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Placenta

Endocannabinoid System in First Trimester Placenta: Low FAAH and High CB1 Expression Characterize Spontaneous Miscarriage

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ABSTRACT

Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) were the first endocannabinoids to be characterized, that bind two G protein-coupled receptors, CB1 and CB2. AEA synthesized by multiple pathways, including NAPE-specific phospholipase D (NAPE-PLD) and degraded by the fatty acid amide hydrolase (FAAH). AEA levels are critical in regulating embryo development and the "window" of implantation. We examined the expression of nape-pld mRNA, CB1 and FAAH in human placenta hypothesizing that their altered signaling may contribute to spontaneous miscarriage. First trimester placentas from women with spontaneous miscarriage (group 1) were matched with placentas from women who underwent termination (group 2). Nape-pld expression was analyzed by RT-PCR; CB1 and FAAH expression by Western blot and immunohistochemistry. Nape-pld mRNA expression was higher in group 2 than in group 1. Western blot analysis revealed higher CB1 expression and lower or absent FAAH in group 1 than in group 2. Immunohistochemistry confirmed CB1 and FAAH signals in group 1 and group 2 placentas, respectively. Human placenta contains the enzymes to synthesize AEA. Moreover, placental tissue represents a target for endocannabinoids whose activity may regulate pregnancy outcome. In particular, very low or absent FAAH and high CB1 levels correspond with spontaneous miscarriage.

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1. Introduction

Endogenous cannabinoids (endocannabinoids, eCBs) are an emerging class of lipid mediators found in both brain and peripheral tissues and activate type 1 and type 2 cannabinoid receptors (CB1 and CB2). These receptors also bind the psychoactive component of marijuana [1]. Two arachidonate derivatives, N-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) are the endocannabinoids whose biological activity has best been characterized to date [2]. Other members, such as N-oleoylethanolamine (OEA), N-palmitoylethanolamine (PEA) are gaining more prominence, but their physiological roles are still being elucidated [2].

Anandamide was the first eCB isolated from brain tissue [1]. It is released from a cell membrane phospholipid precursor (N-arachidonoylphosphatidylethanolamine, NAPE) in response to depolarizing agents, hormones, and neurotransmitters in part via a NAPE-specific phospholipase D (PLD) [2,3]. Other biosynthetic pathways have also been described [4]. The levels of AEA appear to be controlled by its cellular uptake through a putative eCB membrane transporter (EMT) and its subsequent enzymatic degradation into arachidonic acid and ethanolamine by a membrane bound fatty acid amide hydrolase (FAAH) [5].

AEA effects are determined by its interaction with both CB1 and CB2 receptors belonging to the superfamily of G protein-coupled receptors. CB1 is most abundant in the central nervous system but is also present in peripheral tissues including the heart, uterus, ovary, testis, placenta, liver and small intestine [2,6]. CB2 is predominantly expressed in glia, spleen and immune cells [2]. Furthermore, although the physiological relevance of these interactions is still being determined, transient receptor potential vanilloid 1 (TRPV1) channels and GPR55 receptors also are activated by eCBs [7,8].

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Several epidemiological and observational studies have been published on the adverse effects of the cannabinoids present in marijuana on pregnancy in the human and animal models. These effects include early fetal loss, fetal growth restriction, and premature birth [9–11]. The consequent discovery of an 'eCB system (ECS)' [4] has enabled a wealth of information on the significance of endogenous cannabinoid signaling in multiple female reproductive events such as placentation, fertilization, preimplantation embryo development, implantation and post-implantation embryonic growth [3,12,13] to be determined.

Recent experimental studies indicate that AEA levels are critical in regulating the normal embryo development and the "window" of implantation by synchronizing trophoblast differentiation and uterine preparation to the receptive state. In mice, a physiological tone of anandamide, created by a balance between its synthesis and degradation, is critical to preimplantation embryo development and oviductal progression, since either silencing or amplification of anandamide signaling *via* CB1 receptors causes oviductal retention or blastocyst incompetence for implantation [12–15]. In the mouse uterus, AEA concentrations are inversely related to uterine receptivity for implantation with elevated AEA and low FAAH levels characterizing uterine refractoriness to blastocyst implantation [12,13,16–18]. In addition, increased FAAH expression and low AEA levels have been demonstrated at the implantation sites; conversely, low FAAH expression and high AEA levels at the inter-implantation sites, prior to successful implantation, have been reported [12,13,19]. *In vivo* and *in vitro* models have also examined the effects of AEA on trophoblast growth. Particularly, AEA is known to have a biphasic growth/survival effect on the mouse blastocyst with low levels stimulating trophoblast outgrowth and high levels being inhibitory [20]. AEA also inhibits BeWo trophoblast cell proliferation, in a dose-dependent manner, *via* the CB2 receptor [21].

