Uncovering a role for endocannabinoid signaling in autophagy in preimplantation mouse embryos

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ABSTRACT: Endocannabinoid signaling plays various roles in directing reproductive processes. Mouse embryos are shown to express high levels of CB1 receptor (CB1R). Low concentrations of anandamide stimulate embryo growth and implantation but at higher concentrations it adversely affects implantation. We tested the hypothesis that high levels of endocannabinoids cause autophagic activation and cell death in preimplantation mouse embryos. We used methanandamide (METH), a selective CB1R agonist, to examine the effect of heightened endocannabinoid signaling on autophagy in mouse embryos. Western blotting, immunofluorescence staining, transmission electron microscopy and TUNEL analysis were performed. We observed that METH treatment in vitro or in vivo up-regulated autophagic response in preimplantation mouse embryos. In blastocysts, apoptosis was also increased after METH injections. At 28 nM, which is considered a high physiological dose to embryonic cells, METH up-regulated autophagic response in preimplantation mouse embryos. In blastocysts, apoptosis was also increased after METH injections. At 28 nM, which is considered a high physiological dose to embryonic cells, METH up-regulated autophagic response in trophoblast stem cells. This work demonstrates for the first time that blastocysts respond to higher than normal levels of endocannabinoids by increasing autophagic activation and apoptosis.

Key words: endocannabinoid / autophagy / embryo / blastocyst / development

Introduction

The cannabinoid receptors belong to the G-protein-coupled receptor superfamily. There are two subtypes of cannabinoid receptors, cannabinoid receptor type 1 (CB1R) and type 2 (CB2R) (Matsuda et al., 1990; Devane et al., 1992; Munro et al., 1993). CB1R is mainly expressed in the brain, while CB2R is expressed in the spleen and immune system. In mammalian systems, the major endogenous cannabinoi

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signaling through CB1R in blastocysts (Wang et al., 2009). Thus, endocannabinoids are potential regulators of autophagy. As the timely production of adequate amount of anandamide is crucial for embryonic development and implantation in mice, untimely increases in anandamide levels may be perceived as ‘suboptimal’ conditions for embryos, leading to their autophagic activation. Thus, we hypothesized that autophagy is modulated in preimplantation embryos by acute increases in the cannabinoid content in vivo. The results show that high levels of endocannabinoid induce autophagic response and cell death in mouse embryos.

Materials and Methods

Reagents and antibodies
Methanandamide (METH) is a CB1-selective agonist (Tocris Bioscience, Bristol, UK) (Abadji et al., 1994; Khanolkar et al., 1996; Sanchez et al., 2012). It was dissolved in ethanol and diluted with phosphate buffered saline (PBS) to a final concentration of less than 2% ethanol. Anti-LC3B antibody was purchased from Abcam (Cambridge, UK). Anti-Atg7 and anti-tubulin antibodies were purchased from Biosensis (Thebarton, Australia) and Sigma (St. Louis, MO, USA), respectively. Antibodies for α-tubulin and β-tubulin were mixed at a ratio of 1:1 and were applied to western blot analysis. TO-PRO-3 iodide was purchased from Invitrogen (Grand Island, NY, USA).

Animals
Five-week old virgin ICR mice were purchased from Orient-Bio (Gyunggi-do, Korea) and housed for 1 week in the animal facility at the SMART Institute of Advanced Biomedical Science, Konkuk University. Adult female mice were subjected to natural mating with male mice of the same strain. All mice were maintained in accordance with the policies of the Konkuk University Institutional Animal Care and Use Committee. Three sets of experiments were designed to test the effects of METH injections on preimplantation mouse embryos (see Fig. 1B, Groups I–III). METH (20 μg/0.1 ml PBS/mouse) was injected intraperitoneally (i.p.) to pregnant mice at indicated times. Control mice received 0.1 ml PBS. Embryos were collected 6 h after the last injection by flushing uteri with warm PBS. When no embryos were found, oviducts were also flushed to check if embryos were trapped in oviducts. Each group of experiment was conducted at least three times with one to two mice receiving PBS or METH injections. Embryos from PBS- or METH-injected mice each time were pooled and were subjected to immunofluorescence staining or transmission electron microscopic observation.

