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Review

Ligand-receptor signaling with endocannabinoids in preimplantation embryo development and implantation

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Abstract

Although adverse effects of cannabinoids on pregnancy have been indicated for many years, the mechanisms by which they exert their actions were not clearly understood. Only recently, molecular and biochemical approaches have led to the identification of two types of cannabinoid receptors, brain-type receptors (CB1-R) and spleen-type receptors (CB2-R), which mediate cannabinoid effects. These findings were followed by the discovery of endocannabinoids, anandamide and 2-arachidonoylglycerol (2-AG). The natural cannabinoids and endocannabinoids exert their effects via cannabinoid receptors and share similar pharmacological and physiological properties. Recent demonstration of expression of functional CB1-R in the preimplantation embryo and synthesis of anandamide in the pregnant uterus of mice suggests that cannabinoid ligand-receptor signaling is operative in the regulation of preimplantation embryo development and implantation. This review describes recent observations and their significance in embryo-uterine interactions during implantation and future research directions in this emerging area of interest.

Keywords: Cannabinoids; Anandamide; CB1-R; Blastocyst; Uterus; Mouse

1. Introduction

One of the major psychoactive components in marijuana, δ^9-tetrahydrocannabinol (THC), produces a wide array of effects in humans including alterations in mood, perception, cognition, memory, psychomotor activity and consciousness (Dewey, 1986). THC has also been reported to account for the majority of the reproductive hazards of marijuana use. For example, chronic marijuana use is associated with decreased plasma testosterone levels, reduced sperm counts and im-

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potency in men (Kolodney et al., 1974, 1976). In women, the chronic use of marijuana is often associated with fetal abnormalities and early pregnancy termination. In addition, early studies indicate that embryotoxicity and specific teratological malformations in rats, hamsters and rabbits are correlated with exposure to natural cannabis extracts during pregnancy (Geber and Schramm, 1969a,b; Persaud and Ellington, 1967). These effects were attributed to the estrogenic nature of this drug.

In 1990, we began a series of experiments on the possible estrogenic effects of THC in mice. We observed that an acute treatment with THC did not exhibit any overt estrogenic effects. However, ovariectomized or hypophysectomized mice subjected to chronic treatment with THC showed a weak proestrogenic effect with respect to uterine cell proliferation as assessed by nuclear DNA synthesis. This proestrogenic effect elicited by THC was considerably inferior to that produced by estradiol (Paria et al., 1992, 1994). Furthermore, an injection of THC also potentiated estradiol-induced transforming growth factor-β mRNA levels, but attenuated estradiol-induced increases in insulin like growth factor-1 and c-myc mRNA levels in the uterus (Das et al., 1993). These results suggested that THC is capable of producing modest proestrogenic and antiestrogenic effects in the mouse uterus. However, the mechanism(s) by which the THC modulates uterine functions were unknown. Also, THC's ability to bind to estrogen receptors in the target organs remained controversial.

2. Cannabinoid receptors and endocannabinoids

The mechanism of cannabinoid effects in various physiological processes remained obscure until specific cannabinoid receptors were identified. The receptors to which THC binds to produce its effects have now been identified, cloned and characterized. Cannabinoid receptors belong to two major subtypes, brain-type receptors (CB1-R) and spleen-type receptors (CB2-R). Both of these receptor subtypes are coupled to inhibitory guanine nucleotide regulatory proteins. CB2-R is primarily expressed in the spleen, while CB1-R is expressed in the brain, testis, spleen and blood leukocytes (Howlett, 1995; Matsuda et al., 1990; Munro et al., 1993). The identification of these cannabinoid receptors initiated the search for endogenous ligands with cannabinomimetric properties, leading to the discovery of two endogenous cannabinoid ligands, *N*-arachidonoylethanolamine (anandamide) and 2- arachidonoylglycerol (2-AG). These endogenous ligands bind with high affinity to CB1-R and CB2-R and mimic most of the effects of THC (Devane et al., 1992; Felder et al., 1992; Mechoulam et al., 1995; Sugiura et al., 1995; Stella et al., 1997). These reports prompted us to study the presence of cannabinoid ligand-receptor signaling in embryo and uterus during early pregnancy.

