

Expression and Subcellular Localization of Endocannabinoid System in Primary Interstitial Glandular Cells *In Situ* of Mice Ovary

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Abstract

Endocannabinoid synthesizing 2-AG and cannabinoid receptors have been reported in some organ of reproductive system and in oocyte of ovary, but little is known about the expression and subcellular localization of CB1 and DAGL- α in interstitial gland tissue of mouse ovary. The aim of this study was to explain the differential distribution of endocannabinoid system in interstitial glandular cell of mouse ovary. The CB1 and DAGL- α -immunoreactivities were detected within cells, which are consisted of smaller mitochondria with non-tubular cristae and fewer lipid droplets within the interstitial glands. Their immunoreactivities were localized in association with small vesicle clustering dispersed throughout the cytoplasm. The identity of the immunoreactive cells within interstitial glands were showed a possibility of co-existence of CB1 and DAGL- α in steroidogenic cells and autocrine or paracrine exertion of eCB signaling as well as involvement of eCB signaling in regulation of cell differentiation were discussed.

Keywords: CB1, DAGL- α , Interstitial glandular cell, Ovary, Mice

Introduction

The endocannabinoids (eCB) are the unsaturated fatty acids derivatives that they were bound with Gi/o protein couple receptors. Endocannabinoid system is consisted of cannabinoid receptors and their ligands, which are composed of 2-arachidonoyl-glycerol (2-AG), anandamide (AEA), enzyme responsible for biosynthesis of the 2 AG as well as diacylglycerol kinases (DAGLs), synthesizer for AEA such as the particular phospholipase D (NAPE-PLD), and contained enzymatic degradation for eCBs like monoacylglycerol lipase (MGLL) and fatty acid amide hydrolase (FAAH) [1-4].

This eCB system was studied in the several organs [5-8]. Several publications have been reported about the role of endocannabinoid signaling in the female reproductive organs. There are many studies for effects of the eCB system on the ovarian events such as folliculogenesis, oogenesis and oocyte maturity until fertilization and preimplantation of embryo [9] and presents the eCB system immunoreactivities by several authors to assume the results of eCB, which localized on the oocyte maturity after restart of meiosis I [8,10,11]. In my recent study, the novel knowledge showed the expression and localization of CB1, DAGL- α and DAGL- β that it was first reported in oocytes of mouse ovary [12]. Additionally, the eCB signaling molecules including CB1, CB2 and enzymes for degradation as well as FAAH and MGLL were studied to locate in the follicle, corpus luteum and corpus albicans of human ovary [2,8].

Considering the information described above, the previously reported on the subcellular localization of the eCBs signaling molecules, especially enzymes synthesis endocannabinoid in cells after ovulation and luteinization of the ovary are remained poorly still unclear. This present study is interesting to evaluate the distribution of those eCB signaling molecules in the interstitial gland tissue, which is an essential steroidogenic structure of mice ovary by immuno-light and electron microscopy technique as well as immunoblotting analysis.

Materials and methods

Animals and tissue preparation

Fifteen ICR female mice at each stage, which are composed of postnatal 4 weeks (P4W) and postnatal 8 weeks (P8W). They were obtained from the National Laboratory Animal Center (NLAC), Bangkok, Thailand. All animals got with the free access to standard food and water until for experiment. The

expression bands for CB1 and DAGL- α showed an equivalent their authentic molecular and no found differences immunoreactivity of 2 molecules among the 4 phases in estrous cycle of adult ovaries in my previous study [12].

The animals were stunned with thiopental sodium anesthesia at 60 mg/kg body weight before sacrificed, individual mice of trial and control were transcatheterially perfused with 0.1 M PB. Left side of ovaries were fixed with 4 % paraformaldehyde in phosphate buffer and postfixed overnight, then tissues are changed into 30 % sucrose in PB for cryoprotection until use for immunohistochemistry observation [13]. The right-side ovaries were extirpated and subsequently put into liquid nitrogen and stored at -80°C freezer for further western blotting analysis. The cerebrums were utilized as the internal control from mice at 8 weeks.

All experiments were manipulated in engagement with the rules for the care and use of laboratory animals at Khon Kaen University. The study was approved by the animal ethics panel of the Khon Kaen University, based on the Ethics of Animal Experimentation of the National Research Council of Thailand (Reference No. IACUC-KKU-18/63).