The implications of the above studies have been further pursued in human clinical studies; these studies found that successful implantation and its progression require low levels of AEA [12,13,22]. During early pregnancy, AEA levels are inversely correlated with FAAH levels in peripheral blood mononuclear cells (PMNCs) and FAAH levels are lower in women who subsequently miscarry compared with those who progress beyond the first trimester [12,13,23]. In addition, in women undergoing *in vitro* fertilization and embryo transfer, low activity of FAAH in PMNCs and high plasma AEA levels after embryo transfer are associated with failure to achieve a successful pregnancy [24]. Recently, it has been suggested that high plasma AEA level can be used as a potential marker of early pregnancy loss in patients with threatened miscarriage [25]. Altogether these data suggest that circulating FAAH and AEA levels play an important role in the establishment and maintenance of early human pregnancy.

Interestingly, if we look at ECS characterization in early human gestational tissues, few results are available. Helliwell et al. (2004) suggested that FAAH acts as a barrier to the AEA maternal-fetal transfer [26]. Furthermore, a recent study demonstrated CB1 expression in the first trimester placenta characterized by a spatial-temporal modulation [21].

At term, lack of FAAH and high CB1 expression characterize placental villous tissue of non-laboring as compared with laboring [27]. However, whether or not human placenta may represent a source of AEA has not yet been documented.

How FAAH and CB1 are expressed in human placenta during first trimester physiological pregnancies (collected from elective termination of normal pregnancies) *vs* placental samples from spontaneous miscarriage has not yet been investigated. Therefore, in the light of the above considerations, in this study we hypothesize that an altered modulation of the ECS may contribute to the spontaneous pregnancy loss and have examined the expression of several components of AEA metabolism in the first trimester placentas from normal woman undergoing elective abortion or follow spontaneous miscarriage.

2. Materials and methods

2.1. Subjects and tissue collection

The study was approved by our institutional review board and experimental procedures were carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Thirty women were enrolled after gaining their informed and written consent for participation in the study. We collected first trimester placenta samples obtained from women ($n = 15$) with spontaneous miscarriage (group 1); for the control group, placental samples were obtained from women ($n = 15$) that underwent voluntary pregnancy termination (group 2). In both groups the presence of gestational sac and embryo (with or without cardiac activity) were visualized by ultrasound.

As determined by ultrasound data and medical history, the women were between 9 and 12 weeks ± 2 days of gestation and were at second or third pregnancy. Since all the women were 25 ± 5 years old, karyotype analysis was not prescribed. We excluded from enrolment women with previous miscarriage or with chronic diseases (diabetes, hypertension, systemic lupus erythematosus); those with uterine anomalies, those undergoing long-term medical treatments (corticosteroids, low-dose aspirin, heparin), and those who smoked marijuana or more than 20 cigarettes/day.

For the study group the diagnosis of spontaneous miscarriage was made in the patients who met the following criteria: progesterone <5 ng per mL (15.9 nmol per L), hCG decrease, and definitive ultrasound criteria of non-viable pregnancy [28]. Right after the diagnosis of spontaneous miscarriage was made, a prompt surgical evacuation was performed in order to avoid the risk of infection and concerns about coagulation disorders that result from retained products of conception. In general, time from embryonic/fetal demise and evacuation took 2 days. This procedure would minimize potential changes of eCBs which may occur post mortem [29].

Placenta specimens (0.5 cm^2), immediately collected after surgical evacuation either for the study or the control group, were washed in physiological solution to remove excess blood and frozen in dry ice or fixed in formalin in order to obtain tissues for RNA and protein extraction or immunohistochemical (IHC) analysis, respectively. For each patient a decidual specimen was obtained through uterine curettage and frozen or fixed in formalin.

Testes from CD1 mice (Charles River Laboratories, Lecco, Italy) were collected as previously described as positive controls [7].

2.2. Total RNA preparation

Total RNA was extracted from human placenta using the guanidinium thiocyanate reagent method [30]. In brief, the samples were homogenized in 5 volumes (500 µl solution/50–100 mg tissue) of solution D (5.1 M guanidinium thiocyanate, 0.5% sarcosyl, 50 mM sodium citrate pH 7.0, β-mercaptoethanol 1 µl/ml). Following homogenization, 50 µl 2 M sodium acetate pH 4.0 was added and the homogenate hand-agitated. After an addition of 500 µl phenol water-saturated and 100 µl chloroform-isoamylalcohol (CIA 49:1), the homogenate was shaken vigorously, incubated for 15 min on ice and centrifuged at $12,000 \times g$ for 15 min at 4 °C. The aqueous phase was transferred into a fresh tube and total RNA was precipitated by mixing with 1 µl glycogen (20 mg/ml) and 2.5 volumes of ethanol. After centrifugation at $12,000 \times g$ for 60 min at 4 °C, the RNA pellet was washed with 70% of ethanol, centrifuged at $12,000 \times g$ for 15 min at 4 °C and dissolved in an appropriate volume of DEPC treated water. Total RNA quality was determined by spectrophotometry at 260/280 nm and by electrophoresis.