3. Cannabinoid receptors in the preimplantation embryo and uterus

In the mouse, both CB1-R and CB2-R, mRNAs are expressed in preimplantation embryos, while only CB1-R mRNA is present in the uterus (Das et al., 1995; Paria et al., 1995). CB1-R mRNA was primarily detected from the four-cell through the blastocyst stages, whereas CB2-R was present from the one-cell through the blastocyst stage (Fig. 1). Numerous autoradiographic binding sites for [^{3}H]anandamide were also evident from the one-cell through blastocyst stages (Fig. 2). However, in eight-cell, morula and blastocyst, the majority of the binding sites were noted in the outer cells (Fig. 2). Scatchard analysis in day 4 blastocysts showed that anandamide binds to a single class of high affinity receptors in blastocysts with an apparent Kd of 1.0 nM and B_{max} of 0.09 fmol/blastocyst.The presence of CB1-R mRNA and protein as detected by immunocytochemistry (Fig. 3) in preimplantation blastocysts correlates well with high affinity anandamide binding sites. The levels of anandamide binding sites in the blastocyst are higher than those in the brain (Yang et al., 1996). Furthermore, blastocyst CB1-R is biologically active, since both THC and anandamide inhibit forskolin-stimulated cAMP formation and this inhibition is prevented by per-

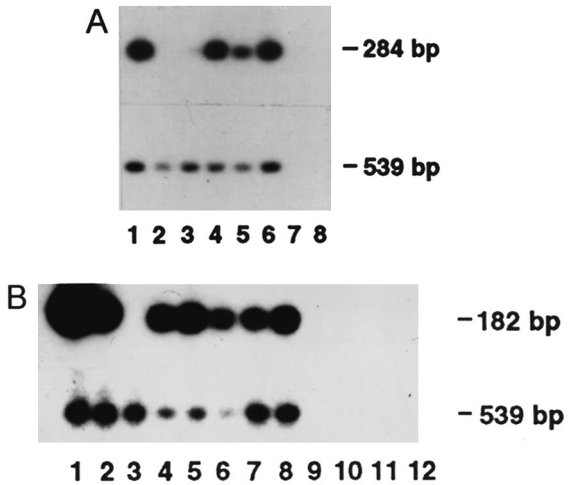

Fig. 1. Analysis of CB1-R and CB2-R transcripts in the preimplantation mouse embryo. (A) Southern blot analysis of RT-PCR-amplified products of CB1-R (284 bp) or β-actin (539 bp). Lanes: 1, mouse brain; 2–6, embryos at one-cell, two-cell, four-cell, eight-cell/morula, and blastocyst stages, respectively; 7, mouse brain RNA without RT reaction; 8, primer control. (B) Southern blot analysis of RT-PCR-amplified products of CB2-R (182 bp) or β-actin (539 bp). Lanes: 1, rat spleen; 2, mouse spleen; 3, day 1 pregnant uterus; 4–8, embryos at one-cell, two-cell, four-cell, eight-cell/morula, and blastocyst stages, respectively; 9–11, rat spleen, mouse spleen and mouse blastocyst RNA without RT reaction; 12, primer control. Reprinted with permission from Ref. Paria et al., 1995.

tussis toxin pretreatment (Das et al., 1995; Paria et al., 1995). The presence of this biologically active CB1-R in the blastocyst suggested that the mouse embryo is a potential target for both endocannabinoids and natural cannabinoids. Indeed, addition of synthetic (CP 55940, WIN 55212-2), natural (THC) or endogenous (anandamide and 2-AG) cannabinoids (Figs. 4 and 5) arrested the development of two-cell embryos into blastocysts in culture (Paria et al., 1995, 1998). A reduction in trophectodermal cell numbers was noted in those blastocysts that escaped the developmental arrest in the presence of cannabinoid agonists (Yang et al., 1996). However, these adverse effects were reversed by simultaneous addition of selective antagonists to CB1-R (SR 141716A, AM 251) with cannabinoid agonists, but not to CB2-R (SR 144528) (Paria et al., 1998). Taken together, these results suggested that cannabinoids mediate their actions in the blastocyst via CB1-R. The role of CB2-R in the blastocyst is yet to be defined.