Immunoblotting analysis

Frozen mice ovaries and cerebrum were homogenized in lysis buffer consisted of 20 mM Tris-HCl (pH 7.5), 10 mM EDTA (pH 8.0), 20 mM KCl, 250 mM sucrose and complete protease inhibitor (Thermo Scientific; Waltham, MA, USA). Then, centrifuge with 12,000 rpm, the protein concentration in supernatant were measured by using the Nano Drop 2000 UV-Vis spectrophotometer (Thermo scientific; Wilmington, DE, USA). Total protein 40 μg from each lysate were individually boiled for 10 min in 2x SDS and subjected to SDS/10 % PAGE electrophoresis. The proteins were electrophoretically transferred to a PVDF membranes (GE Healthcare; Buckinghamshire, UK). 5 % skimmed-milk in TBS/0.3 % tween-20 were used to block non-specific binding, and membranes were incubated overnight at 4°C with each antibody at (0.1 $\mu\text{g}/\text{mL}$) in 5 % skimmed-milk in TBS/0.1 % tween-20, then treated with HRP-goat anti-rabbit IgG (Invitrogen; Camarillo, CA, USA) for 1 h, at room temperature. Immunoreactive bands were detected by using the Immobilon Forte Western HRP substrate (Merck KGaA; Darmstadt, Germany). Goat polyclonal antibody β -actin (Santa Cruz Biotechnology; Texas, USA) was used as the internal control. Statistical differences among the mean values were examined with Tukey's Multiple Comparison Test in the ANOVA. Differences with a P value less than 0.05 were considered significant [12].

Immunohistochemistry

The cryosection at 20 μm in thickness of samples were made on a cryostat and putted on coated glass slides. Then, the tissue on slides were incubated with 0.3 % H_2O_2 in methanol for 10 min. to inhibit intrinsic peroxidase activity and added 10 % normal goat serum in PBS for 30 min. to stop non-specific antibody binding. Several sets of 2 adjacent sections were made to examine the possibility of co-localization of DAGL- α with CB1 because the 2 antibodies utilized in this study were generated in rabbits, which does not allow us to utilize the double immunofluorescence microscopic method. Sections were incubated at 4°C overnight with rabbit polyclonal anti-DAGL- α antibody or anti-CB1 antibody at 1 $\mu\text{g}/\text{mL}$. The specificities of CB1 and DAGL- α antibodies were confirmed previously using brains of gene knockout mice for DAGL- α and CB1 [14-16]. The sections were incubated for 1 h at room temperature with biotinylated goat anti-rabbit IgG (Abcam; Cambridge, MA, USA). Diaminobenzidine (DAB) were used for detection of the antigen-antibody reaction area. Then, sections were treated with ABC kit (Vector Laboratories; Burlingame, CA, USA).

For immunohistochemistry-DAB electron microscopy, the sections on plastic slides were postfixed with 0.5 % OsO_4 in 0.1 M PB. After, en-bloc staining with 0.1 % uranyl acetate. Then, sections were embedded with Epon at 60°C in oven for 30 - 40 h. Ultrathin sections were observed under a JEM1010 transmission electron microscope (Jeol; Tokyo, Japan). In control group for the immunoreactivities of DAGL- α and CB1 antibodies, the individual antibodies were pre-absorbed by using synthetic antigens (100 $\mu\text{g}/\text{mL}$) at overnight. Sections were incubated with pre-absorption solution for 1 h at room temperature and followed in the same protocol as the regular immunoreaction described above [12].

Results and discussion

Protein expression of CB1 and DAGL- α in 4W and 8W female ovaries

In immuno-blotting analysis, the major band with a size of approximately 51 kDa for CB1 and 110 kDa for DAGL- α are corresponded to those authentic as reported by others and my laboratory. There have been dramatically increased in densities at P8W in comparison with the P4W for all both proteins. The band

with an equivalent size of cerebrum had significantly highest expression in comparison with the ovaries (**Figure 1**).