2.3. Messenger RNA detection by RT-PCR

Total RNA was reverse transcribed to prepare cDNA. The reverse transcription was carried out using 2 µg total RNA, 0.5 µg oligo dT, 10 mM dNTP, 0.01 M DTT, 1× first strand buffer (Invitrogen Life Technologies, Paisley, UK), 40 U RNase Out (Invitrogen Life Technologies, Paisley, UK), 200 U SuperScript-III RnaseH− reverse transcriptase (Invitrogen Life Technologies, Paisley, UK) in a final volume of 20 µl, following the manufacturer's instructions. As a negative control, total RNA not incubated with reverse transcriptase was used.

PCR reaction was carried out using 1 µl cDNA and 0.2 pmol oligonucleotide primers specific for the amino-terminus domain of human *nape-pld* (sense: 5′-agatggctgataatggagaa-3′; antisense: 5′-ttctcctcccaccagtc-3′; amplicon predicted size 463 bp) in the PCR mix [0.2 mM dNTP, 1× PCR buffer (Invitrogen Life Technologies), 1.5 mM MgCl$_2$, 1.25 U Taq Polymerase (Invitrogen Life Technologies, Paisley, UK)], using an Applied Biosystem Thermocycler. PCR conditions were: 94 °C for 5 min, 1 cycle; 94 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min, 35 cycles; lastly 72 °C for 5 min, 1 cycle. In order to normalize the signals, 1 µl human cDNA was mixed with 10 pMol specific primers for human actin (sense 5′-ctcttccagccttccttcct-3′;

antisense 5′-ctgcttgctgatccacatc-3′ amplicon predicted size 298 bp) with the previously described PCR mix. PCR conditions were: 94 °C for 5 min, 1 cycle; 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, 35 cycles; lastly 72 °C for 5 min, 1 cycle. Possible contaminations among samples were evaluated using as negative controls samples prepared without cDNA. Finally, 25 μl PCR amplification mixtures were analysed by electrophoresis on 1.3% agarose gel in 1× Tris-acetate (TAE) buffer and stained with 0.5 μg/ml ethidium bromide.

2.4. Tissue protein preparation

Spontaneous miscarriage (group 1) and voluntary pregnancy termination placentas (group 2) were homogenized in five-fold (w/v) excess lysis buffer (25 mM Hepes pH 7.9, 1 mM EDTA, 6 mM MgCl2) in the presence of protease inhibitors (4 μg/ml of leupeptin, aprotinin, pepstatin A, chymostatin, and 5 μg/ml of TPCK). Homogenates were centrifuged for 15 min at 800 × g to exclude nuclei. Supernatants were collected and stored at −80 °C. Protein concentration of the supernatants, consisting of total proteins without the nuclear one was determined using the Lowry method [31]. To assess FAAH and CB1 expression in human placenta, 60 μg of extracted proteins were used for Western blot analysis.

2.5. Western blot analysis

Proteins were separated using 9% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filter (Amersham Pharmacia Biotech, UK) at 280 mA for 2.5 h at 4 °C, in order to evaluate FAAH and CB1 immunoreactivity. Filters were treated for 3 h, to prevent non-specific binding, with a blocking solution (5% non-fat powdered milk, 0.25% Tween20 in Tris-buffered saline, TBS, pH 7.6) and then incubated with the primary antibody (anti-FAAH Ab and anti-CB1 Ab diluted 1:1000; anti-MAPK1 Ab diluted 1:500) in PBS 3% non-fat powdered milk solution overnight at 4 °C on an orbital shaker. Filters were washed in TBS-0.25% Tween20, incubated with 1:1000 horseradish peroxidase-conjugated IgG (Dako Corp., Denmark) in TBS-1% normal swine serum (NSS; Dako Corp., Denmark) and then washed three times in TBS-0.25% Tween20. The immune complexes were detected using the ECL-Western blotting detection system (Amersham Pharmacia Biotech, England) following the manufacturer's instructions. The membranes, stripped at 60 °C for 30 min in stripping buffer (100 mM 2-mercaptoetanol, 2% SDS and 62.5 mM Tris–HCl, pH 7.6), were re-probed with MAPK1 antibody to normalize for loading.

2.6. Immunohistochemistry

Placenta tissues were immediately fixed in 4% formaldehyde and after 12 h were treated in 70% ethanol before embedding in paraffin.

