Endocannabinoid Degradation and Human Fertility

M. Bari
N. Battista
A. Cartoni
G. D'Arcangelo
M. Maccarrone

SUMMARY. Anandamide (AEA) impairs mouse pregnancy and embryo development. Here, we overview the role of AEA in sexual function, focusing on AEA degradation during human pregnancy. Human peripheral lymphocytes express the AEA-hydrolyzing enzyme fatty acid amide hydrolase (FAAH), which decreases in miscarrying women. FAAH is regulated by progesterone and Th1/Th2 cytokines, whereas the AEA transporter and the AEA binding cannabinoid receptors are not affected. Taken together, our results appear to add the endocannabinoids to the hormone-cytokine array

M. Bari, BS, and N. Battista, BS, are Research Assistants, and M. Maccarrone, PhD (E-mail: Maccarrone@med.uniroma2.it), is Assistant Professor at the Department of Experimental Medicine and Biochemical Sciences, University of Rome "Tor Vergata," Via Montpellier 1, I-00133 Rome, Italy.

A. Cartoni, PhD, is Research Assistant and G. D'Arcangelo is Technical Assistant at the Department of Chemical Sciences and Technologies, University of Rome "Tor Vergata," Italy.

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involved in the control of human pregnancy, and suggest that FAAH might be a useful diagnostic marker for large scale, routine monitoring of gestation in humans.

KEYWORDS. Anandamide, cytokines, endocannabinoids, human fertility, sex hormones

INTRODUCTION

Endocannabinoids are an emerging class of lipid mediators, isolated from brain and peripheral tissues (Devane et al. 1992; Mechoulam et al. 1998), which mimic some of the psychotropic, hypnotic and analgesic effects of cannabinoids (Calignano et al. 1998; Meng et al. 1998). The latter compounds, and in particular Δ^9-tetrahydrocannabinol, were reported to have adverse effects on reproductive functions, including retarded embryo development, fetal loss and pregnancy failure (Das et al. 1995; Ness et al. 1999). A major endocannabinoid, anandamide (*N*-arachidonoylethanolamine, AEA), has been shown to impair pregnancy and embryo development in mice (Paria et al. 1996). Down-regulation of anandamide levels in mouse uterus has been associated with increased uterine receptivity, which instead decreased when AEA was up-regulated (Schmid et al. 1997). AEA is an endogenous ligand for both the brain-type (CB_1R) and the spleen-type (CB_2R) cannabinoid receptors, mimicking several actions of cannabinoids on the central nervous system and in peripheral tissues (Di Marzo 1998). CB_1R activation is detrimental for mouse preimplantation and development (Yang et al. 1996; Wang et al. 1999), but appears to accelerate trophoblast differentiation (Paria et al. 2000). A recent study has shown that sex steroids control the expression of the CB_1R gene in the anterior pituitary gland of both male and female rats, leading to the speculation that such a regulatory mechanism might be operational also in the reproductive organs (Gonzales et al. 2000). Moreover, the role of progesterone receptor in Δ^9-tetrahydrocannabinol modulation of sexual receptivity in female rats has been also demonstrated (Mani et al. 2001) and dysregulation of cannabinoid signalling has been shown to disrupt uterine receptivity for embryo implantation in mice (Paria et al. 2001). The effect of AEA via CB_1R and CB_2R depends on its concentration in the extracellular space, which is controlled by a two-step process: (i) cellular uptake by a specific AEA membrane transporter (AMT), and (ii) intracellular degradation by the AEA-hydrolyzing enzyme fatty acid amide hydrolase (FAAH). Since the first report showing an AEA-degrading enzyme (Deutsch and Chin 1993), AMT and FAAH have been characterized in

several mammalian cell lines (Di Marzo et al. 1999; Beltramo et al. 1997; Hillard et al. 1997) and more recently in human cells in culture, in brain (Maccarrone et al. 1998), in platelets (Maccarrone et al. 1999) and in mastocytes (Maccarrone et al. 2000a).