Embryo collection
Random cycling female mice were bred with stud male mice of the same strain. When vaginal plugs were evident the next morning, the mice were considered pregnant (Day 1 of pregnancy). 2-cell stage embryos were collected by flushing the oviducts on Day 2 of pregnancy and blastocysts were collected from uteri on Day 4 of pregnancy (10 a.m.). Collected embryos were added to a 25 μl drop of synthetic oviductal medium enriched with potassium (KSOM) media supplemented with amino acids (KSOM + AA, Millipore, Billerica, MA, USA) covered with mineral oil, and were cultured in 37°C in 5% CO2 incubator.

Culture of trophoblast stem cell
To establish the trophoblast stem (TS) cell line, mitomycin C (Sigma)-treated mouse embryonic fibroblasts (MEFs) were plated in four-well plates (SPL Lifesciences, Pocheon, Korea) the day before collecting blastocysts (Day 1). The next day, blastocysts were collected, and were singly placed on the four-well plates containing TS + F4H medium, and were
cultured in 37 °C in 5% CO₂ incubator (Days 2–3). Hatched blastocysts outgrew on the plates after 2–3 days. The outgrowth was disaggregated by 0.1% trypsin/EDTA (100 μl) for 5 min at 37 °C and 0.5 ml medium was added to stop trypsinization. The medium was changed every other day (Himeno et al., 2008). TS cells were maintained in the presence of fibroblast growth factor (FGF4) and other soluble factors secreted from MEFs. Mitomycin C-treated MEFs were cultured in the heparin TS medium for 3 days and the conditioned media (CM) were saved for TS cell culture. TS cells were cultured on the MMC-MEF feeder cells in TS + F4H [25 ng/ml Human recombinant FGF4 (PeproTech, FL, USA) plus 1 μg/ml heparin (Sigma)], or in 70% MEF conditioned media (MEF-CM) plus FGF4 and heparin without the feeder layer. TS cell colonies have the appearance of refractive and bright coins. This condition is observed while cells are healthy and at early passages (Tanaka et al., 1998; Himeno et al., 2008).

Total RNA extraction
Total RNA was extracted from cultured TS cells by using TRizol Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) following the manufacturer’s protocol. The resuspended RNA was treated with ribonuclease (RNase)-free deoxyribonuclease (DNase, Takara Korea, Daejeon, Korea) for 1 h at 37 °C to remove any contaminated DNA. The concentration and purity were evaluated by the ratio of optical density (OD 260:OD 280) which was determined using a spectrophotometer (ND-1000, Thermo Scientific, Wilmington, DE, USA).

Protein preparation and western blotting
Blastocysts (n = 50) or cultured TS cells were lysed in lysis buffer (50 mM Tris–HCl, 1% NP-40, 150 mM NaCl, 10% Glycerol, EGTA) with 1 mM phenylmethylsulfonylfluoride (PMSF) and Protease Inhibitor Cocktail (Roche Korea, Seoul, Korea), and were sonicated. Lysates were centrifuged at 12,500 g for 15 min at 4 °C. The supernatant was taken for 1 h at 37 °C to remove any contaminated DNA. The slides were incubated with 2% BSA in PBS for 60 min and were incubated with primary antibodies in 2% BSA in PBS for 1 h. The slides were rinsed three times with 2% BSA in PBS. Alexa Fluor™-488-labeled anti-rabbit IgG (Life Technologies, Grand Island, NY, USA) were used as the secondary antibody to detect primary antibody. Embryos were incubated with the secondary antibody at 1:250 in 2% BSA for 40 min at room temperature in the dark and were washed three times. Embryos were counter-stained with TO-PRO-3-iodide (Life Technologies) at 1:500 in PBS for 20 min, and were rinsed three times in PBS. Cover slips were mounted onto slides with a drop of Antifade mounting medium (Life Technologies) and were sealed with nail polish.