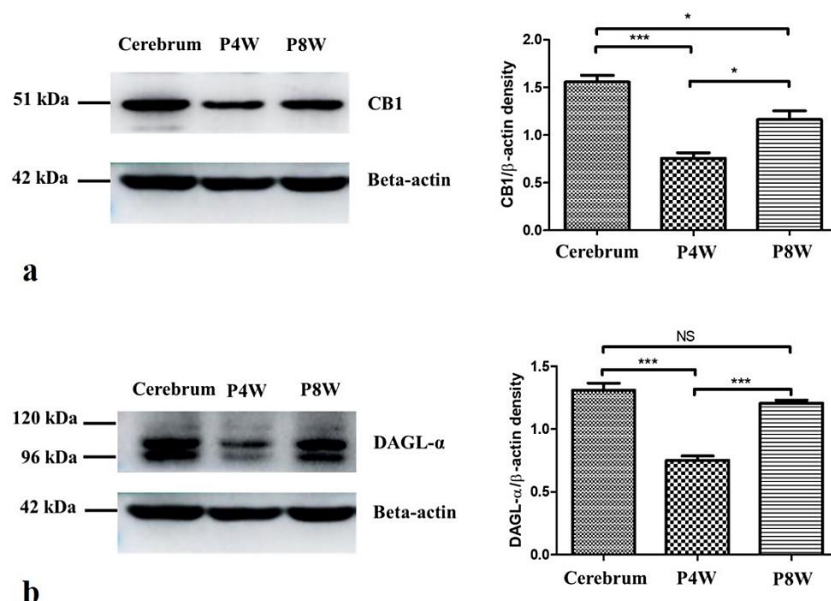


Figure 1 Immunoblot for CB1 (a) and DAGL-α (b) in ovary of mice at puberty (P4W) and adult stage (P8W) are compared with cerebrum of mice at P8W as the internal control (*: sig. $P < 0.05$; ***: $P < 0.001$; NS: not significant).

Localization of CB1 and DAGL-α in interstitial tissue light microscopy

In immuno-light microscopy, the immunohistochemistry staining for CB1 at P8W in **Figure 2(b)** was presented the more intense cells than P4W in **Figure 2(a)** that these cells were sparsely distributed with small vacuole and small dot like appearance within the cytoplasm of interstitial tissue when observed at the high magnification in **Figures 2(c) - 2(e)**. The immunoreactivities were absent within the nuclei and within the follicular cells of ovary. Moreover, the other cells within the corpora lutea in **Figure 2(e)** represented weak intensity when compared with the interstitial gland cells in **Figures 2(c) and 2(d)**.

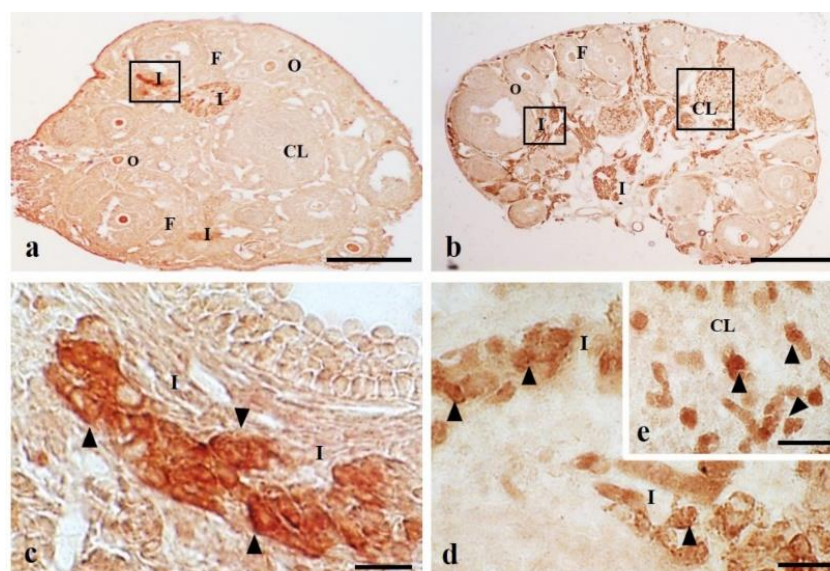


Figure 2 Immuno-light micrographs of adjacent sections of the mouse ovary for CB1 at P4W shows in (a) and P8W shows in (b). The higher magnification micrographs of interstitial areas for CB1 at P4W shows by rectangles in (c). The higher magnification of interstitial gland areas and corpora lutea areas at P8W show in (d, e). Scale bars represent: 1 mm in (a, b) and 50 μm in (c, d, e).

