epithelial cells, while high doses inhibited blastocyst attachment and outgrowth *in vitro* [72]. Concentrations of AEA were higher in non-implantation sites compared to implantation sites [6]. AEA expression was the highest on day-5 during the non-receptive phase in pseudopregnant rats [65]. The increased AEA expression during the non-receptive phase may mediate embryotoxic effects of the uterus observed at this time [65]. 2-AG was present in the peri-implantation mouse uterus at levels 200-fold greater than AEA with similar expression patterns being low at implantation sites and higher at inter-implantation sites [36]. Approximately half of all conceptuses are lost in humans during IVF and embryo transfer and these losses are associated with decreased FAAH and a high frequency of failure of implantation resulting in spontaneous abortion [48,49].

Similar losses of pregnancy occur in naturally mated or artificially inseminated ewes and cows with 30–40 percent of pregnancies lost during the first trimester of pregnancy and an additional 8–10 percent being lost during the second trimester [74–77]. A possible cause for lost pregnancies in ewes and cows may result from a deficiency in progesterone secretion by the corpus luteum [78–83]. Progesterone is obligatory for implantation and to maintain pregnancy throughout gestation [84,85]. In rats and mice, estradiol-17β, PGE_2, and NO increase in uterine tissue on day-4 and are obligatory for implantation [84–86]. In ewes and cows, loss of progesterone secretion by the corpus luteum at the end of the estrous cycle is via uterine endometrial secretion of $PGF_{2\alpha}$ [74–78]. $PGF_{2\alpha}$ is synthesized from the polyunsaturated fatty acid arachidonic acid, which is released from phospholipids by phospholipase A_2 for prostaglandin synthesis. Arachidonic acid is then converted to the 2 series of prostaglandins by cyclooxygenase 1 or 2 (COX_1; COX_2) [75]. PGE_1 and PGE_2 are embryonic/endometrial secretory products that increase in uterine tissue and venous drainage to prevent loss of progesterone secretion by the corpus luteum [74–78]. While the negative effects of endocannabinoids on pregnancy in mice, rats, and women are thought to be via inhibition of implantation