4. Endocannabinoids in the pregnant uterus

The presence of high affinity CB1-R in the blastocyst led us to look for the presence of endogenous cannabinoid ligands in the reproductive tracts of mice. There is evidence for the synthesis of anandamide via enzymatic condensation of free arachidonic acid and ethanolamine in rat, bovine and rabbit brains (Deutsch and Chin, 1993; Devane and Axelrod, 1994; Kruszka and Gross, 1994; Ueda et al., 1995). We also provided evidence that the mouse oviduct and uterus have the enzymatic capacity to synthesize anandamide in a similar way (Paria et al., 1996). This biosynthetic pathway may be of significance in mouse uterus in vivo because, anandamide is not only present in very high amounts (142–1345 pmol/μmol lipid P), but also represents the major component (up to 95%) of all *N*-acylethanolamines. In contrast, *N*-arachidonate is only minor components ($< 5\%$) of mouse uterine *N*-acyl PE. We, therefore, proposed that the generally accepted "transacylation-phosphodiesterase" pathway of *N*-acylethanolamine synthesis via *N*-acyl PE may not be operative in mouse uterus (Schmid et al., 1997). Also, the levels of uterine anandamide fluctuate with changes in the pregnancy status (Fig. 6) (Schmid et al., 1997). Successful implantation is the result of an intimate "cross-talk" between the active blastocyst and the receptive uterus (Paria et al., 1993). Anandamide content is lower in the receptive uterus on day 4 of pseudopregnancy as compared to its levels in the nonreceptive uterus on days 5 and 6 of pseudopregnancy (Schmid et al., 1997) (Fig. 6).

5. Ligand-receptor signaling with cannabinoids during the periimplantation period

The higher levels of anandamide in the nonreceptive uterus correlate well with the embryotoxic effect of the nonreceptive uterine environment. This is also consistent with our observation of