Terminal deoxynucleotidyl transferase dUTP nick end labeling assay
To observe apoptotic cells in blastocysts, we used a DeadEnd fluorometric terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) system (Promega, Madison, WI, USA). Blastocysts from PBS- or METH-injected mice were placed on slides (3–5 embryos per slide). Slides were equilibrated with equilibration buffer for 10 min. After equilibration, slides were incubated with recombiant terminal terminal deoxynucleotidyl transferase incubation buffer in a 37 °C incubator for 1 h covered with plastic coverslips. Sections were incubated with 2× saline-sodium citrate buffer for 15 min and washed with PBS three times. Sections were counterstained with TO-PRO-3-iodide (1:500 in PBS), and were rinsed three times in PBS. Sections were mounted with coverslips with a drop of the Antifade mounting medium and were sealed with nail polish.

Confocal microscopy
Images were obtained using the Olympus Fluoview™ FV1000 Confocal Microscope equipped with laser lines at 488, 543 and 633 nm wavelengths (Tokyo, Japan). Fluorescence intensity was quantified using the software Fluoview version 1.5, a platform associated with the confocal microscope.

Transmission electron microscopy
To examine ultrastructural changes, the embryos were fixed with 2.5% glutaraldehyde (Sigma–Aldrich) in PBS for 2 h at room temperature, and were washed with fresh PBS. The embryos were placed in agar chips, and were post-fixed in 1% osmium tetroxide (Sigma–Aldrich) in PBS. After dehydration and infiltration, the embryos were embedded in Epon 812. Thin sections were cut with a diamond knife in an Ultramicrotome (Reichert Supernova, Vienna, Austria) and were placed on copper grids. The sections were double stained with uranyl acetate and lead citrate, and were then examined under a JEM-1010 transmission electron microscope (80 kV, JEOL, Tokyo, Japan).

Statistical analysis
Statistical significance was examined by a Student’s t-test (two tails) using Microsoft Excel program, or by ANOVA analysis using SPSS 16.0 program (IBM, Armonk, NY, USA).

Results
METH regulates the autophagic response in preimplantation mouse embryos in vitro
It is established that adequate production of anandamide in the uterus sets up the receptive uterine environment for implantation (Fig. 1A) (modified from the ref. Schmid et al., 1997). The perturbation of endocannabinoid levels during this period would produce negative effects on embryo development as well as placentaion (Schmid...
et al., 1997; Wang et al., 2003; Sun et al., 2010). We hypothesized that autophagy is one mechanism of embryonic response to the overexposure to cannabinoids, because the embryos may perceive the high-level cannabinoids as a ‘suboptimal’ environment. To examine the effects of endocannabinoid on autophagic activation on embryos in vitro, we used METH (Khanolkar et al., 1996). METH has been used as a selective agonist for CB1R (Abadji et al., 1994; Sanchez et al., 2012), because this receptor type was shown to mediate the effects of cannabinoids on preimplantation mouse embryos (Paria et al., 1998). We first tested the efficacy of this CB1R agonist on mouse embryos in culture, and METH showed a slight negative effect on hatching at 28 and 100 nM, while 7 nM did not affect hatching (Fig. 2). Thus, this agonist shows similar effective dose ranges like other agonists that were previously used on embryos (Paria et al., 1995; Wang et al., 2003; Turco et al., 2008).