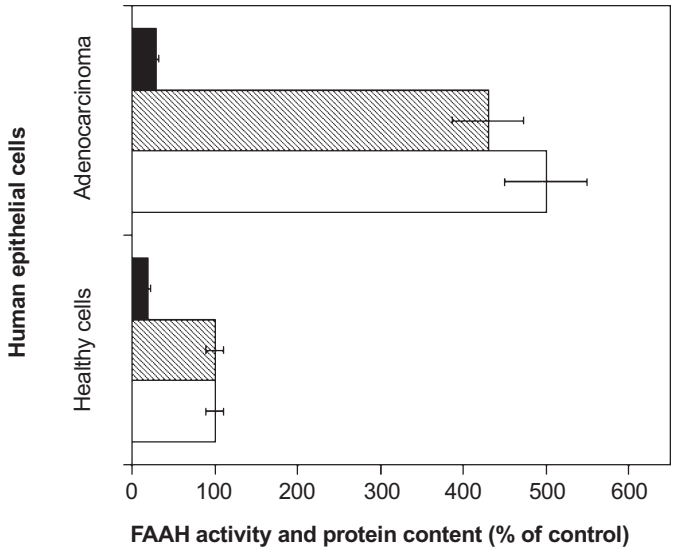
Despite the growing evidence that AEA adversely affects uterine receptivity and embryo implantation in mice (reviewed by Paria and Dey, 2000) and that AEA degradation by FAAH may have physiological significance in these processes (Paria et al. 1996; Paria et al. 1999; Maccarrone et al. 2000b), the regulation of FAAH during early pregnancy is still obscure. Recently, we observed down-regulation of FAAH expression in pseudopregnant mice, suggesting that FAAH modulation was independent of the presence of embryos in the uterus, and found that sex hormones like progesterone and estrogen down-regulate FAAH activity by reducing gene expression (Maccarrone et al. 2000b).

DISTRIBUTION OF FAAH AND AMT

FAAH was localized in the luminal and glandular epithelia of non pregnant mouse uterus (Maccarrone et al. 2000b). *In situ* hybridization consistently detected FAAH mRNA primarily in uterine luminal and glandular epithelial cells (Paria et al. 1999). Also human uterine epithelial cells had a remarkable FAAH activity, which increased more than five times in human adenocarcinoma cells (Maccarrone et al. 2000b). These findings, summarized in Figure 1, are consistent with an epithelial localization of FAAH also in the human endometrium. In this context, it is noteworthy that the Km values of FAAH from mouse or human uterus (approximately 7 μM) were comparable to those recently reported for human brain and for human neuroblastoma and lymphoma cell lines, whereas apparent Vmax values varied (Maccarrone et al. 1998; Maccarrone et al. 2000b). Therefore, it can be proposed that the same enzyme is differently expressed in various species or in different tissues of the same species. Sequence homology between rat, mouse and human FAAH genes (Giang et al. 1997) suggests that indeed FAAH gene is highly conserved. Therefore, the hormonal regulation of FAAH observed in mouse uterus might hold true also for the human counterpart.

FAAH activity was also demonstrated and characterized in mouse blastocysts (Maccarrone et al. 2000b). In order to be hydrolyzed by FAAH, AEA must be transported into the cell. Recent experiments performed on rat neuronal and leukemia cells (Bisogno et al. 1997), on human neuronal and immune cells (Maccarrone et al. 1998) and on human endothelial cells (Maccarrone et al. 2000c), clearly showed the presence of a high-affinity AEA membrane transporter (AMT) in the cell outer membranes. A similar AMT was found in mouse blastocysts (Maccarrone et al. 2000b). The affinity of this transporter was comparable to that of AMT in rat astrocytes (Km = 320 nM) (Beltramo et al. 1997) and

FIGURE 1. *FAAH activity and expression in human uterus*. FAAH activity (white bars) and content (hatched bars) were significantly increased in human adenocarcinoma cells compared to healthy epithelial cells. Antigen competition ELISA (black bars) validated the specificity of FAAH quantitation. 100% = 600 ± 50 pmol.min^{-1}.mg protein^{-1} (activity) or 0.500 ± 0.050 A_{405} units (content).



human cells (Km = 130-200 nM) (Maccarrone et al. 1998). The blastocyst's AMT and FAAH might play a critical role in implantation, because nanomolar concentrations of AEA were found to inhibit embryo development and blastocysts hatching *in vitro* (Schmid et al. 1997; Paria et al. 1998; Maccarrone et al. 2000b). Both detrimental effects of AEA were inhibited by a CB_1R antagonist, in line with the hypothesis that they were mediated by this receptor (Yang et al. 1996).

AEA AND THE INDUCTION OF APOPTOSIS

Interestingly, AEA was found to induce apoptosis in blastocysts, and this effect was not prevented by CB_1R or CB_2R antagonists (Maccarrone et al. 2000b). This rules out the involvement of either cannabinoid receptor in the induction of programmed cell death by the endocannabinoids, and suggests that the arrest of embryo development and blastocyst hatching by AEA did not involve the deployment of apoptotic programmes (Afford et al. 1996; Tonnetti et al. 1999).