Microtome sections (5 μm thick) were mounted on electrostatic glass slides, de-waxed in xylene for 5 min and re-hydrated in graded alcohol for 5 min and finally in PBS 0.01 M pH 7.4. Endogenous peroxidase activity was then blocked by incubation in 0.3% H_2O_2 in PBS 0.01 M pH 7.4 for 20 min. Blocking of non-specific protein binding sites was performed by incubation in PBS 0.01 M pH 7.4 with 10% normal goat serum for 20 min at room temperature. Then sections were incubated overnight with primary antibodies: rabbit anti-human N-terminus CB1 (dilution 1:500 in 0.01 M PBS, 1% Triton-X100, 10% BSA) or rabbit anti-FAAH (dilution 1:1000 in 0.01 M PBS, 0.1% Triton-X100). Immunoreactivity was revealed using the avidin-biotin complex (ABC) system and H_2O_2/DAB (3,3′-diaminobenzidine-tetrahydrochloride) as substrate/chromogen, following the manufacturer's instructions (Vector Laboratories, Burlingame, CA). Slides were finally washed in distilled water for 5 min, then dehydrated in graded alcohols and cleared in xylene for 5 min before mounting with Eukitt mounting medium (BDH, Poole, UK).

2.7. Antibodies

Polyclonal antibodies directed against human N-terminal CB1 [32] or rat C-terminal FAAH (Alexis Biochemicals; Lausen, Switzerland) were raised in rabbit. Specificity of the antibodies have already been extensively investigated in several species [33,34] and checked here again by pre-incubation with an excess amount (2 μg) of the cognate peptide. Anti-MAPK1 (sc-154; Santa Cruz, Biotechnology, Inc., Milan, Italy) was a commercial antibody. CD1 mice testis (Charles River Laboratories, Lecco, Italy) was used as positive control for both CB1 and FAAH immunoreactivity.

2.8. Data presentation and statistics

RT-PCR and Western blot signals were quantified by densitometry analysis carried out using GELDOC 1.00-UV system (BIORAD, Hercules, CA). Signals were plotted as quantitative densitometry analysis corrected on the basis of invariable actin (RT-PCR) or MAPK1 (Western blot) content. Values were expressed as optical density (OD) units.

ANOVA and Student's "t"-test have been carried out to evaluate the significance of differences.

3. Results

3.1. Expression of the nape-pld gene in human placenta

The expression of *nape-pld* mRNA in human placenta was analyzed by RT-PCR in 15 patients of each experimental group and representative results, from one patient of spontaneous miscarriage and one from voluntary pregnancy termination are shown. A positive control (mouse testis) was also used (Fig. 1A). Data indicate that placental villi of both groups expressed *nape-pld*. Quantitative densitometry analysis of *nape-pld* signals, normalized to *actin* content, indicates a significantly higher expression of the enzyme in samples from group 2 ($P < 0.01$) than from samples of group 1 (Fig. 1B).

3.2. Expression of CB1 and FAAH proteins in human placenta

The expression of CB1 and FAAH proteins was analyzed by Western blot in 15 patients from each experimental group and one representative result from each group is shown. A positive control
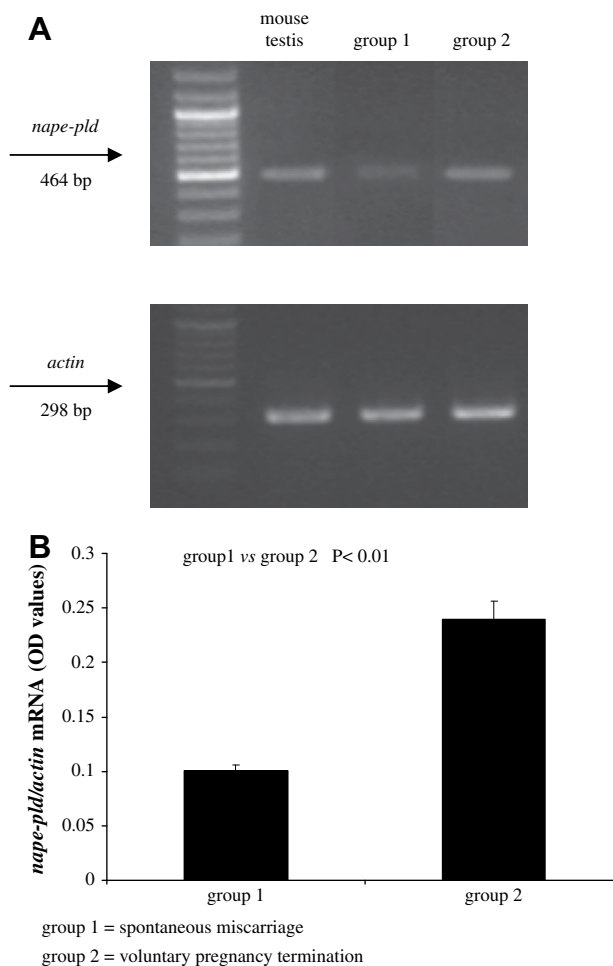
Fig. 1. RT-PCR analysis of *nape-pld* expression in human placenta. *Nape-pld* mRNA expression in the first trimester placenta samples from women ($n = 15$) who underwent spontaneous miscarriage (group 1) and women ($n = 15$) who underwent voluntary pregnancy termination (group 2) **(A)**. *Actin* was used to normalize the signals observed by RT-PCR **(B)**. The figure shows a representative sample from each group. Values for *nape-pld* relative to *actin* are expressed in optical density (OD) units and are representative of three separate cDNA preparations for each observation. Data are expressed as the mean values relative to 15 samples from each group ±SEM (group 1 vs group 2 $P < 0.01$).

