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ARTICLE

Expression and localization of cannabinoid receptors in human immature oocytes and unfertilized metaphase-II oocytes

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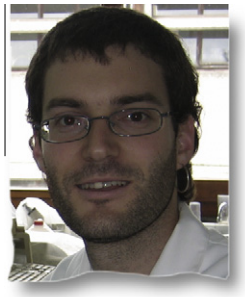

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Abstract Endocannabinoid anandamide and cannabinoid receptors have been described in some organs of the female reproductive system, but little is known about the expression of these receptors in human oocytes. The aim of the study was to describe the expression of cannabinoid receptors in human oocytes and to investigate their differential distribution at various stages of meiotic resumption in human oocytes. A total of 750 human oocytes from 214 patients were analysed by Western blot, immunocytochemistry and PCR. For this study, oocytes that were not suitable for intracytoplasmic sperm injection (ICSI) (germinal-vesicle and metaphase-I stages), as well as metaphase-II oocytes that had not developed into an embryo after ICSI were used. Western blot analysis revealed the presence of CB1 and CB2 receptor proteins in human oocytes. CB1 and CB2 receptor immunostaining patterns changed during the various stages of meiotic resumption. Localization of CB1 receptor was peripheral at germinal-vesicle stage, homogeneous over the entire oocyte at metaphase I and peripheral at mature metaphase II. CB2 receptor localization was peripheral at germinal-vesicle and metaphase-I stages but homogeneous over the entire cell at metaphase II. This finding suggests a possible role for endocannabinoids, acting via receptors, in the maturation of female gametes and fertilization.

KEYWORDS: cannabinoid receptor, CB1, CB2, human oocyte, meiotic resumption

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Introduction

Endocannabinoids are fatty acid derivatives that exert their effects by binding to membrane receptors. To date, two cannabinoid (CB)-specific Gi/o protein-coupled receptors have been cloned and characterized from mammalian tissues. They show very distinctive distribution profiles (Howlett et al., 2002): CB1 receptor (Matsuda et al., 1990) is expressed in a wide variety of tissues, being particularly abundant in the brain, whereas CB2 receptor (Munro et al., 1993) was originally described in the immune system.

Several papers have been published concerning the role of cannabinoids in the female reproductive system. The main psychoactive component of marihuana, delta-9-tetrahydrocannabinol, induces a marked decrease in plasma prolactin and LH concentrations in rats (de Miguel et al., 1998). More recently, it has been reported that anandamide (AEA), an endogenous cannabinoid ligand, also decreases serum LH and prolactin concentrations in rats (Wenger et al., 1999, 2001).

The CB receptors have been found in various parts of the mammalian female reproductive system. In the mouse reproductive tract, only CB1 receptor is expressed in the uterus and oviduct (Das et al., 1995; Paria et al., 2001; Wang et al., 2004), whereas both CB1 and CB2 receptors have been found in preimplantation embryos. CB1 receptor mRNA was detected from the 4-cell stage to the blastocyst stage, while CB2 receptor was present from the 1-cell stage to the blastocyst stage (Paria et al., 2001). The presence of CB1 receptor protein in blastocysts has been confirmed by immunohistochemistry and it has been demonstrated that it is biologically active (Das et al., 1995; Paria et al., 2001). The expression of CB receptors has also been described in the human uterus (Dennedy et al., 2004) and placenta (Habayeb et al., 2008; Helliwell et al., 2004; Park et al., 2003) during pregnancy.

Bisogno et al. (1997) showed that ovaries from the sea urchin contain AEA as well as enzyme activities potentially responsible for their biosynthesis and degradation. Results obtained in mice have shown that endocannabinoid signalling through CB1 receptor, and the AEA tone created in mouse oviduct and embryos because of synthesis and degradation enzymes (Wang et al., 2006a), is crucial to various female reproductive events including development of preimplantation embryos (Paria et al., 2001), their oviductal transport (Wang et al., 2004) and their implantation in the receptive uterus (Paria et al., 1995; Sun and Dey, 2008, 2009; Wang et al., 2003, 2006b). Finally, Wang et al. (2008) have suggested that CB1 receptor regulates labour in mice by interacting with the corticotrophin-releasing hormone-driven endocrine axis.

It should be highlighted that AEA is also present in human follicular fluid (Schuel et al., 2002; Wang et al., 2003) and, as components of the endocannabinoid system have been recently identified in the ovarian medulla and cortex, it is possible that cannabinoid signalling may be involved in the regulation of follicular maturation and development (Bagavandoss and Grimshaw, 2010; El-Talatini et al., 2009). However, the role of the endocannabinoid system in oocyte maturation is still unknown. Therefore, this study aimed to characterize in depth, using a variety of experimental methods, the differential expression and distribution of the two cannabinoid receptors in human oocytes at various stages of meiotic resumption.

Materials and methods

Oocyte collection

Human oocytes were obtained from 214 patients (aged 25—40 years) undergoing intracytoplasmic sperm injection (ICSI) at the Human Reproduction Unit of the Cruces Hospital and 750 oocytes from 214 patients were analysed. The main ICSI indications were male factor (79.9%) and failure of intrauterine insemination. The main female-associated conditions were endometriosis (9.8%) and tubal factor (11.2%). Patients had no previous history of cannabinoid drug consumption.