Consistently, AEA has been shown to inhibit cancer cell proliferation (De Petrocellis et al. 1998), and to induce apoptosis in lymphocytes (Schwarz et al. 1994), neuronal cells (Maccarrone et al. 2000d), and brain tumors (Galve-Roperh et al. 2000). These findings are in keeping with the notion that Δ^9-tetrahydrocannabinol promotes apoptosis in glioma cells, through a CB_1R independent mechanism (Sànchez et al. 1998).

Collectively, our findings lead to a general picture suggesting that a decreased FAAH activity in mouse uterus during early pregnancy might allow higher levels of AEA, which can be instrumental in modifying endometrium during pregnancy. However, the toxic effects of AEA to the blastocysts are prevented by the activity of AMT and FAAH in these cells, which rapidly scavenge the endocannabinoid. These events are under hormonal control, showing an interplay between endocannabinoids and sex hormones in regulating fertility in mammals. In this line, a recent report has demonstrated that FAAH promoter has a putative estrogen receptor binding site (Puffenbarger et al. 2001), further strengthening the concept of a common hormone-endocannabinoid network. From this stand-point, we sought to ascertain the role of endocannabinoid degradation in human fertility.

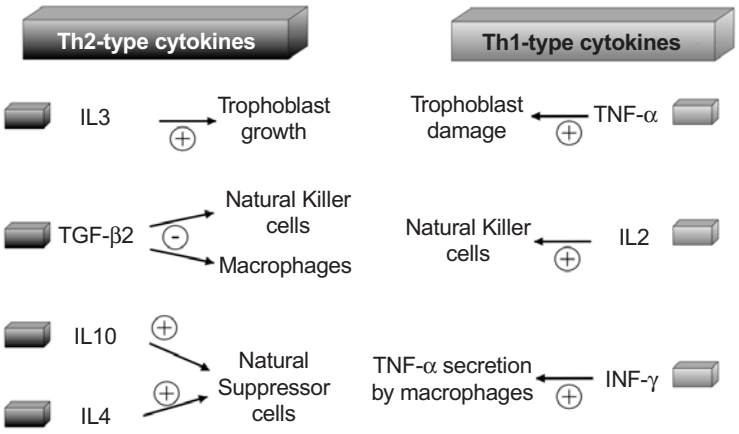
ENDOCANNABINOID DEGRADATION AND HUMAN FERTILITY

Spontaneous abortion is the most common adverse outcome of pregnancy, associated with considerable pain, suffering and medical costs (Kline et al. 1989; Sozio and Ness 1998). Early markers of miscarriage have long been sought for their clinical relevance, though they have not yet been identified (Goldstein et al. 1994; Redline et al. 1994). Little is known about the influence of lifestyle on spontaneous abortion, although cigarette smoking and the use of illicit drugs have been implicated as adverse factors (Walsh 1994; Ness et al. 1999).

Peripheral lymphocytes play a critical role in embryo implantation and successful pregnancy in humans (Piccinni et al. 1998). These cells produce leukemia inhibitory factor (LIF) and immunomodulatory proteins, which favor fetal implantation and survival (Szekenes-Bartho and Wegmann 1996; Stewart and Cullinan 1997; Duval et al. 2000). More generally, lymphocytes regulate a hormonal-cytokine network at the fetal-maternal interface, and a defect in the integrity of this network may result in fetal loss (Szekenes-Bartho and Wegmann 1996; Stewart and Cullinan 1997; Piccinni et al. 1998; Duval et al. 2000). Progesterone (P), a hormone essential for the maintenance of pregnancy, is also known to modulate immune function (Correale et al. 1998) and to elicit an immunological response critical for normal gestation (Szekenes-Bartho and Wegmann 1996; Szekenes-Bartho et al. 1996). Indeed, P has been shown to favor the development

of human T lymphocytes producing type 2 T-helper (Th2) cytokines (interleukins 3, 4 and 10, and transforming growth factor β2), which inhibit the anti-fertility Th1-type cytokines (tumor necrosis factor α, interleukin-2 and interferon-γ), thus allowing the survival of fetal allograft and successful pregnancy (Piccinni et al. 1995; Piccinni et al. 1996). The interactions between this cytokine network and the trophoblast are depicted in Figure 2. More recently, the P-induced Th2 bias has been found to stimulate the release of leukemia inhibitory factor (LIF) from T lymphocytes, mediated by IL-4 (Piccinni et al. 1998). Clinical data, showing that women with unexplained recurrent abortions have a reduced LIF production, suggest that the latter is indeed critical for implantation and maintenance of fetus in humans (Piccinni et al. 1998; Sharkey 1998; Taupin et al. 1999). FAAH might limit the pathophysiological effects of AEA and the other congeners by hydrolyzing them (Giang et al. 1997; Goparaju et al. 1998). Therefore, FAAH activity in lymphocytes might be involved in controlling pregnancy failure by regulating the level of AEA in uterus. In particular, it can be proposed that endocannabinoids may interfere with the lymphocyte-dependent