(mouse testis) was also used. The pre-absorbed antiserum failed to detect the signals demonstrating antibody specificity (Figs. 2A–D). Quantitative densitometry analysis of CB1 signals (Fig. 2A), corrected for MAPK1 content, indicates a significantly higher expression of the receptor ($P < 0.01$) in samples representing placentas from spontaneous miscarriage (Fig. 2B). In contrast, FAAH Western blotting indicates that enzyme expression (Fig. 2C) was exclusively present in placentas from voluntary pregnancy termination (Fig. 2D).

3.3. Localization of CB1 and FAAH proteins in human placenta by immunohistochemistry

To validate and extend the above results, CB1 and FAAH in placental tissue were localized by immunohistochemistry.

A clear and strong immunopositivity for CB1 was found in trophoblastic cells of placental tissue of spontaneous miscarriage. In particular, cytotrophoblastic and some areas of syncytiotrophoblast had strong CB1 immunoreactivity (Fig. 3A, inset), while there was also immunoreactivity in the stromal compartment (Fig. 3A). Conversely, no immunoreactivity was found in the trophoblastic cells of voluntary pregnancy termination (Fig. 3B). Specificity of the signal was confirmed through the use of pre-absorbed antibody (Fig. 3C). Worthy of note is that CB1 immunoreactivity was similar in decidual tissue collected from both study groups. A clear cytoplasmatic signal was observed in decidual cells of glandular epithelium (Figs. 3D,E). Again, absence of the signal was found using the pre-absorbed antibody (Fig. 3F).

Analysis of FAAH indicates the absence of expression in trophoblastic cells of spontaneous miscarriage (Fig. 3G). On the contrary, a strong signal was localized in cytotrophoblast and

syncytiotrophoblast of voluntary pregnancy termination (Fig. 3H, inset). Specificity of the FAAH signal was tested once again through the use of the pre-absorbed antibody (Fig. 3I).

4. Discussion

This study provides evidence for a markedly altered activity of some of the ECS components in the human placental tissue from first trimester spontaneous miscarriage. Specifically, we detect a local placental expression of *nape-pld* mRNA, the transcript synthesizing NAPE-PLD enzyme, showing a lower expression level in spontaneous miscarriage as compared with physiologically normal pregnancies.

Our results allow inference for a critical role of eCBs in early placental physiology. Previous studies have demonstrated the detrimental effects of high AEA plasma levels on successful pregnancy progression [12,13,23]. Indeed, the AEA blood determination has been proposed as a new early marker of human spontaneous abortion [12,13,25] possibly acting through CB2 on impairment of the developing trophoblast [21]. During early pregnancy, blood AEA levels were inversely correlated with FAAH levels in peripheral blood mononuclear cells (PMNCs). Maternal FAAH levels were significantly lower in women who subsequently miscarried compared with those who progressed beyond the first trimester [12,13,23]. In addition, a suppression of systemic levels of plasma AEA was required to achieve an ongoing pregnancy after *in vitro* fertilization (IVF) treatment and embryo transfer [24]. Animal models suggest that low uterine AEA levels, maintained by high FAAH expression, are important for successful implantation [15,17]. The detection of FAAH in the trophoblast layers of first trimester human placenta indicate that FAAH could act as a barrier to protect