On the other hand, the immunoreactive in cells for DAGL- α were less numerous reactivity than those for CB1 in the interstitial glandular cells and corpora lutea, while DAGL- α were not found immunoreactivity in follicles and nuclei almost like those for CB1. Moreover, the immunoreactivities at P4W stages in **Figure 3(a)** shows lower intensity less than P8W in **Figure 3(b)**. In addition, cells immunoreactive for CB1 and those for DAGL- α in the interstitial glands were stable in their population density among the 4 estrous stages, while those in the corpus luteum were changed with an approximate tendency that they show a relatively more numerous population density at the diestrus stage. Because of this tendency details remain to be further analyzed and will be reported elsewhere.

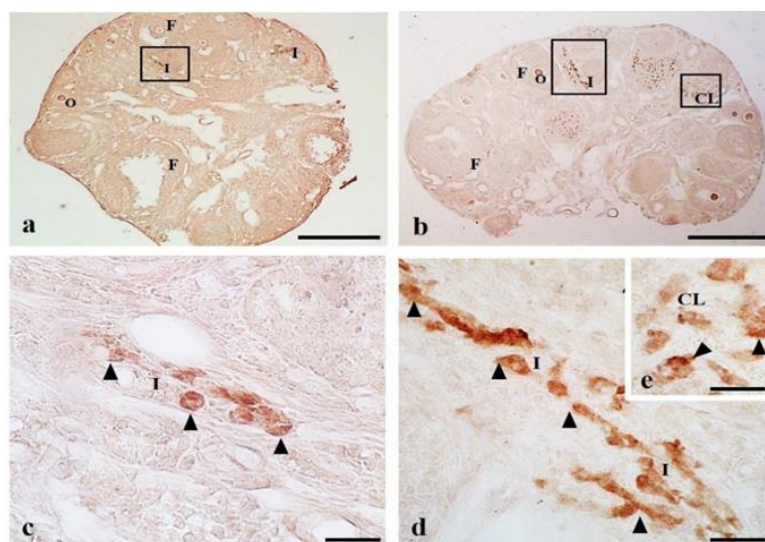


Figure 3 Immuno-light micrographs for DAGL- α at P4W shows in (a) and P8W shows in (b). The high magnification of interstitial areas of (a) shows in (c). The high magnification of interstitial areas and corpora lutea areas of (b) are represented in (d, e). Note cells immunoreactive for CB1 are more numerous than those for DAGL- α in the same interstitial gland. Scale bars represent: 1 mm in (a, b) and 50 μ m in (c, d, e).

In the contrast, in negative control study group shows no significant immunoreactivity in **Figure 4(a)**, following examination and description in immuno-electron microscopy was confined to the interstitial glands in the present study.

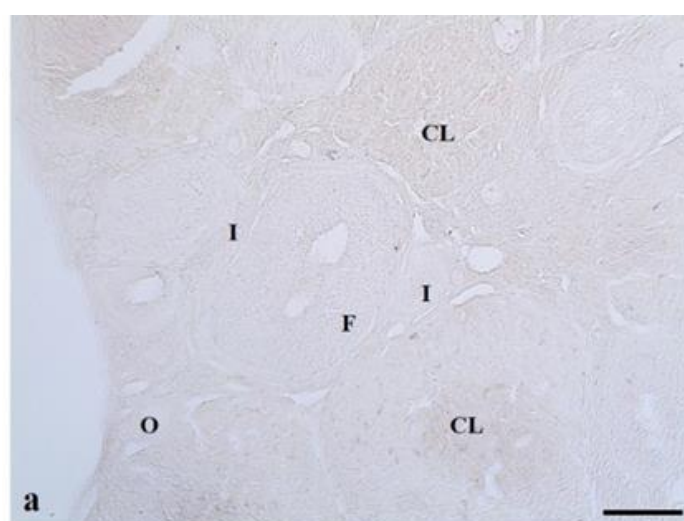


Figure 4 The immunoreactivity shows no significant in any portion of ovary by the antigen pre-absorption examination. CL: Corpus lutea; F: Follicular cells; I: Interstitial glandular cells; O: Oocyte. Scale bar represent: 200 μ m.

Subcellular localization of CB1 and DAGL- α in interstitial glandular cells immuno-DAB electron microscopy

In immuno-electron microscopy, the immunoreactive for CB1 represented a minor cell type and characterized by smaller mitochondria containing lamellar cristae, fewer numerous and smaller lipid droplets. The CB1-immunoreactive materials were deposited on small aggregations of a few vesicles with size about 150 - 300 nm, and were presented adjacent cytoplasm. These immunostaining of vesicle aggregates were dispersed all over the cytoplasm of immunoreactive cells in **Figures 5(c)** and **5(d)**. No immunoreactive materials were deposited on mitochondrial membranes within the cells in **Figure 5(b)**.