This study used all the oocytes that had been discarded because of failure to continue the reproductive cycle. Thus, the metaphase-II (MII) oocytes undergoing ICSI in which fertilization failed, as well as all the immature (germinal vesicle (GV) and metaphase I (MI)) oocytes, were collected. After removing the corona cumulus cells, they were stored and transported in minimal media in micro-centrifuge tubes at 4°C, to the laboratory where they were processed according to the protocol for each technique.

Of the 750 oocytes studied, 187 were at GV stage, 128 at MI and 435 at MII. MII (unfertilized) oocytes were collected on day 2 after oocyte retrieval. GV and MI oocytes were collected on the same day as oocyte retrieval in ICSI cases. In two patients, the oocytes ($n = 21$) corresponded to cases where IVF/ICSI could not be attempted because of failure to obtain a sperm sample (one case of total inability to achieve ejaculation and the other because of failure to obtain viable spermatozoa after thawing the spermatozoa obtained by testicular biopsy). During the period of study in the ICSI programme, the fertilization rate of MII oocytes was 64%. In addition, this study analysed 50 unfertilized MII oocytes from the IVF non-ICSI programme for a complementary analysis to validate the PCR results obtained.

Ethical approval was obtained from the Clinical Research Ethical Committee of the Basque Health System (Osakidetza; CEIC reference number E07/54, 3/2008). Informed consent was obtained from all patients.

The IVF cycle management has been previously described (Matorras et al., 2002, 2004, 2009). Briefly, it consists of down-regulation with the gonadotrophin-releasing hormone analogue triptorelin acetate (Decapeptyl; Laboratorios Lasa, Madrid, Spain) using a long protocol and ovarian stimulation with recombinant FSH (Gonal F; Merck Serono, Spain) and highly purified urinary menopausal gonadotrophins (Menopur; Ferring, Spain) or recombinant LH, ovulation being triggered with 250 mg recombinant human chorionic gonadotrophin (Ovitrelle; Merck Serono). The transvaginal ultrasound-guided oocyte retrieval was scheduled 36 h after human chorionic gonadotrophin.

All ICSI procedures and assessments were performed by members of the research group. With regard to ICSI, the cumulus—oocyte—complexes were scored under an

inverted microscope at 100× magnification. When a cumulus—oocyte—complex was found, the stage of maturity was assessed by noting the volume, density and condition of the surrounding coronal and cumulus cells, according to published criteria (Veeck, 1988) and classified into one of the four categories: (i) mature; (ii) slightly immature; (iii) completely immature; or (iv) slightly hyper-mature. The oocytes were cultured in medium for 2 h after collection. The preincubation medium consisted of Universal IVF Medium (Medicult, Denmark) equilibrated in 5% CO_2, at 37°C for a minimum of 2 h prior to use. Immediately prior to micromanipulation, the cumulus corona cells were removed and each oocyte was examined under the microscope to assess its maturation stage and integrity. MII oocytes were defined by the absence of the germinal vesicle and the presence of an extruded polar body (Matorras et al., 2009). Fertilized oocyte scoring involved careful analysis of the pronuclei and the nucleoli within the nuclei during a single examination 16—18 h following fertilization.

On the following day the oocytes were examined for fertilization, as indicated by appearance of two pronuclei and extrusion of the second polar body. Normal fertilization was defined as observation of two pronuclei 16—18 h post insemination and visualization of two polar bodies in the perivitelline space.

A sample of human brain cortex was kindly donated by Dr. Leyre Urigüen (University of the Basque Country, Spain) and Jurkat cells were kindly donated by Dr. Guillermo Velasco (Complutense University of Madrid, Spain), for use as positive controls for the CB1 receptor and CB2 receptor, respectively.

Reverse-transcription PCR analysis

RNA from oocytes (~60 oocytes, from a mix with oocytes at all the possible maturation stages, GV, MI, MII; $n = 3$), cerebral cortex and Jurkat cells were isolated with the RNAqueous-Micro Micro Scale RNA Isolation Kit (Ambion, Austin, TX, USA), including a DNase digestion step using an RNase-free DNase kit (Ambion) to exclude possible contamination by genomic DNA. The procedure for obtaining the cDNA was performed with the ImProm-II Reverse Transcription System (Promega, Madison, WI, USA). Briefly, about 150 ng of RNA and random primers were heated at 65°C for 10 min and chilled on ice for 5 min. Then, once added, the reverse transcription mix was annealed at 25°C for 5 min. First-strand synthesis reaction was carried out at 55°C for 60 min and reverse transcriptase was inactivated at 70°C for 15 min. The primers used for PCR were as follows: human CB1 receptor, 5′-CGTGGGCAGCCTGTTCCTCA-3′ and 5′-CATGCGGGCTTGGTCTGG-3′ (408 bp product); human CB2 receptor, 5′-CGCCGGAAGCCCTCATACC-3′ and 5′-CCT CATTCGGGCCATTCCTG-3′ (522 bp product). Human β-actin (5′-TCCCTGGAGAAGAGCTACGA-3′ and 5′-ATCTGCTGGAA GGTGGACAG-3′; 362 bp product, exon spanning) was used as an internal control.

PCR reactions were performed using the following parameters: 95°C for 5 min, 40 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 1 min, followed by a final extension step at 72°C for 5 min. The mixture was electrophoretically separated on a 2% agarose gel.