group1 = spontaneous miscarriage

group2 = voluntary pregnancy termination
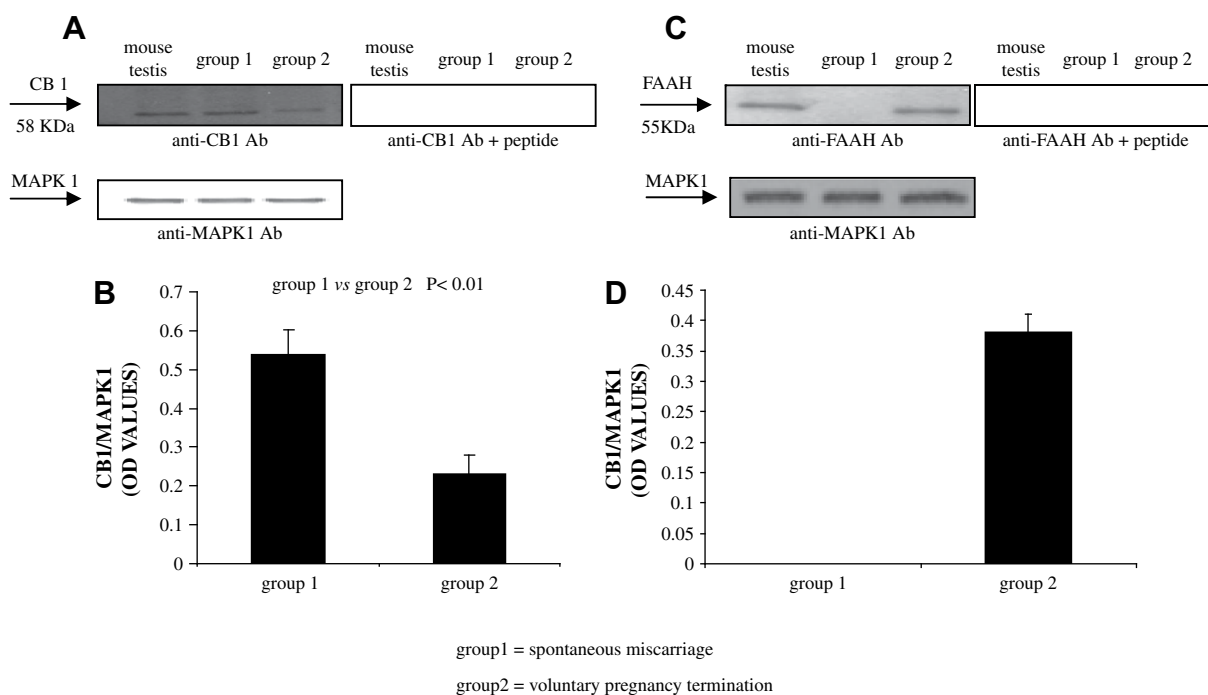
Fig. 2. Expression of CB1 and FAAH proteins in human placenta by Western blot. CB1 detection in the first trimester placenta samples from women ($n = 15$) who underwent spontaneous miscarriage (group 1) and women ($n = 15$) who underwent voluntary pregnancy termination (group 2). Mouse testis was used as positive control and a representative sample from each group is shown. Specificity was demonstrated by the use of pre-absorbed antibody (**A**). CB1 levels were quantified by densitometry analysis of CB1/MAPK1 signals. Values are expressed in optical density (OD) units. Data are expressed as the mean values relative to 15 samples from each group ±SEM (group 1 *vs* group 2 $P < 0.01$) (**B**). FAAH detection in the first trimester placenta samples exclusively from women ($n = 15$) who underwent voluntary pregnancy termination (group 2). Women ($n = 15$) who underwent spontaneous miscarriage (group 1) were also examined. Mouse testis was used as positive control and a representative result from each group is shown. Specificity was demonstrated by the use of pre-absorbed antibody (**C**). FAAH levels were quantified by densitometry analysis of CB1/MAPK1 signals. Values are expressed in optical density (OD) units. Data are expressed as the mean values relative to 15 samples from each group ±SEM (group 1 *vs* group 2 $P < 0.01$) (**D**).
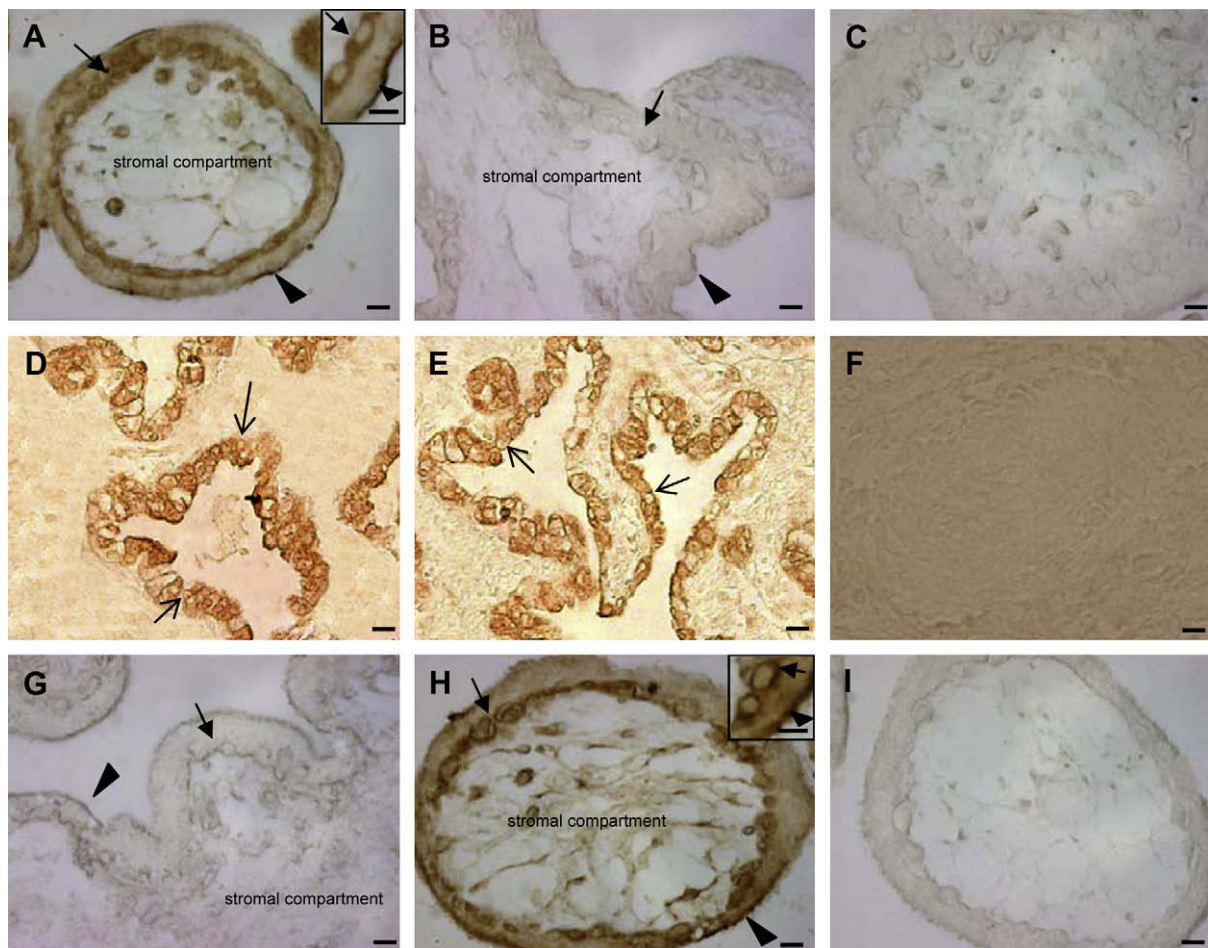
Fig. 3. Representative localization of CB1 and FAAH proteins in human placenta by immunohistochemistry. Detection of a strong immunopositivity for CB1 in trophoblastic cells from placental tissue of women ($n = 15$) who underwent spontaneous miscarriage (group 1) (**A**). The inset shows enlargement of syncytiotrophoblasto and of cytotrophoblastic cells with unstained nuclei. No immunoreactivity was seen in trophoblastic cells of placentas of women ($n = 15$) who underwent voluntary pregnancy termination (group 2) (**B**). Specificity of the signal was tested through the use of the pre-absorbed antibody (**C**). CB1 localization was also demonstrated in decidual tissue of both groups (**D, E**). Specificity of the signal was tested through the use of the pre-absorbed antibody (**F**). Absence of FAAH immunoreactivity in trophoblastic cells of women who underwent spontaneous miscarriage (group 1) (**G**). Localization of FAAH in trophoblastic cells of women who underwent voluntary pregnancy termination (group 2) (**H**). The inset shows enlargement of syncytiotrophoblasto and of cytotrophoblastic cells with unstained nuclei. Specificity of the signal was tested through the use of the pre-absorbed antibody (**I**). Arrows indicate immunopositive cytotrophoblastic cells; arrows heads indicate immunopositive syncytiotrophoblastic cells; open arrows indicate unstained nuclei in decidual tissue. Scale bar: 20 μm.