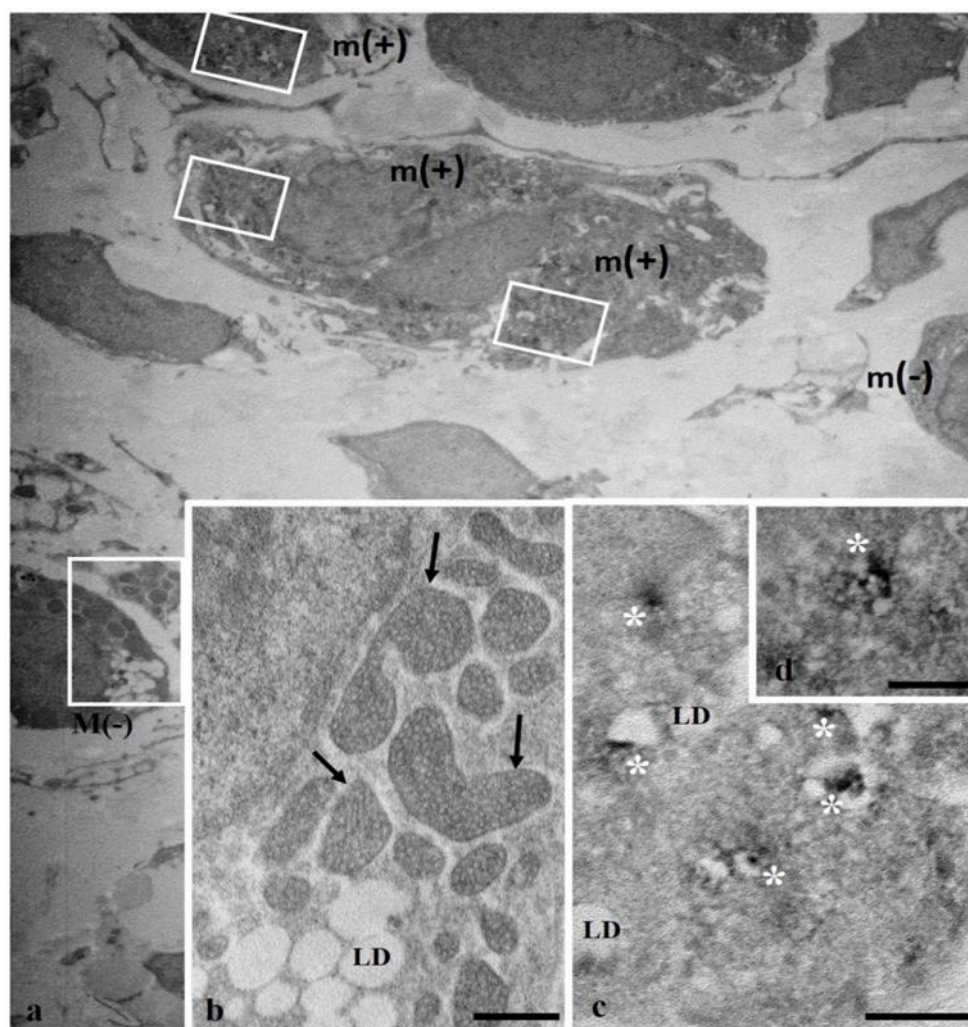


Figure 5 Immuno-electron micrographs of aggregates of cells immunoreactive for CB1 (m+) and immunonegative cells (m-), both contained smaller mitochondria with lamellar crista, together with another aggregate of cells (M-) contained mitochondria with tubular cristae (a). At (b) was a higher magnification view of an area enclosed by single rectangle in (a), showing the cells contain tubular crista-mitochondria. At (c, d) were higher magnification view of an area enclosed by triple rectangle in (a), showed that the immunoreactivity appeared in forms of small vesicle aggregations (*). Scale bars represent: 4 μ m (a) and 1 μ m (b, c, d).

Moreover, electron-dense immunoreactive materials for DAGL- α were deposited on small aggregations of a few vesicles with size about 150 - 300 nm, and were presented adjacent cytoplasm in cells of ultrastructural features similar to those of the type immunoreactive for CB1 marked by small mitochondria, which contained lamellar cristae in **Figures 6(a)** and **6(b)**.

