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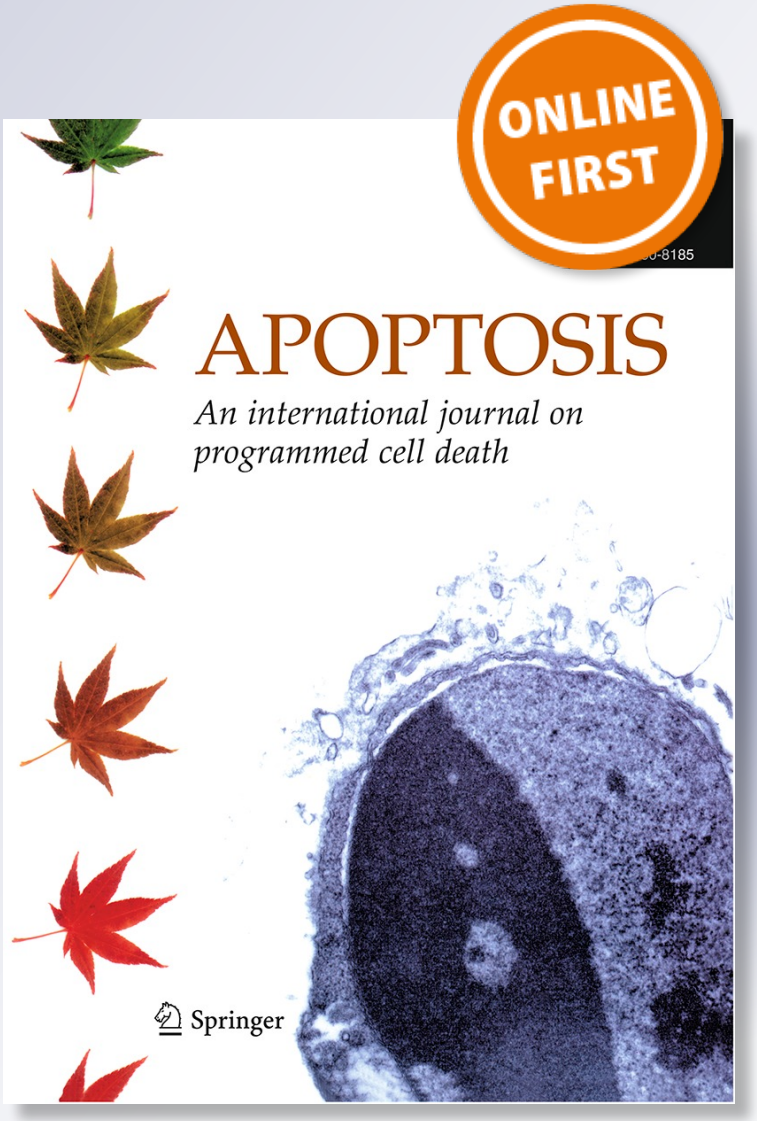

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Heparin exerts anti-apoptotic effects on uterine explants by targeting the endocannabinoid system

Ana Inés Salazar[1] · Claudia Vercelli[1,2] · Victoria Schiariti[1] · Carlos Davio[3] · Fernando Correa[1] · Ana María Franchi[1]

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Abstract Miscarriage caused by Gram-negative bacteria infecting the female genital tract is one of the most common complications of human pregnancy. Intraperitoneal administration of LPS to 7-days pregnant mice induces embryo resorption after 24 h. Here, we show that LPS induced apoptosis on uterine explants from 7-days pregnant mice and that CB1 receptor was involved in this effect. On the other hand, heparin has been widely used for the prevention of pregnancy loss in women with frequent miscarriage with or without thrombophilia. Besides its anticoagulant properties, heparin exerts anti-inflammatory, immunomodulatory and anti-apoptotic effects. Here, we sought to investigate whether the administration of heparin prevented LPS-induced apoptosis in uterine explants from 7-days pregnant mice. We found that heparin enhanced cell survival in LPS-treated uterine explants and that this effect was mediated by increasing uterine FAAH activity. Taken together, our results point towards a novel mechanism involved in the protective effects of heparin.