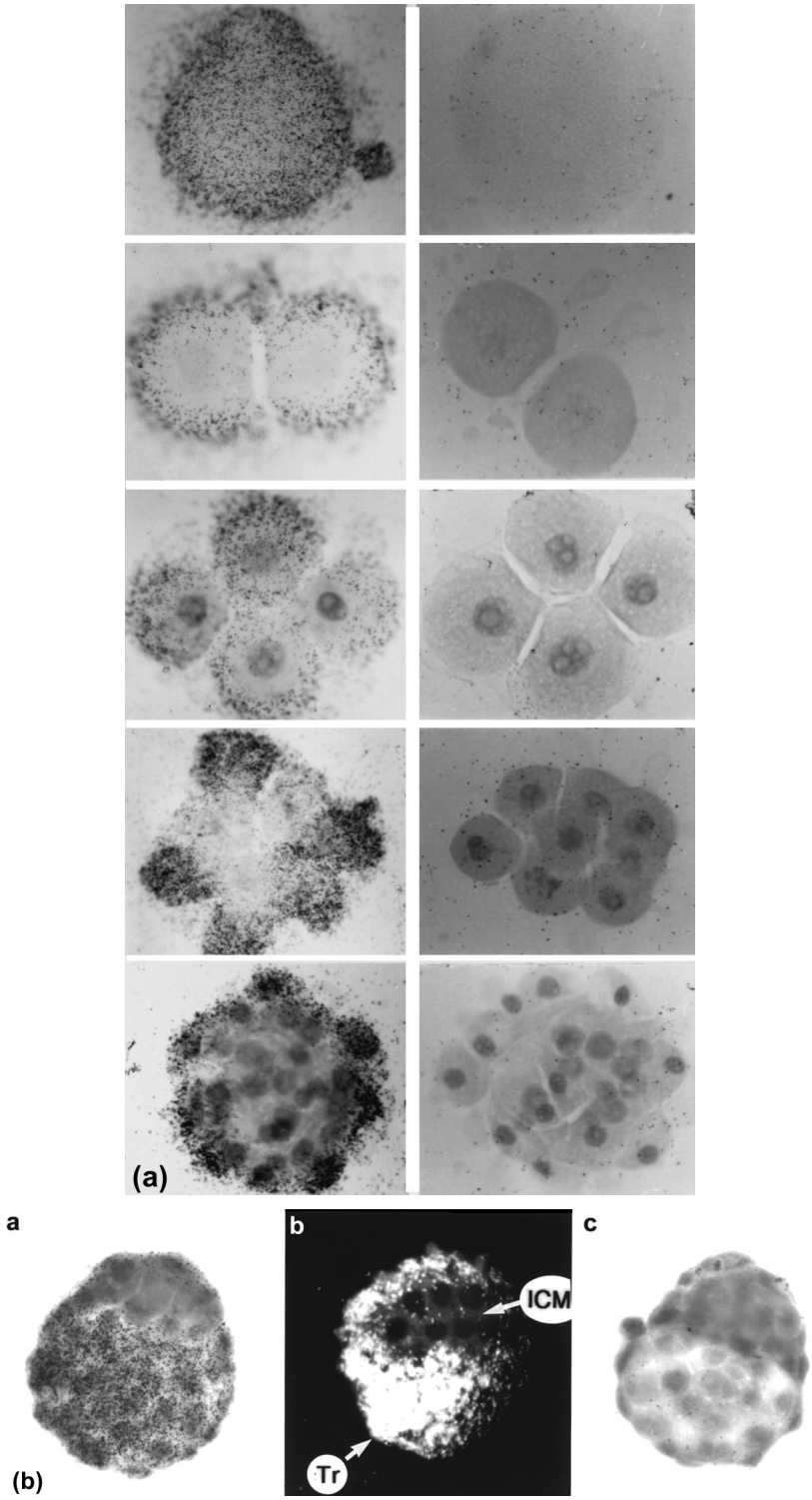

Fig. 2

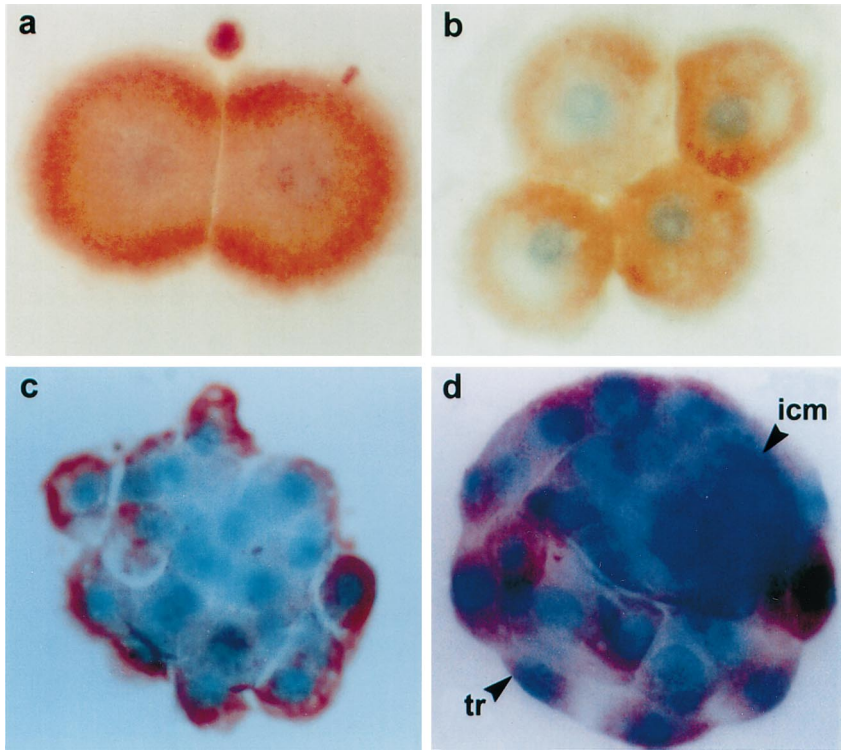

Fig. 3. Immunocytochemistry of CB1-R in preimplantation mouse embryos. Bright field microphotographs (X 400) of representative (a) two-cell; (b) four-cell; (c) morula and (d) blastocyst are shown. Red deposits indicate positive immunostaining. Tr, trophectoderm; icm, inner cell mass. Reprinted with permission from Ref. Yang et al., 1996).

anandamide-induced inhibition of embryo development and zona-hatching of blastocysts (Paria et al., 1995, 1998; Schmid et al., 1997; Yang et al., 1996). However, the lower levels of anandamide in the receptive uterus on day 4 of pregnancy suggest its involvement in normal embryo development and implantation. In this regard, we have provided evidence that the effects of cannabinoids are differentially executed depending on the embryonic stage and cannabinoid levels in the uterine environment. Blastocysts exposed in culture to low levels of cannabinoid agonists exhibit accelerated trophoblast differentiation and outgrowth, while inhibition of trophoblast differentiation is observed with higher doses of these agonists (Wang et al., 1999). Thus, levels of anandamide may be critical in regulating the ''window'' of implantation by synchronizing trophoblast differentiation and uterine preparation to the receptive state. We observed that the higher levels of anandamide at the inter-implantation sites are compared with those at the implantation sites. Higher levels of anandamide at the inter-implantation sites could be responsible for the inhibition of trophoblast proliferation to these regions, while lower levels may help trophoblast proliferation at

Fig. 2. Autoradiographic anandamide binding sites in the preimplantation mouse embryos. (A) Autoradiographic binding sites (bright field; black grains) in representative one-cell embryos through morula. (Left) Binding sites in the presence of [^{3}H]anandamide. (Right) Nonspecific binding sites in