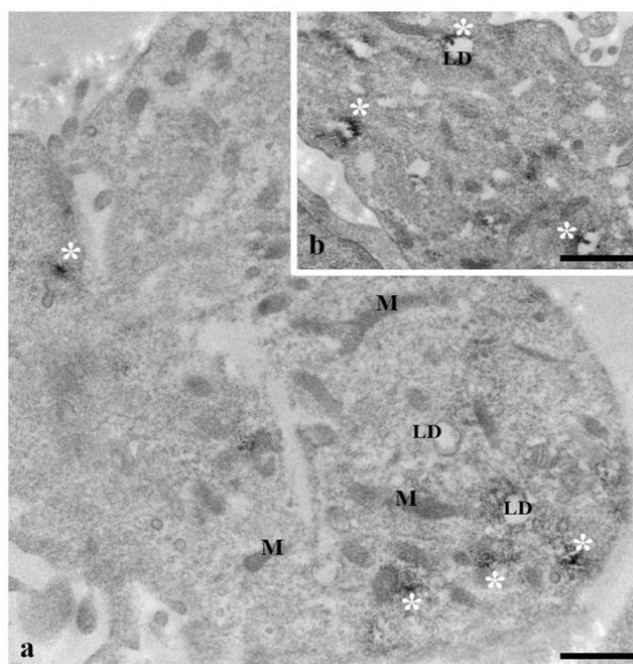


Figure 6 Immunoelectron micrographs of cells immunoreactive for DAGL- α (m+) contained smaller mitochondria with lamellar cristae (M) and fewer lipid droplets (LD) in (a, b). The immunoreactivities were appeared in forms of small vesicle aggregations (*). Scale bars represent: 600 nm.

On the other hand, in the electron microscopic evaluation of negative control study group in interstitial gland cells show no significant immunoreactivity with dark brown color of DAB-EM particles that were detected in association with plasma membrane of vesicle in the cytoplasm of interstitial gland cells in **Figure 7(a)**.

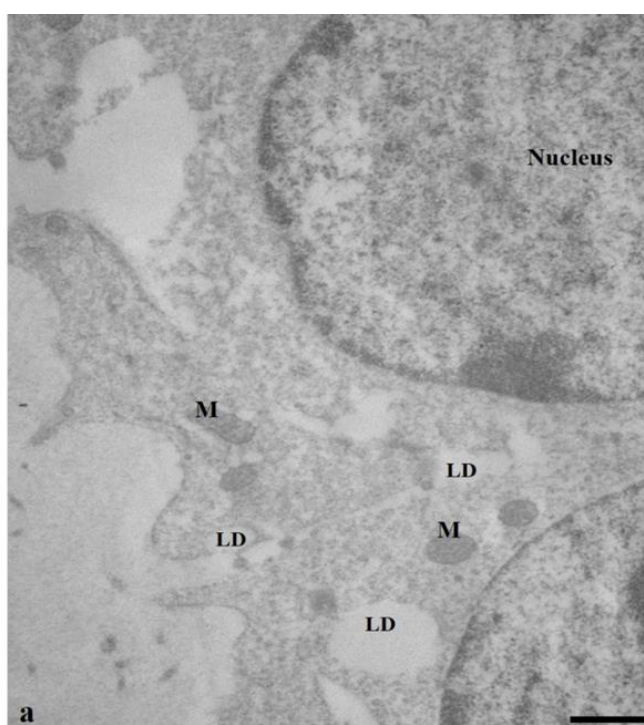


Figure 7 Immuno-electron micrographs of cells immunonegative contained smaller mitochondria with lamellar cristae (M) and fewer lipid droplets (LD). Scale bar represent: 600 nm.

The endocannabinoid system plays a really important role in our body. Now, this system is interested for scientist. To the best of knowledge, this is the first time presents the detection in interstitial gland cells of immunoreactivities for CB1 and DAGL- α in association of small vesicle aggregates within a population of cells containing mitochondria with non-tubular cristae and relatively fewer lipid droplets in the interstitial glandular tissue.

The immunoreactivities in this present for CB1 and DAGL- α signaling molecules were regularized to be described to authentic molecules in mice ovaries, the immunoblot band sizes for both molecules were presented compatible with those molecules that were detected in the brain and a number of other tissues [14,15,17].