Keywords Miscarriage · Cell survival · Endocannabinoid system · Pregnancy loss · FAAH

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Abbreviations

AA	Arachidonic acid
AEA	Anandamide
CB1	Cannabinoid receptor type 1
CB2	Cannabinoid receptor type 2
eCS	Endocannabinoid system
FAAH	Fatty acid amide hydrolase
IL	Interleukin
LMWH	Low molecular weight heparins
LPS	Lipopolysaccharide
m-AEA	R(+)-methanandamide
PBMC	Peripheral blood mononuclear cells
TUNEL assay	Terminal deoxynucleotidyl transferase-mediated dUTP-biotin DNA-nick end labeling assay

Introduction

Miscarriage is the spontaneous loss of pregnancy before 24 weeks of gestation and it has been shown to occur in one in five pregnancies [1]. There is evidence that infections account for up to 15 % of early miscarriages (before 12 weeks) and up to 66 % of late miscarriages (from 12 to 24 weeks) [1, 2]. Infectious agents can access the choriodecidual space early in gestation and invade the fetal membranes, placenta, amniotic fluid and the fetus via different pathways, including ascending through the vagina and cervix, haematogenous dissemination after maternal infection or iatrogenically introduced after an amniocentesis [3, 4]. Gram-negative bacteria are responsible of almost 90 % of genital infections associated with miscarriage and lipopolysaccharide (LPS), a major component of their bacterial wall, is considered to be highly abortogenic [5]. We have developed a LPS-induced early pregnancy

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loss animal model were intraperitoneal administration of the endotoxin to 7-days pregnant mice leads to embryonic resorption followed by fetal expulsion [6]. Previous works from our lab have highlighted the participation of the endocannabinoid system (eCS) on LPS-triggered deleterious effects in reproductive tissues [7, 8]. Moreover, cumulative evidence supports the role of the eCS in key processes during pregnancy. Thus, the human endometrium expresses both CB1 and CB2 receptors [9] and low levels of anandamide (AEA) are necessary for an adequate implantation and trophoblast outgrowth [10, 11]. Conversely, increased concentrations of AEA have been shown to be embryotoxic, leading to arrested embryo development and pregnancy failure [12]. Accordingly, high plasma AEA levels and low FAAH activity in peripheral lymphocytes have been shown to correlate with frequent miscarriage in humans [13]. Moreover, high plasma levels of AEA have been associated with early pregnancy loss in humans [14]. In this sense, it has been reported that AEA induces apoptosis in different reproductive tissues. Fonseca et al. [15] have shown that AEA induced programmed cell death in primary rat decidual cells whereas Contassot et al. [16] reported a similar finding in uterine cervix cancer cells. The fact that administration of progesterone to LPS-treated mice restores FAAH activity and protein expression and prevents embryo resorption [17], supports the hypothesis that excessive production of AEA exerts deleterious effects during pregnancy. Moreover, CB1 receptor activation seems to be involved in AEA effects since it has been shown that CB1-*KO* mice are resistant to LPS-induced embryo loss [8]. In contrast, little is known regarding the participation of CB2 on AEA-induced apoptosis in reproductive tissues.

Heparin is a complex glycosaminoglycan [18] that has been extensively used in obstetric practice for the prevention of pregnancy complications associated with thrombophilic disorders as well as in women with unexplained frequent miscarriages [19, 20]. In addition to its anticoagulant properties, heparin has been shown to exert anti-inflammatory and immunomodulatory functions [reviewed in 21]. Moreover, recent works points toward heparin having anti-apoptotic effects. It has been reported that heparin prevented TNF-α- and INF-γ-induced apoptosis on primary first trimester villous trophoblast [22] and protected the BeWo trophoblast cells [23] whilst fractioned heparins promoted survival of decidual cells [24]. Although the molecular mechanisms by which heparin exerts these anti-apoptotic effect remain poorly understood, cumulative evidence suggests that heparin may bind different growth factors enhancing their ability to activate their cognate receptors [reviewed in 25].