FIGURE 2. *Interaction between Th1/Th2 cytokines and trophoblast*. Type 2 T-helper (Th2) cytokines (interleukin (IL)-3, IL-4, IL-10 and transforming growth factor β2, TGF-β2) favor blastocyst implantation and successful pregnancy, by promoting, either directly or indirectly: (i) trophoblast growth, (ii) inhibition of natural killer (NK) cell activity, and (iii) stimulation of natural suppressor cells. Conversely, type 1 T-helper (Th1) cytokines (tumor necrosis factor α (TNF-α), IL-2, IL-12 and interferon (INF)-γ) impair gestation, by causing direct damage to the trophoblast, by stimulating NK cells and by enhancing TNF-α secretion by macrophages.



cytokine network which regulates the development and maintenance of successful pregnancy in humans (Piccinni et al. 1998).

FAAH IN MATERNAL LYMPHOCYTES AND HUMAN GESTATION

In this line, we have recently demonstrated that decreased activity and expression of FAAH in peripheral lymphocytes is an early (< 8 weeks of gestation) marker of human spontaneous abortion (Maccarrone et al. 2000e). Indeed, in a clinical study, we measured FAAH activity, [^{3}H]AEA uptake by AMT and [^{3}H]CP55.940 binding to CBR in lymphocytes isolated from 100 healthy women at 7-8 weeks of gestation (Maccarrone et al. 2001). This is the earliest time in gestation where the difference between FAAH content in women who miscarried and those who did not was found to be significant (Maccarrone et al. 2000e). The *a posteriori* association between the gestation outcome and the FAAH activity and expression, AMT activity or CBR binding, showed that FAAH activity and protein were lower in all the 15 women who miscarried than in the 85 who did not, whereas AMT activity and CBR binding were similar in both groups (Table 1). These observations point towards a key-role for FAAH, but not for AMT or CBR, in lymphocyte-mediated control of the hormone-cytokine network at the fetal-maternal interface. Since FAAH might indirectly control AMT, by maintaining the concentration gradient which drives AEA facilitated diffusion through AMT itself (Deutsch et al. 2001), it can be speculated that by controlling FAAH the cell controls also the transport of AEA, and hence its activity in the extracellular space. In this frame, we further investigated how FAAH might be regulated by fertility-related signal molecules.

We found that *in vitro* treatment of human lymphocytes with P, at the concentrations found in serum during pregnancy (from 0.02 to 0.30 μg/ml) (Piccinni et al. 1995), enhanced FAAH activity and gene expression in a dose-dependent manner, as did treatment of human lymphocytes with Th2-type cytokines IL-4 or IL-10. Conversely, treatment with Th1-type cytokines IL-12 or IFN-γ reduced FAAH activity and expression (Maccarrone et al. 2001). We also found that treatment of lymphocytes with P, IL-4, IL-10, IL-12, or IFN-γ did not quite affect AMT activity, neither did it affect [^{3}H]CP55.940 binding to CBR (Maccarrone et al. 2001).

High FAAH activity should lower the level of its substrate, and indeed in a following study we have shown that healthy women (with higher lymphocyte FAAH) have lower blood AEA compared to aborting patients (Maccarrone et al. 2002). As noted above, peripheral lymphocytes play a critical role in human pregnancy by producing LIF (Sharkey 1998). Therefore, we tested whether the endocannabinoids would affect LIF release from peripheral T cells. We found that

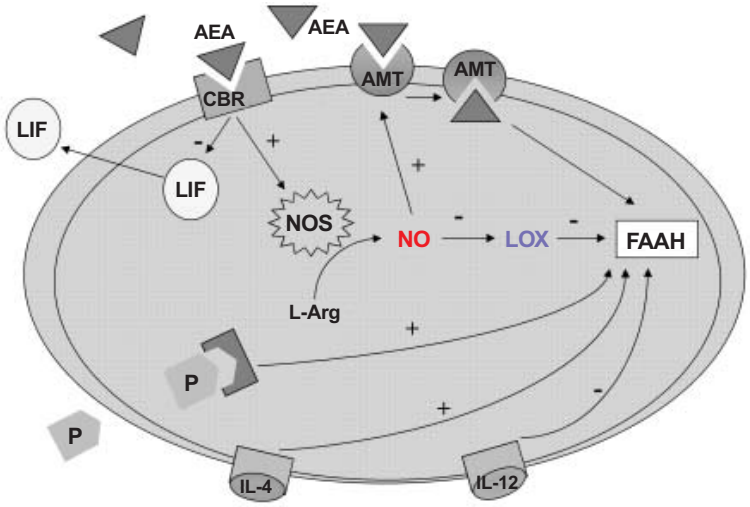
TABLE 1. CBR Binding, AMT Activity, and FAAH Activity and Content in Women Who Miscarried and Those Who Did Not