Regarding to localize of immunoreactivities for CB1 and DAGL- α in association of small vesicle represented aggregates in a minor population of cells. The identification of the cells should be first considered. It is known that the primary interstitial cells are differentiated from fibroblasts at pre-natal stages and early postnatal stages, while the secondary interstitial cells are derived from theca interna cells after atresia of their surrounding follicles [18]. The histological features of the secondary interstitial cells are well-known. However, there have been few studied that they were described in detail of ultrastructure and immunohistochemistry features of the primary interstitial cells. As far as we all know, no studies are available about the description on occurrence of the cells under discussion here. Judging from an arrangement of cell groups containing tubular crista-mitochondria nearby the cell aggregations under discussion here, and fewer numerous lipid droplets and smaller mitochondria with non-tubular cristae in the cells, it is attraction to identify the cells as the primary interstitial gland cells or, even if not, intrinsic precursor cells for cells typical of steroidogenic characteristics including mitochondria with tubular cristae.

Although the primary interstitial cells are considered to be involuted with the postnatal development [18,19], It is difficult to judge about substantial the morphological evidence for the involution. Further, studies remain to be elucidated about the reliable identification of the immunoreactive cells and the origin tracing of the primary interstitial cells in the prenatal ovary in order to confirm the present supposed identification of the cells with non-tubular crista-mitochondria as the primary interstitial cells.

The localization patterns of immunoreactivities for the 2 eCB signaling molecules in the cells are the same as those reported for an equivalent molecule in oocytes of mice in my previous study [12]. The immunoreaction of electron microscopy was presented on intracellular membrane of vesicle with aggregates distribution all over the cytoplasm. In the contrast, the localization of immunoreactivity of light microscopic level were found the dark brown color of DAB reaction with diffuse feature all over the cytoplasm of interstitial gland cell. From these results, it can be described as follows the difference of thickness sections between light microscopic level (20 μm) and electron microscopic level (less than 0.1 μm). At the ultrathin sections for EM technique presented distribution of trivial immunoreaction and corresponded under light microscopic level, which made the thicker sections to show diffuse feature and high reaction of their immunoreactivities all over the cytoplasm within cells more than at EM level [12]. Regarding to the functional exertion of CB1 and DAGL- α on the vesicular membranes, a rather conventional formation as already recognize in neurons [5] is extreme likely, that an eCB 2-AG is synthesized at the vesicular membranes by DAGL- α and crosses the cells by membrane trafficking routes to the plasma membranes where it is extracellularly released, while CB1 is transported by vesicles to the plasma membranes in which it is incorporated and worked. If CB1-immunoreactive cells and DAGL- α immunoreactive cells are verified to be the same by the immuno-light microscopic double staining, which is not possible because of non-availability at my hand, unfortunately, of the 2 antibodies derived from 2 different animals, the autocrine signaling is possible in the interstitial gland. If CB1 and DAGL- α immunoreactive cells are discrete, the paracrine signaling is the case in the gland. Regardless of autocrine or paracrine signaling, what is the functional significance of eCB signaling on the cells of a minor population? Although any conclusions cannot be drawn until the identification of the cells at present on the possibility that they are the primary interstitial cells or intrinsic precursor cells for the secondary interstitial cells, it is tempting to speculate that the eCB signaling is involved in regulation of cell differentiation considering some recent findings by others in the brain [20,21]. From this present study, it is critical to examine the immunoreactivities in the ovary of CB1 and DAGL- α gene knockout of mutant mice for confirmation of the immunoreaction to understand the specific role function of endocannabinoid and compare with the endocannabinoid disfunction in interstitial gland cells of mice ovary, due to mutant mice were not available at my hand. In addition to my findings via immunohistochemistry and immunoblotting, it is necessary to examine biochemically whether or not the progesterone production is modulated by endocannabinoids. The possibility, once validated by further biochemical, molecular biological and clinical examinations to confirm specific important roles of endocannabinoid in interstitial glandular cells.

Conclusions

Endocannabinoid signal molecule presents different expression and localization detail in mice ovaries at puberty and young adult stages. Thus, based on the information described above, I propose that these isoforms may have specific roles in ovarian function. The isoform particularly of endocannabinoid system must be further studied to clarify mechanisms of ovulation and luteinization. The evidence of this signaling molecules particularly for specific ovarian functions will be permitted to utilize the molecule specific inhibitors or activators to compensate ovarian dysfunction which is 1 among major causes of female infertility.

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