In this study, we demonstrate that addition of heparin to uterine explants from 7-days pregnant mice enhances tissue survival by rescuing cells from LPS- or AEA-triggered apoptosis. We also provide evidence for heparin exerting its anti-apoptotic effects via enhancing FAAH activity in uterine explants. Collectively, we show for the first time that heparin interacts with the uterine eCS and that this interaction is important for the anti-apoptotic effects of heparin.

Materials and methods

Reagents

LPS (serotype 05:B55) from *Escherichia coli*, R(+)-methanandamide, anandamide, arachidonic acid, Hoechst 33342 and heparin were purchased from Sigma Chemical Co. (St Louis, MI, USA). URB-597, AM251 and AM630 were obtained from Tocris Cookson Inc. (Ellisville, MO, USA). Dulbecco's Modified Eagle Medium (DMEM) and Penicillin–Streptomycin Solution (ATB) were from Invitrogen (Life Technologies, Argentina). Foetal bovine serum (FBS) was from Internegocios SA (Mercedes, Buenos Aires, Argentina). [5,6,8,9,11,12,14,15-^{3}H]-anandamide (172.4 Ci/mmol, 100 μCi/ml) were provided by PerkinElmer Life and Analytical Sciences, Inc. (Walthman, MA, USA). TLC aluminium silica gel plates were purchased from Merck KGaA (Darmstadt, Germany). All other chemicals were analytical grade.

Animals and uterine explants preparation

Eight to twelve-week-old virgin female BALB/c or CD1 (*wild-type* [*WT*] or CB1-*knockout* [CB1-*KO*]) mice were paired with eight to 12-week-old BALB/c or CD1 (*WT* or CB1-*KO*) males respectively. The day of appearance of the coital plug was taken as day 0 of pregnancy. Animals were housed in cages under controlled conditions of light (12 h light, 12 h dark) and temperature (21–25 °C) and received murine chow and water ad libitum.

On day 7 of pregnancy, BALB/c, CD1 *WT* or CD1 CB1-*KO* mice were euthanized by cervical dislocation. The uteri were excised and the implantation sites were removed. Next, decidual and embryo tissues were separated from uterine tissue and discarded. Uterine explants were then cultured in DMEM supplemented with 10 % FBS and 1 % penicillin/streptomycin (v/v) in a humidified environment containing 5 % CO_2 and held at a constant temperature of 37 °C.

Ethics statement

The experimental procedures reported here were approved by the Animal Care Committee of the Center for

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Pharmacological and Botanical Studies of the National Research Council (CEFYBO–CONICET) and by The Institutional Committed for the Care and Use of Laboratory animals from the School of Medicine (University of Buenos Aires), and were carried out in accordance with the Guide for Care and Use of Laboratory Animals (NIH).

Tissue processing for histology

After each experiment, uterine explants were fixed in a neutral buffered 10 % formalin solution for 24 h. Next, uterine tissues were dehydrated through successive passages of increasing concentrations of ethanol (from 70 to 100 %). Dehydrated uterine tissues were embedded in paraffin wax and sliced in 5 μm thick sections. Previous to TUNEL assay or Hoechst 33342 staining, paraffin wax was removed and tissues were rehydrated.

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin DNA-nick end labeling (TUNEL) assay

Terminal deoxynucleotidyl transferase dUTP-biotin DNA-nick end labeling (TUNEL) assay is a well-established method for detecting DNA fragments occurring during apoptosis. TUNEL assay kit was purchased from Roche (Mannheim, Germany) and manufacturer's recommendations were followed. Briefly, uterine tissue sections were treated with proteinase K for 30 min to allow tissue permeabilization. Next, sections were incubated with "TUNEL reaction solution" for 1 h at 37 °C followed by three washes of 5 min each with PBS. Positive control was obtained by incubating permeabilized tissue sections with DNAse I (3000 U/ml in Tris–HCl 50 mM buffer pH 7.5 containing BSA 1 mg/ml,) previously to the TUNEL assay. Negative control was obtained by incubating permeabilized tissue sections with the label reagent in the absence of the terminal transferase (TdT) enzyme.