Parameter	Women with normal gestation	Women who miscarried
CBR binding	20380 ± 1930	20400 ± 1795
(cpm·mg protein^{-1})	(100%)	(100%)
AMT activity	50 ± 4	49 ± 4
(pmol·min^{-1}·mg protein^{-1})	(100%)	(100%)
FAAH activity	133 ± 9	48 ± 5
(pmol·min^{-1}·mg protein^{-1})	(100%)	(36%)
FAAH content	0.250 ± 0.030	0.130 ± 0.020
(A_{405} units)	(100%)	(52%)

treatment of human lymphocytes with AEA reduced the production of LIF, an effect counteracted by SR141716, but not by SR144528 nor by capsazepine, a selective antagonist of vanilloid receptors (Zygmunt et al. 1999). Therefore, inhibition of LIF release by AEA was mediated by CB_1 receptors only.

Altogether, these data suggest that a low FAAH activity, and hence higher AEA levels, can lead to spontaneous abortion by reducing LIF production. This unprecedented effect of AEA is consistent with its adverse effects on embryo implantation and development in mouse (Paria et al. 1996; Schmid et al. 1997; Yang et al. 1996; Di Marzo 1998; Wang et al. 1999; Maccarrone et al., 2000b). Moreover, keeping in mind the role of LIF in regulating growth and differentiation of neurons and endothelial cells (Taupin et al. 1999), a wider implication of the present findings can be anticipated. The interplay among P, cytokines, FAAH, endocannabinoids and LIF is depicted in Figure 3. It is shown that P, by interacting with its receptor, increases the synthesis of FAAH, which in turn reduces the extracellular concentration of AEA by driving its import through the AMT transporter. In this way the effect of AEA on LIF release by binding to type 1 cannabinoid receptors is reduced. FAAH activation by P is further enhanced by interleukin-4. This cytokine can also directly activate FAAH, as does interleukin-10, whereas interleukin-12 or interferon-γ inhibit FAAH activity. The scheme also shows that nitric oxide (NO), produced from L-arginine by CBR-activated nitric oxide synthase, stimulates AEA degradation, by (i) enhancing AMT activity (Maccarrone et al. 2000c), and (ii) preventing the inhibition of FAAH by lipoxygenase (Maccarrone et al. 1998; Maccarrone et al. 2000a). This is noteworthy, because of the manifold roles of NO in male and female fertility (Chwalisz and Garfield 2000; Kuo et al. 2000; Sikka et al. 2001; Herrero et al.

FIGURE 3. *AEA, progesterone and leukemia inhibitory factor in human lymphocytes*. Progesterone (P), by interacting with its intracellular receptor, increases the synthesis of FAAH, which in turn reduces the extracellular concentration of AEA by driving its import through the AEA membrane transporter (AMT). In this way the effect of AEA on leukemia inhibitory factor (LIF) release by binding to type 1 cannabinoid receptors (CBR) is reduced. FAAH activation by P is further enhanced by interleukin-4 or interleukin-10 (omitted for the sake of clarity), whereas it is partly prevented by interleukin-12 or interferon-γ (omitted for the sake of clarity). Also nitric oxide (NO), produced from L-arginine (L-Arg) by CBR-activated nitric oxide synthase (NOS), stimulates AEA degradation, by enhancing AMT and preventing the inhibition of FAAH by lipoxygenase (LOX) activity.



2001), thus adding a further player in the endocannabinoids/hormone/cytokine network regulating the reproductive function.

CONCLUSIONS

The reported findings give a biochemical ground to the previous observation that low FAAH activity correlates with spontaneous abortion in humans (Maccarrone et al. 2000e). They represent the first evidence of a link between the hormone-cytokine network responsible for successful pregnancy and the peripheral endocannabinoid system, and suggest that FAAH, but not anandamide transporter or CB receptors, might be critical for this link. These results might represent also a useful framework for the interpretation of a novel interaction be-

tween P and exogenous cannabinoids, recently shown to regulate female sexual receptivity (Mani et al. 2001). They also suggest that quantitation of FAAH protein in lymphocytes might be an accurate marker of spontaneous abortion in humans, easy to measure in routine analyses.

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