the growing embryo from the detrimental effects of maternal AEA [12,13,26]. Therefore, taking into account the above results, our hypothesis was that if high levels of maternal AEA were indeed detrimental for early placental and fetal development, FAAH expression would be low or absent in the placenta of spontaneous early pregnancy loss. Western blot analysis of FAAH levels between 9 and 12 weeks of gestational age supported our hypothesis about FAAH absence in placental samples of spontaneous miscarriage as compared with uncomplicated early pregnancy. Interestingly, FAAH immunoreactivity became undetectable in trophoblast layers of placental villi from early spontaneous miscarriage, while it was present in syncytiotrophoblast and overall in cytotrophoblast of normal placental tissues of matched gestational age. Our data about normal human early placenta are in accordance with those of Helliwell et al. (2004). They described in the human first trimester placenta that FAAH levels increase from 9 weeks, peak between 10 and 11 weeks and decline by 12 weeks and onward [26]. Moreover, it has also been found that in maternal plasma FAAH activity peaks at 9–10 weeks of gestation [23]. Therefore, our data are consistent with the notion that FAAH may have a role as a placental barrier against the detrimental effects of maternal

AEA [26]. Recently, high FAAH immunoreactivity unusually located in the nucleus of trophoblast cells has been found in placental tissue of recurrent miscarriage [35]. Whether this is a specific feature of the phenomenon of recurrent miscarriage should be further investigated.

Moreover, the aforementioned alterations in the ECS encountered in the placental specimens of spontaneous miscarriage could be related to chromosome abnormalities, which account for ∼60% of all spontaneous abortions [36], Indeed, as these variations of the ECS could be a consequence of chromosome aberrations, further studies investigating the karyotype of the miscarried tissues could clarify this point. Furthermore, also possible effects due to post mortem changes should be taken into consideration [29].