Tissue sections were then analyzed with a fluorescence microscope and images were obtained with a digital camera (Nikon, Japan) coupled to the microscope. TUNEL positive cells were quantified counting the number of bright green cells in ten random non-overlapping fields from tissue sections from each treatment.

Hoechst 33341 staining

Rehydrated uterine tissue sections were stained with Hoechst 33341 (1:1000 v/v from a stock solution of 1 μg/ml) for 10 min at room temperature followed by three washes of 5 min each with PBS. Tissue sections were then analyzed with a fluorescence microscope and images were obtained with a digital camera (Nikon, Japan) coupled to the microscope.

Caspase 3/7 activity assay

The activity of caspases 3 and 7 were assayed together in cultured uterine explants using the Caspase-Glo® 3/7 Assay kit from Promega (Madison, WI, USA) and following the manufacturer's instructions. The reaction was measured in a microplate luminometer/fluorometer FLUOstar Omega (BMG Labtech; Ortenberg, Germany) and caspase 3/7 activity is expressed as relative luminescence units (RLU) per mg of wet tissue (RLU/mg wet tissue).

Determination of fatty acid amide hydrolase (FAAH) activity

FAAH (EC 3.5.1.4) activity was assayed as described by Paria et al. [10]. The hydrolyzed [^{3}H]-AA was resolved in the organic layer of a solvent system of ethyl acetate:hexane:aceticacid:distilled water (100:50:20:100 v/v) mixture. The plate was exposed to iodine to identify the zones corresponding to AA. The distribution of radioactivity on the plate was counted in a scintillation counter by scraping off the corresponding spots detected in the plate. The area of each radioactive peak corresponding to AA was calculated and expressed as a percentage of the total radioactivity of the plates. Protein concentration was determined by the method of Bradford. Enzyme activity is reported as nmol [^{3}H]-AA/[(mg protein).h]. The optimal reaction conditions were previously determined (data not shown).

Statistical analyses

From each 7-days pregnant mouse, enough uterine explants were obtained for all the treatments in each experiment. Therefore, statistical analysis was performed in blocks when necessary. Data were analyzed by means of one-way or two-ways ANOVA procedures and means were compared by Tukey post hoc test. Differences between means were considered significant when p value was 0.05 or less. Different letters indicate significant differences between means. Normality and homoscedasticity were tested by Shapiro–Wilk (modified) and Levene test, respectively. Statistical analysis was performed using the software Infostat (Córdoba, Argentina).

Results

LPS and AEA induce apoptosis in uterine explants: involvement of cannabinoid receptors

Previous works from our lab show the participation of the eCS in the molecular mechanisms involved in LPS-

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triggered uterine tissue damage [7] and embryonic resorption [8]. Here, we sought to investigate whether LPS (1 μg/ml) produced tissue damage in the uterus from 7-days pregnant BALB/c mice by activating the pro-apoptotic machinery. Implantation sites were obtained from 7-days pregnant BALB/c mice, decidual and embryo tissues were eliminated and uterine explants were incubated in DMEM containing 10 % FBS and 1 % antibiotics. Next, LPS (1 μg/ml) was added to the medium and caspase 3/7 activity was measured using Caspase-Glo® 3/7 Assay kit at 6, 9 and 12 h post-stimulation. As shown in Fig. 1, apoptosis happened in both control and LPS-treated conditions in a time-dependent manner. However, uterine explants treated with LPS (1 μg/ml) showed increased caspase 3/7 activity at each time-point when compared to their respective control timepoints, suggesting that LPS induced apoptosis in uterine explants by activating caspase signaling pathways.

Since the endocannabinoid system has been shown to mediate LPS-induced injury in uterus and decidua [7, 8], we decided to evaluate the effects of R(+)-methanandamide (m-AEA), a non-hydrolyzable analog of anandamide (AEA), on uterine explants. Since we observed in the previous experiment that the difference in caspase 3/7 activity between control and LPS-treated uterine explants was maximal at 9 h post-treatment, we chose this time-point for the next experiment. Uterine explants from 7-days pregnant BALB/c mice were incubated with different concentrations of m-AEA (1–100 nM) for 9 h followed by caspase 3/7 activity quantification. As shown in Fig. 2a, m-AEA induced an increase in caspase 3/7 activity in an inverted-U fashion, with the concentration of m-AEA 10 nM having the strongest pro-apoptotic effect. Therefore, this concentration was used in the following experiments. Figure 2b, c show the increased apoptosis induced by m-AEA 10 nM in uterine explants from 7-days pregnant BALB/c mice, quantified by TUNEL assay. A similar finding was observed when uterine explants from 7-days pregnant BALB/c mice were treated with m-AEA 10 nM and then stained with Hoechst 33342 (Fig. 2d).