Real-time quantitative PCR analysis

The RNA isolation and the process of obtaining the cDNA were performed as previously described for classical reverse-transcription PCR analysis. Real-time quantitative PCR was performed in three replicates using the Applied Biosystems 7300 Real-Time PCR System (Foster City CA, USA) and formulation of sequence-specific primers (unlabelled) and TaqMan MGB probe (6-FAM dye-labelled) (CB1 receptor: Hs01038522_s1, CB2 receptor: Hs00361490_m1) as a double-stranded DNA-specific fluorescent dye. The gene-specific primers and fluorescent Taqman probes were used according to the manufacturer's protocols (Applied Biosystems). The thermal profile of real-time quantitative PCR consisted of a holding stage of 95°C for 20 s and 50 cycles of 95°C for 1 s and 60°C for 20 s. β-actin (Hs99999903_m1) was used as an endogenous control gene. Amplifications were run in a StepOne Real-Time PCR System (Applied Biosystems).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis and immunoblotting

About 100 denuded human oocytes (from a mix with oocytes at all the possible maturation stages, GV, MI and MII; $n = 3$) were collected in sodium dodecyl sulphate (SDS) sample buffer (50 mmol/l Tris—HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.1% bromophenol blue). Human cerebral cortex and Jurkat cell membranes were prepared as previously described (Agirregoitia et al., 2006). Triplicate experiments were performed.

Proteins were heated at 100°C for 5 min and were loaded into 12% resolving gels and separated by one-dimensional SDS polyacrylamide gel electrophoresis (~80 oocytes per lane; 30 μg grey matter of the human prefrontal cerebral cortex; 30 μg Jurkat cells). Proteins were then transferred to polyvinylidene difluoride membranes (Amersham Biosciences, Little Chalfont, UK), using the Mini Trans-Blot electrophoretic transfer system (Bio-Rad Laboratories, Hercules, CA, USA). Blotted membranes were treated and revealed as previously reported (Luconi et al., 1998). The membrane was incubated with a primary rabbit polyclonal anti-CB1 receptor antiserum for the first 99 amino acid residues of human CB1 receptor (1:250; Affinity BioReagents, Golden, CO, USA) and anti-CB2 receptor antiserum for the human CB2 receptor amino acids 20—33 (1:200; Cayman Chemicals, Ann Arbor, MI, USA), overnight at 4°C. Both antibodies had been validated in a previous study (Agirregoitia et al., 2010). The membrane was incubated for 2 h at room temperature with a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Affinity BioReagents) diluted 1:2500. Immune complexes were detected with enhanced chemiluminescent reagent.

Immunofluorescence

After meiotic-stage evaluation, in order to localize the receptors immunocytochemically, oocytes were suspended in phosphate-buffered saline (PBS) and bovine serum albumin (BSA; 1 μg/μl) and smeared onto a slide coated with poly-L-lysine. They were all fixed with 3% paraformaldehyde

for 10 min. Then, slides were washed three times in PBS and incubated for 20 min in PBS and 10% (v/v) bovine foetal serum at room temperature. For indirect immunofluorescence staining, slides were incubated with anti-CB1 receptor and anti-CB2 receptor antisera at a dilution of 1:400 overnight at 4°C. Slides were then washed in PBS + BSA (1 μg/μl) three times, incubated with Alexa Fluor 488 goat anti-rabbit IgG secondary antibody (Molecular Probes; Eugene, OR, USA) for 2 h at 37°C in the dark, washed in PBS + BSA (1 μg/μl) three times (in all cases, nuclei were stained with Hoechst 33342 during the second wash to facilitate the determination of maturational stage of each oocyte), assembled with Fluoromount G (EMS; Hatfield, England) and finally examined by confocal microscopy. Negative controls were performed by omitting the primary antibody before secondary antibody addition (data not shown).

Results

Reverse-transcription PCR and real-time quantitative PCR analysis of cannabinoid receptor mRNA

The presence of CB1 receptor but not CB2 receptor transcripts in human oocytes were detected using both reverse-transcription PCR and real-time quantitative PCR. The expected 408-bp fragment for the CB1 receptor was detected in samples of grey matter of human prefrontal cerebral cortex (positive control) and in oocytes. The 522-bp fragment corresponding to the CB2 receptor was detected in Jurkat cells (positive control) but not in oocytes. The housekeeping gene β-actin was detected in all tissues (**Figure 1**). The data obtained by real-time quantitative PCR (**Table 1**) confirm these results. Even so, it must be noted that the quantity of CB1 receptor mRNA is very low. The controls in the absence of reverse transcriptase or in the absence of template were negative (data not shown).

As the presence of CB1 receptor and CB2 receptor mRNA has been described in spermatozoa (Agirregoitia et al., 2010), in order to confirm that the detection of CB1 receptor mRNA in MII oocytes unfertilized by ICSI was not because of the previously injected single spermatozoon, 50 MII oocytes which failed to fertilize by conventional IVF were subjected to the same analysis and the results were consistent with the results explained above (data not shown).

Table 1 Cycle thresholds for real-time quantitative PCR using specific primers for CB1 and CB2 receptors and β-actin.

Gene	*Tissue*	*Cycle threshold*
CB1 receptor	Oocyte	40 ± 0.3
	Cortex	25 ± 0.1
CB2 receptor	Oocyte	Unknown
	Jurkat	27 ± 0.2
β-Actin	Oocyte	39.8 ± 0.3
	Cortex	21 ± 0.2
	Jurkat	25 ± 0.2

Values are mean ± SEM. Experiments were performed in triplicate.