the presence of 500-fold molar excess of unlabelled anandamide. (B) Autoradiographic binding sites in representative blastocysts. (a) Binding sites (bright field; black grains) in the presence of [^{3}H]anandamide. (b) Confocal microscopy of a (white grains). (c) Nonspecific binding (bright field) in the presence of 500-fold molar excess of unlabeled anadamide. Tr, trophecoderm; ICM, inner cell mass. Reprinted with permission from Ref. Paria et al., 1995.

the implantation sites, suggesting dual functions of anandamide depending upon its local concentration (Schmid et al., 1997; Wang et al., 1999).

6. Anandamide hydrolysis in the pregnant uterus

The lower levels of anandamide at the implantation area may be due to rapid turnover of anandamide. A tissue amidohydrolase, also known as fatty-acid-amide hydrolase (FAAH), is capable of hydrolyzing anandamide and oleamide. Recently, the FAAH gene has been cloned in several species, including mice (Cravatt et al., 1996; Giang and Cravatt, 1997). The mRNA for FAAH is mainly localized in uterine luminal and glandular epithelia on days 1–4 of pregnancy. A recent investigation shows that mouse uterine FAAH activity is also modulated by sex steroids, progesterone and estrogen (Maccarrone et al., 2000a). Furthermore, using whole uterine homogenates, these investigators have shown that FAAH activity decreases during pregnancy in mice. We have shown that FAAH mRNA is primarily retained in the remaining epithelial cells in the endometrial bed with the progression of implantation (days 5–8). Furthermore, FAAH mRNA is also expressed in preimplantation and implanting embryos (Paria et al., 1999). The presence of FAAH mRNA in the uterus and embryo is consistent with anandamide amidase activity (Paria et al., 1999; Maccarrone et al., 2000a). Contrary to the anandamide levels, the amidase activity is higher at the implant site than at the inter-implantation site (Paria et al., 1996). Thus, these results suggest that both the uterus and