To determine the effects of anandamide on the autophagy of blastocysts in vitro, blastocysts were obtained at 10 a.m. on Day 4 of pregnancy and were cultured in 28 nM METH for 12 or 24 h. As shown in Fig. 3A, the LC3-II form is increased in blastocysts treated with 28 nM METH for 12 h, suggesting a heightened autophagic response. LC3 immunofluorescence staining also showed similar results, as LC3 puncta around nuclei increase in 28 nM METH-treated blastocysts (Fig. 3B). In blastocysts treated with 7 or 28 nM METH for 24 h, we investigated protein localization of Atg7, an enzyme mediating the conjugation of Atg5 and Atg12 as well as the activation of LC3 during autophagic activation. As shown in Fig. 4, Atg7 puncta increase near nuclei of trophectodermal cells of 28 nM METH-treated blastocysts, but not in 7 nM METH-treated blastocysts (Fig. 4). This is in accordance with a previous finding that 7 nM is a low physiological dose of

![Figure 2](image-url)

**Figure 2** Effectiveness of METH as CB1R agonist on embryo hatching. Mouse embryos were collected on Day 2 of pregnancy and cultures for 96 h until hatching. The total number of embryos used in the experiment is shown in the Supplementary data, Table S1. METH was added to KSOM + AA media at 7, 28 or 100 nM. Control, vehicle only (1% EtOH in PBS). ANOVA analysis was done to examine the statistical significance. *Control versus 7 nM, P = 0.025; **control versus 28 nM, P = 0.013; ***control versus 100 nM, P = 0.005.

![Figure 3](image-url)

**Figure 3** Autophagic activation in mouse embryos treated with 28 nM METH in vitro. (A) Blastocysts were incubated in 28 nM METH or in vehicle (1% EtOH in PBS) for 12 h and were then subjected to western blotting with anti-LC3 antibody. Blastocysts (50–60 embryos/group) were directly collected in the RIPA buffer containing 1% SDS. Five micrograms of lyastes was loaded in each lane. The ratio of LC3-II form/αβ-tubulin signals was used to analyze the data. The normalized value of the control group was set as 1 and the relative values of the other group were plotted. Data show the mean of four independent experiments. Error bars represent standard deviation. *P < 0.05. (B) Immunofluorescence staining of LC3 in blastocyst cultured in METH in vitro for 12 h. Green puncta are aggregated LC3 proteins and they represent autophagic activation. Blastocysts (3–5 per group) were used in each experiment, and three independent experiments generated similar results. Green, LC3; blue, TO-PRO-3; scale bar, 20 μm.
cannabinoid agonists for preimplantation embryos, while 28 nM is a high physiological dose with adverse effects (Wang et al., 2003; Turco et al., 2008). The perinuclear localization of large Atg7 puncta has also been previously observed in dormant blastocysts when autophagy is highly activated (Lee et al., 2011). These results suggest that the exposure to high concentration of METH leads to autophagic activation in blastocysts in vitro.

Administration of METH to pregnant mice induces autophagic activation in blastocysts in vivo

Next, we investigated whether higher than normal levels of anandamide affect autophagic activation in preimplantation embryos. As represented in Fig. 1A, the uterine level of anandamide falls on Day 4 of pregnant mice when the uterus becomes receptive for implantation (Schmid et al., 1997). To produce an environment with higher than normal levels of anandamide in the uterus, we gave intraperitoneal injection of 20 μg METH to Day 3 pregnant mice at 10 p.m. and embryos were obtained 6 h later (Fig. 1B, Group I). As shown in Fig. 5, embryos retrieved from METH-injected mice showed dramatic increases in LC3 puncta formation around nuclei. Another group of mice received two injections of METH, at 10 p.m. on Day 3 and at 4 a.m. on Day 4, and embryos were collected at 10 a.m. on Day 4 (Fig. 1B, Group II). Again, the increased number of LC3 puncta was noted in blastocysts from METH-injected mice (Fig. 6A). Ultrastructural analysis further confirmed increased autophagy in them (Fig. 6B). As indicated by open arrows, the presence of numerous AVs within blastomerses is clearly shown by transmission electron microscopy (Fig. 6B).