Immuno-identification of cannabinoid receptor proteins

Figure 2 shows representative Western blots using human oocytes, human prefrontal cerebral cortex grey matter and Jurkat cells. The anti-CB1 receptor polyclonal antiserum labelled a band at 58 kDa in oocyte and cortex protein extracts. The anti-CB2 receptor polyclonal antiserum recognized two major bands at 42 and 46 kDa in oocytes and in Jurkat cells. When anti-CB1 receptor or anti-CB2 receptor antisera were omitted before secondary antibody addition, no bands appeared (data not shown).

Immunocytochemical localization of cannabinoid receptors

Immunofluorescence analysis revealed that both the CB1 and CB2 receptors were present in the human oocytes (**Figure 3**).

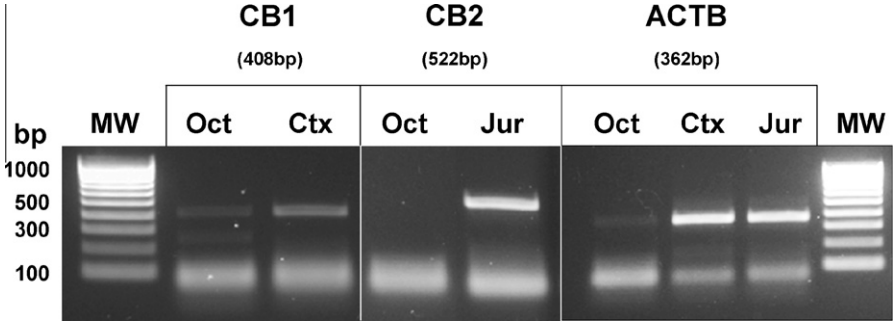

Figure 1 Reverse-transcription PCR products for cannabinoid receptors CB1 and CB2 and β-actin (ACTB) in human oocyte (Oct), grey matter from the human prefrontal cerebral cortex (Ctx) and Jurkat cells (Jur). CB1: amplified fragment using primers specific for the human CB1 cannabinoid receptor (408 bp). CB2: amplified fragment using primers specific for the human CB2 cannabinoid receptor (522 bp). β-actin was used as an internal control (362 bp). Agarose electrophoresis gel (2%) stained with ethidium bromide. MW = molecular weights (bp). Experiment was performed in triplicate: representative products are shown.

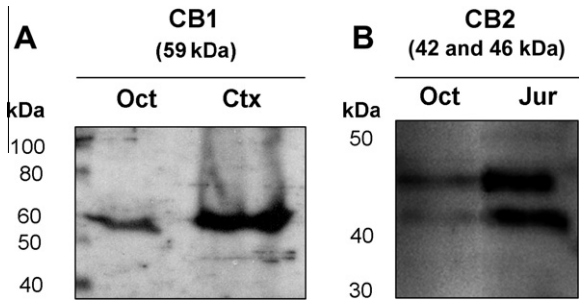

Figure 2 Western blot analysis of (A) CB1 receptor in human oocytes (Oct) and grey matter from the human prefrontal cerebral cortex (Ctx) and (B) protein extracts from human oocytes and Jurkat cells (Jur) using rabbit antiserum against CB2 receptor. Experiment was performed in triplicate with ~80 oocytes: representative blots are shown.

However, the localization of each type of receptor was different at the different stages of resumption of meiosis in mature human oocytes. On the one hand, CB1 receptor localization was homogeneous over the entire oocyte at MI stage contrasting with the peripheral localization of this receptor at GV and MII stages. On the other hand, CB2 receptor immunostaining was found homogeneously in oocytes at MII stage, while at GV and MI stages the CB2 receptor localization was peripheral. When the anti-CB1 receptor and anti-CB2 receptor antisera were omitted before secondary antibody addition, specific fluorescence was not evident (Neg; **Figure 3**).

Cannabinoid receptors and clinical conditions

GV and MI oocytes studied corresponded to the whole population of immature oocytes, thus representing the status of immature oocytes in IVF programmes. No differences were observed in cannabinoid receptor analysis regarding clinical parameters (age, body mass index, smoking habit, ovarian stimulation response and associated conditions), since the cannabinoid receptor findings were very constant in all the cases studied (data not shown).

Regarding the MII oocytes analysed, since they corresponded only to unfertilized MII oocytes, some concern could exist concerning the extrapolation of findings for this group of oocytes to the 'normal' MII population. Even so, no differences were observed regarding the aforementioned clinical parameters or regarding the suspected cause of ICSI failure (male, female or mixed) (data not shown). Neither were there differences when results were compared with the two cases where ICSI could not be attempted because of the lack of availability of spermatozoa.

Discussion

In recent years, the role of the endocannabinoid system in mammalian reproductive events, such as spermatogenesis, sperm motility, fertilization, preimplantation embryo development, implantation and post-implantation embryonic growth has been widely studied (Taylor et al., 2007; Wang et al., 2006a). Specifically, El-Talatini et al. (2009) recently published a study on the role of the endocannabinoid system in the human ovary.

The aim of the present study was to gather further data on the presence and localization of cannabinoid receptors during the resumption of meiosis in human oocytes, in order to build a platform for explaining a possible role of cannabinoids in this process.

Western blot analysis revealed the presence of CB1 receptor and CB2 receptor proteins in human oocytes. With regard to CB1 receptor, the band of about 58 kDa (positive control: grey matter from the human prefrontal cerebral cortex) accords in size with previous reports (Agirregoitia et al., 2010; De Jesus et al., 2006; Rossato et al., 2005; Xu et al., 2005). In the case of CB2 receptor, the bands of approximately 42 and 46 kDa (positive control: Jurkat cells) correspond to its theoretical molecular mass (Filppula et al., 2004) and is in agreement with previous reports (Zhang et al., 2007; Agirregoitia et al., 2010).