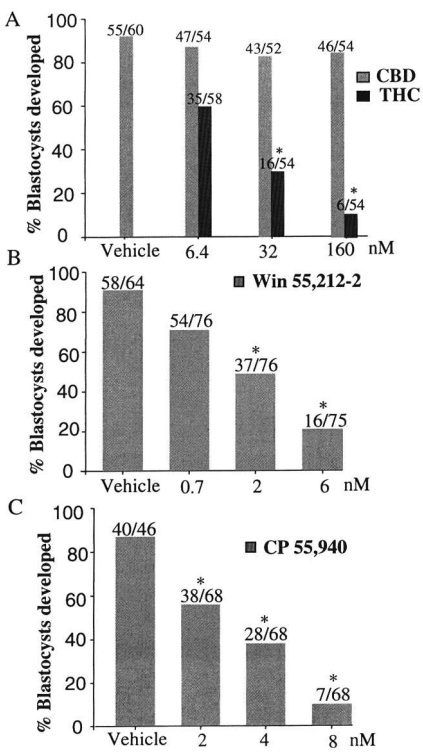

Fig. 4. Effects of natural and synthetic cannabinoid agonists on preimplantation embryo development. Effects of THC (δ^9-tetrahydrocannabinol) and CBD (cannabidiol) (A), WIN 55,212-2 (C) and CP 55,940 (D). The numbers above the bars indicate the number of blastocysts that developed/total number of two-cell embryos cultured. Each experiment was repeated 5 or 6 times with controls run simultaneously (statistical analysis by χ^2 and Fisher exact tests; $^*P < 0.05$, when compared to controls). Reprinted with permission from Ref. Paria et al., 1995.

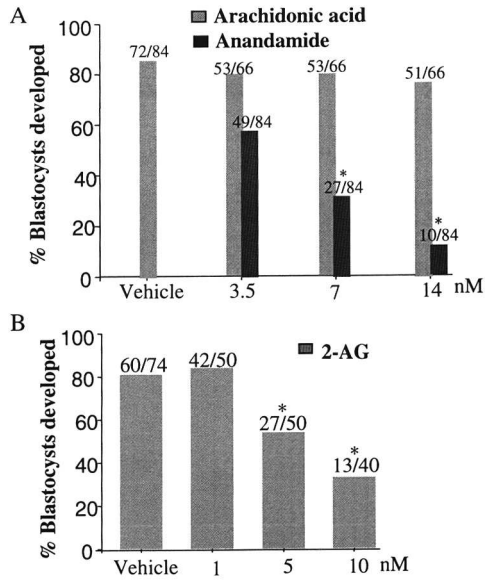

Fig. 5. Effects of endogenous cannabinoid ligands on preimplantation embryo development. Effects of anandamide and arachidonic acid (A) and 2-AG (B). The numbers at the top of each bar indicates that the number of blastocysts were developed/total number of two-cell embryos cultured. $^*P < 0.05$ compared with controls (χ^2 test). Reprinted with permission from Refs. Paria et al., 1995, 1998.

embryo have anandamide hydrolyzing capacity for modulating the local concentration of anandamide conducive to embryo development and implantation. In this respect, a recent report has been shown that the decreased anandamide hydrolase levels in peripheral lymphocytes are correlated with spontaneous abortion in women, suggesting that higher anadamide levels have adverse effects on pregnancy (Maccarrone et al., 2000b).