There is growing evidence that a major transition in placental physiology occurs at approximately 10 weeks with the dissipation of trophoblast plugs from the spiral arteries allowing maternal blood to perfuse the placenta for the first time [37,38]. Thus, at about 10 weeks of gestation, there would be a need for the human fetus to protect itself from circulating maternal AEA. Substances in the maternal blood must first pass through the syncytiotrophoblast and then the villous cytotrophoblast, before crossing the remainder

of the placenta and enter the fetal circulation *via* the placental capillaries. The high levels of FAAH observed in villous cytotrophoblast from normal placenta and expression in the syncytiotrophoblast confirm that high FAAH levels form (during normal first trimester pregnancy) a barrier between fetal and maternal blood flow by isolating the fetus-placental unit from the systemic AEA fluctuations. In addition, non-pregnant mouse uterine tissue expresses high levels of AEA, which must decrease for successful implantation [12,13,16,17]. As a consequence, we speculate that the strong expression of FAAH in the cytotrophoblast of the invasive trophoblast columns, that enter in direct contact with uterus, may also reflect the need to maintain low AEA levels at implantation sites for successful pregnancy. The fact that FAAH was not detectable in placental tissues of spontaneous early miscarriage indicates that, in pathological conditions, the fetus-placental unit may be exposed unconditionally to maternal detrimental levels of AEA with consequent adverse effect on successful pregnancy.

As far as CB1 detection is concerned, Western blot analysis enabled a quantification of CB1 receptor levels in the first trimester human placenta, showing that they are lower in uncomplicated pregnancy as compared with the abortion. When we localize the signal by immunohistochemistry, we did not find CB1 immunoreactivity in trophoblast layers of placenta from normal pregnancy, in accordance with the findings by Helliwell et al. [26] and partially with findings by Habayeb et al. [21] since they found null or poor immunoreactivity, respectively. Furthermore, results showed while a strong immunoreactivity was present in syncytiotrophoblast and cytotrophoblast of placenta from spontaneous miscarriage. A decidual contaminant may explain why there is CB1 protein detection in homogenized placental tissues from normal pregnancy. This is a likely explanation as we clearly found CB1 immunoreactivity in the decidual tissues of both study groups. Therefore, as it stands, in human placenta absence (or a very scanty level) of FAAH correlates with high CB1 expression, which may allow circulating AEA to achieve miscarriage [25]. The molecular mechanism by which AEA-CB1 signaling exerts this effect is unclear and needs additional studies.

Increasing evidence suggests that the bio-effectiveness of AEA depends on its local concentration (in the extracellular space), which is regulated by the balance between degradation (by FAAH) and synthesis (by NAPE-PLD, and other enzymes) as well as by the presence of signaling competent CB1 receptors, with appropriate spatio-temporal regulation [2]. To further address the underlying mechanism by which differential placental AEA levels are under different pregnancy status, it is natural to speculate as to whether AEA is produced in the human placenta and whether the placenta itself represents a potential target site for the action of maternal or placental eCBs in physiological and pathological conditions.

Our demonstration of *nape-pld* transcript in the first trimester human placenta provides for the first time evidence of a potential endogenous production of AEA. Additionally, we have demonstrated that *nape-pld* transcript levels are higher in placental tissues of uncomplicated early pregnancy as compared with spontaneous miscarriage. Therefore, in the first trimester placenta, as it has already been found in the early stages of embryo development [12,13,15,39], we hypothesize that a critical balance between AEA synthesis by NAPE-PLD and degradation by FAAH may create locally an appropriate "anandamide tone", protected from fluctuations of maternal plasma eCB levels, indispensable to proper growth and development of the fetus. The role of AEA-CB1 signaling has been characterized overall in preimplantation and perimplantation embryo development [12–15,18–20,33], while the effects on the following placental development and fetus ontogeny have not been extensively studied. However a number of observations suggest that endocannabinoid signaling may play a key role in neuronal development from the earliest stages and that CB1 receptors have a wide expression pattern in the developing nervous system following neuronal differentiation [40,41]. Cannabinoid receptors have been identified by quantitative autoradiography in the human brain at 33 weeks gestation [42], whereas in the rat embryo, receptors have been detected at embryonic day 11 [43]. Recently, AEA was detected in human amniotic fluid at 16 weeks gestation [44].

In conclusion, our results clearly indicate that to allow pregnancy progression it may be necessary a fine regulation of the AEA levels in the placental environment. Furthermore, placental tissues from spontaneous miscarriage are characterized by very low (if any) FAAH levels and high CB1 expression. Since both progesterone and estradiol activate FAAH promoter [45,46], further research on the cascade of events leading to placental FAAH down regulation should be carried out in the future. Furthermore, since AEA inhibits chorioncarcinoma growth in culture [21] *via* CB2, also additional mechanisms due to CB2 activity might be taken into account.

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