Some comments should be made regarding the extrapolation of the current data. Immature oocytes correspond

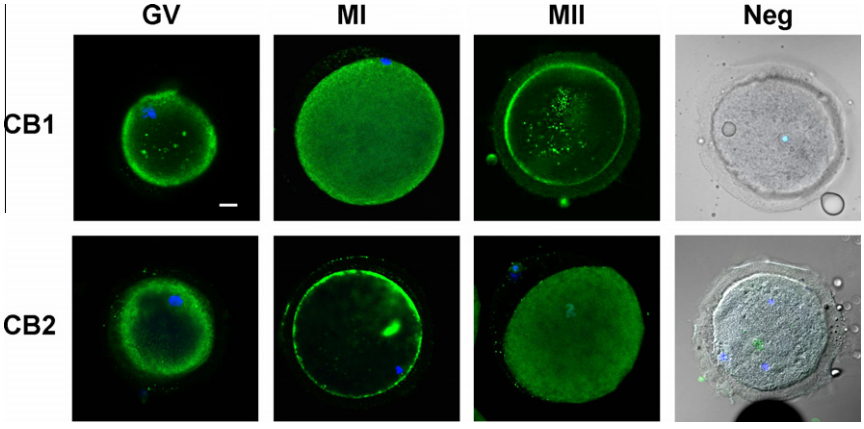

Figure 3 Immunofluorescence analysis of cannabinoid receptors in human oocytes. The distribution of CB1 and CB2 cannabinoid receptors at germinal vesicle (GV), metaphase I (MI) and metaphase II (MII) is shown. Neg = phase-contrast image of control, consisting of omission of the primary antiserum, $n = 15$ per stage. Representative photomicrographs are shown. Bar = 20 μm.

to all the immature oocytes obtained from the patient population during the period of the study, thus a selection bias was precluded. However, the MII oocytes were non-fertilized oocytes, so it could be speculated that the results obtained for these MII oocytes could not correspond to 'normal' MII oocytes. But, taking into account that there are a number of reasons for fertilization failure (women's advanced age, poor sperm quality, poor oocyte quality and methodological difficulties) and considering that the findings were consistently observed among the different patients/oocytes, it is reasonable to suggest that they adequately represent the status of the human MII oocyte, at least during ICSI procedures. Indeed in the very small subset of cases (two patients) where ICSI could not be performed because of failure to obtain spermatozoa for the procedure, the same receptor distribution was obtained. Immunofluorescence analysis of human oocytes confirmed the presence of CB1 and CB2 receptors in these cells. In this regard El-Talatini et al. (2009) recently localized CB1 receptor immunostaining in oocytes from primordial to secondary follicles but not in tertiary follicles and, in contrast, intense CB2 receptor staining has been observed in oocytes during all follicle maturation stages. These results are not consistent with those of the current study, but as it was carried out in oocytes separated from follicles and the presence of CB1 receptor has been corroborated using various techniques, the non-detection of this receptor in oocytes from tertiary follicles could be because of relatively low level of expression of CB1 receptor rather than its absence.

The CB1 and CB2 receptor immunostaining patterns changed during the various stages of meiosis resumption, as occurs with other proteins (Kume et al., 1997) and also with the CB2 receptor in mouse spermatogenesis (Grimaldi et al., 2009). Specifically, the localization of CB1 receptor was peripheral at the GV stage, homogeneous over the entire oocyte at MI stage and peripheral again at mature MII stage. CB2 receptor localization was peripheral at GV and MI stages but homogeneous over the entire cell at MII. This differing receptor relocalization would suggest that each cannabinoid receptor has a distinct role at each stage of oocyte meiosis. Moreover, it has already been reported that in mammals all of the qualitative and quantitative changes in protein synthesis occurring during oogenesis take place at the moment of the resumption of meiosis (Picton et al., 1998). Additionally, Ji et al. (1997) have observed modifications in the human oocyte membrane protein pattern during pre-ovulatory maturation. However, in the case of CB1 and CB2 receptors, the reason for their relocalization in meiosis has not been studied at all.

According to the classical theory of G-protein-coupled receptor (GPCR) functionality, they have to reach the cell surface to act. After agonist binding, GPCR undergo a rapid desensitization and the ligand—receptor complex is internalized prior to being recycled back to the cell surface or being degraded (Cahill et al., 2007). Therefore, taking into account the current data for the CB1 receptor, it could be postulated that it could be activated at GV stage (when the CB1 receptor is localized at the plasma membrane) and subsequently internalized when the oocyte reaches the MI stage (when the CB1 receptor is localized within the cytoplasm). Then, the receptor could be recycled or degraded, being localized at the plasma membrane once again when the oocyte is blocked at the MII stage. As this study has shown the presence of CB1 receptor mRNA in oocytes, the possibility of *de novo* synthesis of the CB1 receptor protein in MII cannot be ruled out. However, this hypothesis seems less probable since during meiosis protein synthesis declines (Picton et al., 1998) because of scarcity of ribosomes (Sathananthan et al., 2006).

In the case of the CB2 receptor, it could be postulated that it is not activated until MI stage and then it is subsequently internalized when the oocyte arrives at the MII stage (when CB2 receptor is localized within the cytoplasm). In this case, the CB2 receptor protein cannot be synthesized *de novo* because of the absence of both rough endoplasmic reticulum in mature oocytes (Sathananthan et al., 2006) and CB2 receptor mRNA, as demonstrated in the present study.