Increased apoptosis in blastocysts retrieved from METH-injected mice

To examine whether higher than normal levels of anandamide affect cell survival of blastocysts preparing to implant on Day 4, mice received two METH injections at 10 a.m. and at 4 p.m. on Day 4 (20 μg each, Group III, see Fig. 1B). Blastocysts were collected at 10 p.m. on Day 4 just prior to implantation, and were subjected to LC3 immunofluorescence staining and TUNEL analysis. At this time, some blastocysts initiate the attachment reaction for implantation and look somewhat deformed after uterine flushing (Fig. 7). As reported previously, the overall LC3 signal is low in blastocysts at 10 p.m. (Lee et al., 2011) (Fig. 7A), but large LC3 puncta have appeared in METH-treated blastocysts (Fig. 7A). Furthermore, blastocysts from METH-injected mice showed a significantly higher number of apoptotic cells (Fig. 7B). These results show that blastocysts exposed to higher than physiological levels of anandamide show a heightened autophagic response and apoptotic cell death prior to implantation.

Regulation of autophagy by METH in TS cell

CB1R is expressed in the trophectoderm of blastocysts and the effects of cannabinoids on mouse embryos are mediated by this receptor type (Paria et al., 1995, 1998). To examine whether the effect of METH mainly targets trophoblasts, we established a TS cell line from a single blastocyst following the established protocol (Tanaka et al., 1998; Himeno et al., 2008). A panel of RT–PCR analyses was performed to check the purity of established TS cell lines. As shown in Fig. 8A, caudal type homeobox 2 (Cdx2), FGF receptor 2 (Fgfr2), eomesodermin (Eomes) and heart and neural crest derivatives

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**Figure 4** Increased expression of Atg7 in METH-treated blastocysts in vitro. Blastocysts were treated with vehicle (Con, 1% EtOH in PBS), 7 nM METH, or 28 nM METH for 24 h in vitro and were subjected to immunofluorescence staining with anti-Atg7 antibody. Blastocysts (3–5 per group) were used in each experiment, and three sets of experiments generated similar results. Green, Atg7; red, TO-PRO-3; scale bar, 20 μm.
expressed 1 (Hand1) genes are expressed, while Oct4 is not (Fig. 8A). Previous work has established that TS cells mainly express CB1R, and that 7 nM is a physiologic dose of anandamide which benefits the proliferation of TS cells (Sun et al., 2010). Thus, we used 7 and 28 nM (as a high dose) to examine the effect of METH on autophagy in these cells. As shown in Fig. 8B, 7 nM METH did not have any significant effect on the conversion of LC3 in TS cells, whereas 28 nM METH caused the increased conversion of LC3 from form I to form II.

**Discussion**

Autophagy is dramatically induced during cell starvation to provide energy and macromolecules to cells (Mizushima, 2007). This process is also involved in several aspects of development. For example, during mammalian embryo development, autophagy is heavily activated in fertilized oocytes. Preimplantation embryos developed from Atg5 deficient oocytes exhibit developmental arrest prior to implantation (Tsukamoto et al., 2008). It is supposed that the lack of autophagy during early embryonic development leads to accumulation of unnecessary maternal proteins and mRNA that are no longer needed after fertilization (Tsukamoto et al., 2008). Autophagy is not only a critical process for normal development and survival but it also serves as a mechanism to protect cells or organisms from suboptimal environments (Mizushima and Levine, 2010). Suboptimal environment is a broad term considering that each cell’s need for survival requires different conditions. For mouse embryos in utero, they face a dramatic change in cellular needs before and after implantation. We previously observed that autophagic activation is maintained at basal levels in blastocysts on Day 4 morning of pregnancy, but it is dramatically reduced around 10 p.m. when the time of implantation is near (Lee et al., 2011). This is in accordance with the levels of uterine anandamide, as its level dramatically reduces at the time of implantation (Schmid et al., 1997). Thus, there is a correlation between the uterine content of anandamide and the status of autophagy in blastocysts. As shown in this investigation, when METH is given to pregnant mice, embryos respond by up-regulating autophagic activation.
Blastocysts also show heightened apoptosis under METH stimulation in vivo, suggesting that higher than normal levels of anandamide overall produce adverse effects on the survival of blastocysts.