7. Cannabinoids interfere with implantation

Synchronized development of the embryo to the blastocyst stage and preparation of the uterus to the receptive stage are essential for implantation (Paria et al., 1993; Psychoyos, 1973). In the mouse, these events occur in the morning of day 4, the pregnancy is followed by the initiation of implantation in the evening (2200–2300 h) of the same day (Das et al., 1994). The factors involved in this process and their mechanisms of action are not clearly defined. The tight regulation of anandamide formation and hydrolysis in the pregnant uterus indicates that the cannabinoids might play an important role in the establishment of successful implantation. Implantation in the mouse can be postponed by ovariectomy prior to preimplantation ovarian estrogen secretion in the morning of day 4, a condition is termed to be delayed implantation. Delayed implantation can be maintained by continued progesterone treatment, but can be terminated by an injection of estrogen with blastocyst activation and initiation of implantation (Paria et al., 1993; Yoshinaga and Adams, 1966). As stated earlier, lower levels of anandamide are associated with uterine receptivity for implantation, while higher levels are correlated with uterine refractoriness (Schmid et al., 1997). Similar observation is noted during the delayed implantation in mice. While higher levels of anandamide are present in the progesterone-treated delayed uterus, the levels markedly diminished after termination of the delayed implantation by estrogen (unpublished results). These results suggest that increased levels of uterine cannabinoid ligands may interfere with the implantation process. Indeed, infusion of CP 55,940 (a synthetic cannabinoid) via miniosmotic pumps during the preimplantation period, prevented implantation and this inhibition was reversed by co-administration of CP 55,940 with SR 141716A (a CB1-R antagonist) (Paria et al., 1998). In contrast, single- or repeated injections of THC or continuous infusion of THC during the periimplantation period failed to inhibit implantation suggesting that either implantation process is not responsive to THC or THC is rapidly metabolized and cleared before it reaches the targets (Paria et al., 1992). The rapid metabolism and clearance of THC appeared to be the case because, infusion of THC together with cytochrome P450 inhibitors interfered with

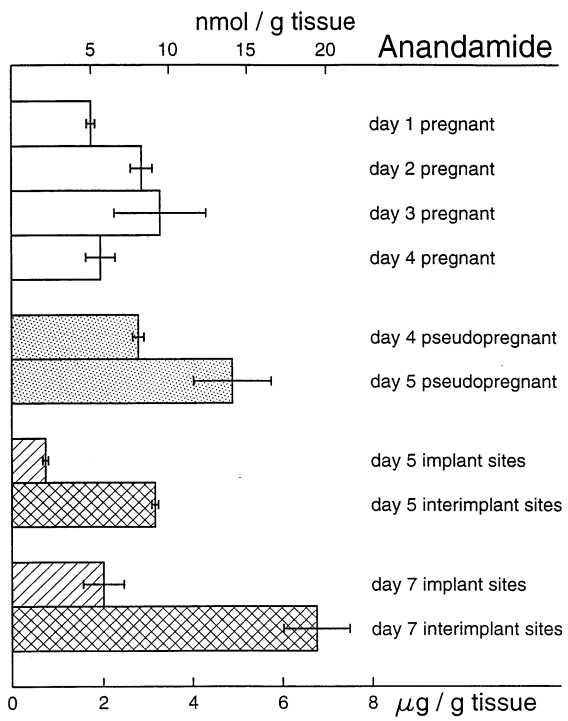

Fig. 6. Levels of endogenous anandamide in the periimplantation mouse uterus. Levels of anandamide in day 5, pseudopregnancy were significantly higher ($P < 0.05$) than those on days 1–4 of pregnancy as well as day 4 of pseudopregnancy (ANOVA followed by student t-test). Levels of anandamide in inter-implantation sites were significantly higher ($P < 0.05$) than those from implantation sites (paired t-test). Reprinted with permission from Ref. Schmid et al., 1997.