In-depth study of these hypotheses will be important for elucidating many aspects of meiotic resumption, as it has been found that vesicular trafficking at the cell membrane of oocytes is a crucial determinant of meiotic arrest (El-Jouni et al., 2007). Although the molecular mechanisms of resumption and arrest of meiosis in oocytes are not fully understood, previous data indicate that GPCR signalling is crucial in maintaining meiotic arrest (Mehlmann, 2005) and there is general agreement that elevated cyclic AMP concentrations occur during meiotic arrest (El-Jouni et al., 2007). It is also known that agonist stimulation of CB1 and CB2 cannabinoid receptors activates a number of signal transduction pathways via the Gi/o family of G proteins, as inhibition of adenylyl cyclase and the consequent inhibition of cyclic AMP production (Wang et al., 2003). Moreover, it has been described that AEA concentration in follicles with mature oocytes is higher than in the immature oocytes (El-Talatini et al., 2009). So, this leads to the hypothesis that endocannabinoid signalling involving AEA and the CB receptors in the oocyte are involved in the resumption of meiosis. This hypothesis of the involvement of the endocannabinoid signal in meiotic phases was reinforced with the work of Grimaldi et al. (2009) where they described a pivotal role of the CB2 receptor in mouse spermatogenesis.

The present study reports the presence of CB1 receptor but not of CB2 receptor mRNA in mature oocytes, although the proteins of both receptors were present and, as has been already mentioned, the protein localization changes during each stage of meiotic resumption. The absence of CB2 receptor mRNA could occur because the transcription becomes silent after the resumption of meiosis until the 4—8-cell stage (Gosden and Lee, 2010) and because the mRNA is selectivity degraded during oocyte maturation (Stitzel and Seydoux, 2007). Moreover, it is known that the timing and pattern of RNA synthesis in the oocyte may not necessarily coincide with translation into an active protein (Picton et al., 1998). Even so, it would be interesting for further studies to check if, in the case of CB1 and CB2 receptors, changes in localization patterns during each stage of meiotic resumption coincide exactly with transcription profiles.

It has been described that the presence or absence of RNA could be important for embryo development since the RNA stored in the oocyte could be necessary for the early stages of post-fertilization development and embryogenesis (Picton et al., 1998). But it is interesting too to note that a study (Agirregoitia et al., 2010) reported the presence of

CB1 receptor and CB2 receptor mRNA in mature spermatozoa because it has been shown that human spermatozoa retain some transcripts (Miller et al., 2005) which are not present in unfertilized human oocytes, and it has been postulated that the spermatozoa deliver these transcripts to the ooplasm at fertilization (Ostermeier et al., 2004). Accordingly, the complementarity of CB2 receptor mRNA (and maybe of CB1 receptor mRNA) could be important in early zygotic and embryonic development as has been reported for other transcripts (Miller and Ostermeier, 2006). In fact, this complementarity could hold the key to the identification of male-derived factors that underlie idiopathic infertility (Mehlmann, 2005).

In conclusion, as far as is known, this is the first report of the presence of the cannabinoid receptors CB1 and CB2 during the various stages of meiotic resumption in human oocytes. This suggests that AEA, which is present in follicular and oviductal fluids (El-Talatini et al., 2009; Schuel et al., 2002), also has an action during the final nuclear maturation of the oocyte. Therefore, it will be interesting to investigate the role of cannabinoid receptors and evaluate the importance of their localization during the maturation of oocyte, since that essential step, in both natural and assisted reproduction, has not yet been studied in humans. It should be remembered that the oocytes studied were obtained in the context of an IVF/ICSI programme, thus perhaps not reflecting the true status of oocyte maturation during spontaneous natural ovarian cycle. Further studies are needed to investigate the cannabinoid receptors in unstimulated cycles.

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References

Agirregoitia, E., Valdivia, A., Carracedo, A., Casis, L., Gil, J., Subiran, N., Ochoa, C., Irazusta, J., 2006. Expression and localization of delta-, kappa-, and mu-opioid receptors in human spermatozoa and implications for sperm motility. J. Clin. Endocrinol. Metab. 91 (12), 4969–4975.

Agirregoitia, E., Carracedo, A., Subiran, N., Valdivia, A., Agirregoitia, N., Peralta, L., Velasco, G., Irazusta, J., 2010. The CB(2) cannabinoid receptor regulates human sperm cell motility. Fertil. Steril. 93 (5), 1378–1387.

Bagavandoss, P., Grimshaw, S., 2010. Temporal and spatial distribution of the cannabinoid receptors (CB1, CB2) and fatty acid amide hydroxylase in the rat ovary. Anat. Rec. (Hoboken) 293 (8), 1425–1432.

Bisogno, T., Ventriglia, M., Milone, A., Mosca, M., Cimino, G., Di Marzo, V., 1997. Occurrence and metabolism of anandamide and related acyl-ethanolamides in ovaries of the sea urchin *Paracentrotus lividus*. Biochim. Biophys. Acta 1345 (3), 338–348.

Cahill, C.M., Holdridge, S.V., Morinville, A., 2007. Trafficking of delta-opioid receptors and other G-protein-coupled receptors: implications for pain and analgesia. Trends Pharmacol. Sci. 28 (1), 23–31.

Das, S.K., Paria, B.C., Chakraborty, I., Dey, S.K., 1995. Cannabinoid ligand-receptor signaling in the mouse uterus. Proc. Natl. Acad. Sci. USA 92 (10), 4332–4336.