As previously demonstrated by Wang et al. (2006a, b), sustained levels of uterine anandamide also lead to aberrant expression genes of lineage specification in embryos. Autophagic activation under high anandamide levels could be another indicator of the compromised quality of preimplantation embryos. Autophagy is considered to be a mechanism of cell survival which occurs under unfavorable conditions, but the prolonged activation can lead to cell death (Lee et al., 2011).

Anandamide in the periimplantation uterus is an established lipid indicator of healthy receptive uterus (Schmid et al., 1997). It is

**Figure 7** LC3 immunofluorescence staining and TUNEL analysis in blastocysts from METH-injected mice (Fig. 1B, Group III). Mice received METH injections at 10 a.m. and 4 p.m. on Day 4 of pregnancy and blastocysts were collected at 10 p.m. on the same day. (A) LC3 immunofluorescence staining. Green, LC3; blue, TO-PRO-3. (B) TUNEL staining. Red, TUNEL-positive cell; blue, TO-PRO-3; Scale bar, 20 μm. (C) The numbers of TUNEL-positive cells and total cells in a visual field were counted and are shown as percentages. *P < 0.01.

**Figure 8** METH-induced autophagic activation in the trophoblast stem (TS) cells. (A) RT–PCR analyses showing the purity of the established TS cell line. Cdx2, caudal type homeobox 2; Eomes, eomesodermin; Fgf2, fibroblast growth factor receptor 2; Hand1, heart and neural crest derivatives expressed 1. GAPDH is used as a control. (B) Western blot analysis of LC-3 in TS cells. The ratio of LC3-II form/αβ-tubulin signals was used to analyze data. The normalized value of the control group was set as 1 and the relative values of the other group were plotted. Data show the mean of two independent experiments. ANOVA was done to examine statistical significance. Control vs 7 nM, not significant; *control vs 28 nM, P = 0.006. (C) A representative figure of LC3 western blot analysis. Increased LC3-II form in TS cells treated with 28 nM METH (high concentration).
presumed that the adequate amount of anandamide is crucial for successful implantation, whereas higher than normal levels of anandamide affect embryonic developmental competence and survival. Embryos and their derivative cells lines, such as TS cells used herein, show high sensitivity to anandamide and their agonists (Wang et al., 2003; Sun et al., 2010). In preimplantation mouse embryos, the effects of cannabinoids on development are mediated by CB1R (Paria et al., 1998). Thus, we used METH as a cannabinoid agonist in our study, which shows high selectivity for this receptor type (Abadji et al., 1994). As we also used in this investigation, 7 nM METH is recognized as the healthy physiological dose for embryos while 28 nM METH produces adverse effects. Sheep embryos also show increased apoptosis when cultured in 28 nM cannabinoid agonist (Turco et al., 2008). While we used i.p. injections of METH to acutely increase higher levels of METH, it was previously shown that continued exposure to high levels of cannabinoids in utero also resulted in increased apoptosis in mouse blastocysts (Wang et al., 2006a, b). Thus, acute increases of in vivo cannabinoids by injections also cause detrimental effects on pre-implantation embryos by increasing cell death response.

Several works have shown that cannabinoids or the non-psychoactive component, cannabidiol, can induce autophagy in cancer cells (Salazar et al., 2009; Shrivastava et al., 2011). Glioma cells also respond to Δ⁹-tetrahydrocannabinol, a widely used cannabinoid agonist, and exhibit increased cell death through autophagic stimulation (Salazar et al., 2009). In these cells, ER stress response is shown to promote autophagy via inhibiting mTOR complex 1. In breast cancer cells, cannabidiol induces both apoptosis and autophagy which are coordinated by beclin 1 (Atg6) (Shrivastava et al., 2011). ER stress is also involved in this phenomenon. Whether a similar mechanism is involved as a signaling mediator of autophagic activation in mouse embryos warrants further investigation.

Supplementary data
Supplementary data are available at http://molehr.oxfordjournals.org/.

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Authors’ roles

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Conflict of interest
None declared.

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