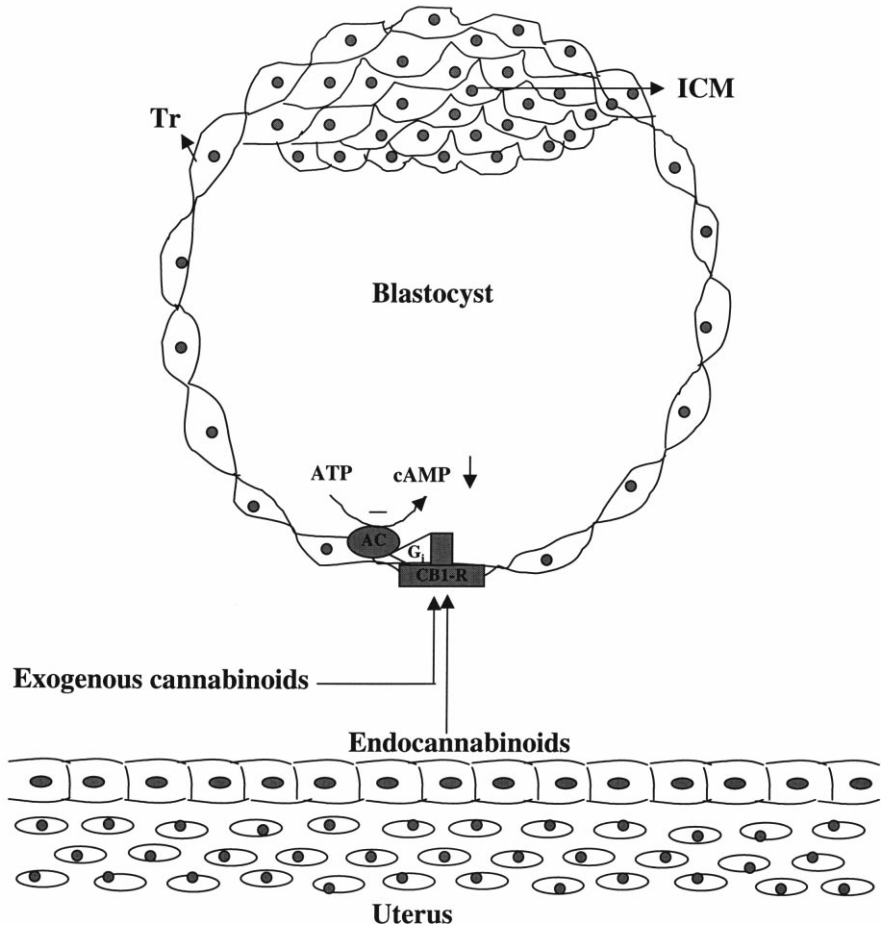

Fig. 7. A schematic diagram is depicting a possible mode of action of endogenous and exogenous cannabinoids on the blastocyst. AC, adenyl cyclase; ATP, adenosine triphosphate; Tr, trophectoderm; ICM, inner cell mass.

the implantation process (Paria et al., 1998). Although, blood levels of THC remained unaltered, accumulation of THC was observed in the uterus in the presence of cytochrome P450 inhibitors. Thus, the effectiveness of cannabinoids depends on their metabolism and turnover by the target organ. Inhibition of implantation by CP 55,940 alone suggests that it is not efficiently metabolized by the cytochrome P450. This inhibition of implantation by cannabinoids could be due to their direct effects on the uterus or embryo. Since this inhibition is reversible by the CB1-R antagonist and blastocysts express functional CB1-R, it is likely that the embryo is the target of cannabinoids during implantation.

8. Concluding remarks and future directions

In conclusion, our work has been clearly shown that, at least in the mouse, ligand-receptor signaling with endocannabinoids is intimately associated with embryo-uterine interactions during implantation (see Fig. 7). The exact physiological significance of this signaling pathway is not yet understood. Further investigation using CB1-R and/or CB2-R knocks-out, mice is required to better understand the roles of endocannabinoids in implantation. In addition, it is also not known whether this signaling pathway is operative in embryo-uterine interaction in other species. Although much emphasis is placed on the roles of

endocannabinoids or natural cannabinoids on neuronal functions, their roles in embryo-uterine interactions during early pregnancy should not be ignored.

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