De Jesus, M.L., Salles, J., Meana, J.J., Callado, L.F., 2006. Characterization of CB1 cannabinoid receptor immunoreactivity in postmortem human brain homogenates. Neuroscience 140 (2), 635–643.

de Miguel, R., Romero, J., Munoz, R.M., Garcia-Gil, L., Gonzalez, S., Villanua, M.A., Makriyannis, A., Ramos, J.A., Fernandez-Ruiz, J.J., 1998. Effects of cannabinoids on prolactin and gonadotrophin secretion: involvement of changes in hypothalamic gamma-aminobutyric acid (GABA) inputs. Biochem. Pharmacol. 56 (10), 1331–1338.

Dennedy, M.C., Friel, A.M., Houlihan, D.D., Broderick, V.M., Smith, T., Morrison, J.J., 2004. Cannabinoids and the human uterus during pregnancy. Am. J. Obstet. Gynecol. 190 (1), 2–9; discussion 3A.

El-Jouni, W., Haun, S., Hodeify, R., Hosein Walker, A., Machaca, K., 2007. Vesicular traffic at the cell membrane regulates oocyte meiotic arrest. Development 134 (18), 3307–3315.

El-Talatini, M.R., Taylor, A.H., Elson, J.C., Brown, L., Davidson, A.C., Konje, J.C., 2009. Localisation and function of the endocannabinoid system in the human ovary. PLoS One 4 (2), e4579.

Filppula, S., Yaddanapudi, S., Mercier, R., Xu, W., Pavlopoulos, S., Makriyannis, A., 2004. Purification and mass spectroscopic analysis of human CB2 cannabinoid receptor expressed in the baculovirus system. J. Pept. Res. 64 (6), 225–236.

Gosden, R., Lee, B., 2010. Portrait of an oocyte: our obscure origin. J. Clin. Invest. 120 (4), 973–983.

Grimaldi, P., Orlando, P., Di Siena, S., Lolicato, F., Petrosino, S., Bisogno, T., Geremia, R., De Petrocellis, L., Di Marzo, V., 2009. The endocannabinoid system and pivotal role of the CB2 receptor in mouse spermatogenesis. Proc. Natl. Acad. Sci. USA 106 (27), 11131–11136.

Habayeb, O.M., Taylor, A.H., Bell, S.C., Taylor, D.J., Konje, J.C., 2008. Expression of the endocannabinoid system in human first trimester placenta and its role in trophoblast proliferation. Endocrinology 149 (10), 5052–5060.

Helliwell, R.J., Chamley, L.W., Blake-Palmer, K., Mitchell, M.D., Wu, J., Kearn, C.S., Glass, M., 2004. Characterization of the endocannabinoid system in early human pregnancy. J. Clin. Endocrinol. Metab. 89 (10), 5168–5174.

Howlett, A.C., Barth, F., Bonner, T.I., Cabral, G., Casellas, P., Devane, W.A., Felder, C.C., Herkenham, M., Mackie, K., Martin, B.R., Mechoulam, R., Pertwee, R.G., 2002. International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. Pharmacol. Rev. 54 (2), 161–202.

Ji, Y.Z., Bomsel, M., Jouannet, P., Wolf, J.P., 1997. Modifications of the human oocyte plasma membrane protein pattern during preovulatory maturation. Mol. Reprod. Dev. 47 (1), 120–126.

Kume, S., Yamamoto, A., Inoue, T., Muto, A., Okano, H., Mikoshiba, K., 1997. Developmental expression of the inositol 1,4,5-trisphosphate receptor and structural changes in the endoplasmic reticulum during oogenesis and meiotic maturation of *Xenopus laevis*. Dev. Biol. 182 (2), 228–239.

Luconi, M., Bonaccorsi, L., Maggi, M., Pecchioli, P., Krausz, C., Forti, G., Baldi, E., 1998. Identification and characterization of functional nongenomic progesterone receptors on human sperm membrane. J. Clin. Endocrinol. Metab. 83 (3), 877–885.

Matorras, R., Urquijo, E., Mendoza, R., Corcostegui, B., Exposito, A., Rodriguez-Escudero, F.J., 2002. Ultrasound-guided embryo transfer improves pregnancy rates and increases the frequency of easy transfers. Hum. Reprod. 17 (7), 1762–1766.

Matorras, R., Mendoza, R., Exposito, A., Rodriguez-Escudero, F.J., 2004. Influence of the time interval between embryo catheter loading and discharging on the success of IVF. Hum. Reprod. 19 (9), 2027–2030.

Matorras, R., Prieto, B., Exposito, A., Mendoza, R., Crisol, L., Herranz, P., Burgues, S., 2009. Mid-follicular LH supplementation in women aged 35—39 years undergoing ICSI cycles: a randomized controlled study. Reprod. Biomed. Online 19 (6), 879—887.

Matsuda, L.A., Lolait, S.J., Brownstein, M.J., Young, A.C., Bonner, T.I., 1990. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346 (6284), 561—564.

Mehlmann, L.M., 2005. Stops and starts in mammalian oocytes: recent advances in understanding the regulation of meiotic arrest and oocyte maturation. Reproduction 130 (6), 791—799.

Miller, D., Ostermeier, G.C., 2006. Towards a better understanding of RNA carriage by ejaculate spermatozoa. Hum. Reprod. Update 12 (6), 757—767.

Miller, D., Ostermeier, G.C., Krawetz, S.A., 2005. The controversy, potential and roles of spermatozoal RNA. Trends Mol. Med. 11 (4), 156—163.

Munro, S., Thomas, K.L., Abu-Shaar, M., 1993. Molecular characterization of a peripheral receptor for cannabinoids. Nature 365 (6441), 61—65.

Ostermeier, G.C., Miller, D., Huntriss, J.D., Diamond, M.P., Krawetz, S.A., 2004. Reproductive biology: delivering spermatozoan RNA to the oocyte. Nature 429 (6988), 154.

Paria, B.C., Das, S.K., Dey, S.K., 1995. The preimplantation mouse embryo is a target for cannabinoid ligand-receptor signaling. Proc. Natl. Acad. Sci. USA 92 (21), 9460—9464.

Paria, B.C., Song, H., Wang, X., Schmid, P.C., Krebsbach, R.J., Schmid, H.H., Bonner, T.I., Zimmer, A., Dey, S.K., 2001. Dysregulated cannabinoid signaling disrupts uterine receptivity for embryo implantation. J. Biol. Chem. 276 (23), 20523—20528.

Park, B., Gibbons, H.M., Mitchell, M.D., Glass, M., 2003. Identification of the CB1 cannabinoid receptor and fatty acid amide hydrolase (FAAH) in the human placenta. Placenta 24 (10), 990—995.

Picton, H., Briggs, D., Gosden, R., 1998. The molecular basis of oocyte growth and development. Mol. Cell Endocrinol. 145 (1—2), 27—37.

Rossato, M., Ion Popa, F., Ferigo, M., Clari, G., Foresta, C., 2005. Human sperm express cannabinoid receptor Cb1, the activation of which inhibits motility, acrosome reaction, and mitochondrial function. J. Clin. Endocrinol. Metab. 90 (2), 984—991.

Sathananthan, A.H., Selvaraj, K., Girijashankar, M.L., Ganesh, V., Selvaraj, P., Trounson, A.O., 2006. From oogonia to mature oocytes: inactivation of the maternal centrosome in humans. Microsc. Res. Tech. 69 (6), 396—407.

Schuel, H., Burkman, L.J., Lippes, J., Crickard, K., Forester, E., Piomelli, D., Giuffrida, A., 2002. *N*-Acylethanolamines in human reproductive fluids. Chem. Phys. Lipids 121 (1—2), 211—227.

Stitzel, M.L., Seydoux, G., 2007. Regulation of the oocyte-to-zygote transition. Science 316 (5823), 407—408.

Sun, X., Dey, S.K., 2008. Aspects of endocannabinoid signaling in periimplantation biology. Mol. Cell Endocrinol. 286 (1—2) (Suppl. 1), S3—S11.

Sun, X., Dey, S.K., 2009. Cannabinoid/endocannabinoid signaling impact on early pregnancy events. Curr. Top Behav. Neurosci. 1, 255—273.

Taylor, A.H., Ang, C., Bell, S.C., Konje, J.C., 2007. The role of the endocannabinoid system in gametogenesis, implantation and early pregnancy. Hum. Reprod. Update 13 (5), 501—513.

Veeck, L.L., 1988. Oocyte assessment and biological performance. Ann. N Y Acad. Sci. 541, 259—274.

Wang, H., Matsumoto, H., Guo, Y., Paria, B.C., Roberts, R.L., Dey, S.K., 2003. Differential G protein-coupled cannabinoid receptor signaling by anandamide directs blastocyst activation for implantation. Proc. Natl. Acad. Sci. USA 100 (25), 14914—14919.

Wang, H., Guo, Y., Wang, D., Kingsley, P.J., Marnett, L.J., Das, S.K., DuBois, R.N., Dey, S.K., 2004. Aberrant cannabinoid signaling impairs oviductal transport of embryos. Nat. Med. 10 (10), 1074—1080.

Wang, H., Dey, S.K., Maccarrone, M., 2006a. Jekyll and hyde: two faces of cannabinoid signaling in male and female fertility. Endocr. Rev. 27 (5), 427—448.

Wang, H., Xie, H., Dey, S.K., 2006b. Endocannabinoid signaling directs periimplantation events. Aaps J. 8 (2), E425—432.

Wang, H., Xie, H., Dey, S.K., 2008. Loss of cannabinoid receptor CB1 induces preterm birth. PLoS One 3 (10), e3320.

Wenger, T., Toth, B.E., Juaneda, C., Leonardelli, J., Tramu, G., 1999. The effects of cannabinoids on the regulation of reproduction. Life Sci. 65 (6—7), 695—701.

Wenger, T., Ledent, C., Csernus, V., Gerendai, I., 2001. The central cannabinoid receptor inactivation suppresses endocrine reproductive functions. Biochem. Biophys. Res. Commun. 284 (2), 363—368.

Xu, W., Filppula, S.A., Mercier, R., Yaddanapudi, S., Pavlopoulos, S., Cai, J., Pierce, W.M., Makriyannis, A., 2005. Purification and mass spectroscopic analysis of human CB1 cannabinoid receptor functionally expressed using the baculovirus system. J. Pept. Res. 66 (3), 138—150.

Zhang, R., Kim, T.K., Qiao, Z.H., Cai, J., Pierce Jr., W.M., Song, Z.H., 2007. Biochemical and mass spectrometric characterization of the human CB2 cannabinoid receptor expressed in *Pichia pastoris* — importance of correct processing of the N-terminus. Protein Expr. Purif. 55 (2), 225—235.

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