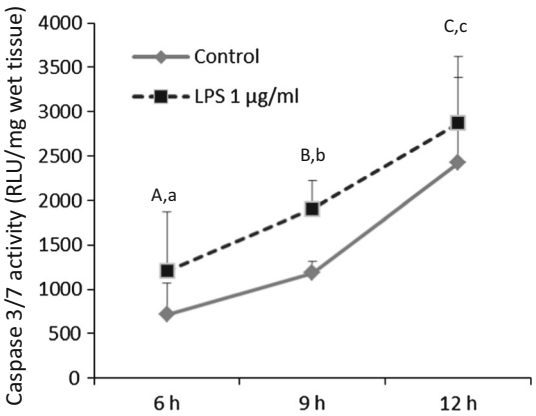

Fig. 1 LPS effect on caspase 3/7 activity at different time points. Uterine explants from 7-days pregnant BALB/c mice were incubated with fresh DMEM (control) or with fresh DMEM containing LPS (1 μg/ml) for 6, 9 or 12 h. After each time point, caspase 3/7 activity was meadured using Caspase-Glo® 3/7 Assay kit. Statistical analysis was performed with blocks design. *Upper-case letters* represent differences among different time points in each treatment. A ≠ B ≠ C, $p < 0.05$. *Lower-case letters* represent differences between treatments. a ≠ b ≠ c, $p < 0.05$. Data are shown as mean ± SEM (n = 8)

In order to elucidate the participation of cannabinoid receptors in LPS- and m-AEA-mediated increase apoptosis in uterine explants from 7-days pregnant BALB/c mice, we performed the next experiments using CB1 and CB2 receptor antagonists. First, we incubated uterine explants from 7-days pregnant BALB/c mice with LPS (1 μg/ml) in the presence or absence of AM251 (10 nM), a CB1 receptor antagonist, and/or AM630 (10 nM), a CB2 receptor antagonist. As shown in Fig. 3a, LPS (1 μg/ml) increased caspase 3/7 activity in uterine explants as expected. However, when CB1 receptor was blocked with AM251 (10 nM), we observed a tendency to revert the pro-apoptotic effect of LPS to control levels. AM630 (10 nM) failed to affect LPS-induced increase of caspase 3/7 activity but, interestingly, when both AM251 and AM630 (each at 10 nM) were used, the reversion of LPS effects on caspase 3/7 activity was complete. Next, when we assessed apoptosis with the TUNEL assay, we observed that AM251 (10 nM) reversed LPS pro-apoptotic effects on uterine explants, AM630 (10 nM) had no effect and when both antagonists were used, they blocked LPS effects (Fig. 3b, c). Similarly, when we assessed apoptosis with the Hoechst 33342 staining, we observed that AM251 (10 nM) prevented LPS-induced apoptosis. AM630 (10 nM) alone failed to revert LPS pro-apoptotic effects on uterine explants, however, when both antagonists were used the reverting effect was complete (Fig. 3d).

Uterine explants from 7-days pregnant CD1 CB1-*KO* mice are resistant to LPS-induced apoptosis when compared to uterine explants from CD1 *wild type* (WT) mice

We have previously shown that CD1 CB1-*KO* mice experienced lower rate of embryo resorption after LPS administration when compared to CD1 *WT* mice [8]. Therefore, we sought to determine whether CD1 CB1-*KO* mice were also resistant to LPS- and m-AEA-induced apoptosis. Uterine explants from 7-days pregnant CD1 *WT* or CB1-*KO* mice were treated respectively with LPS (1 μg/ml) or m-AEA (10 nM) and apoptosis was

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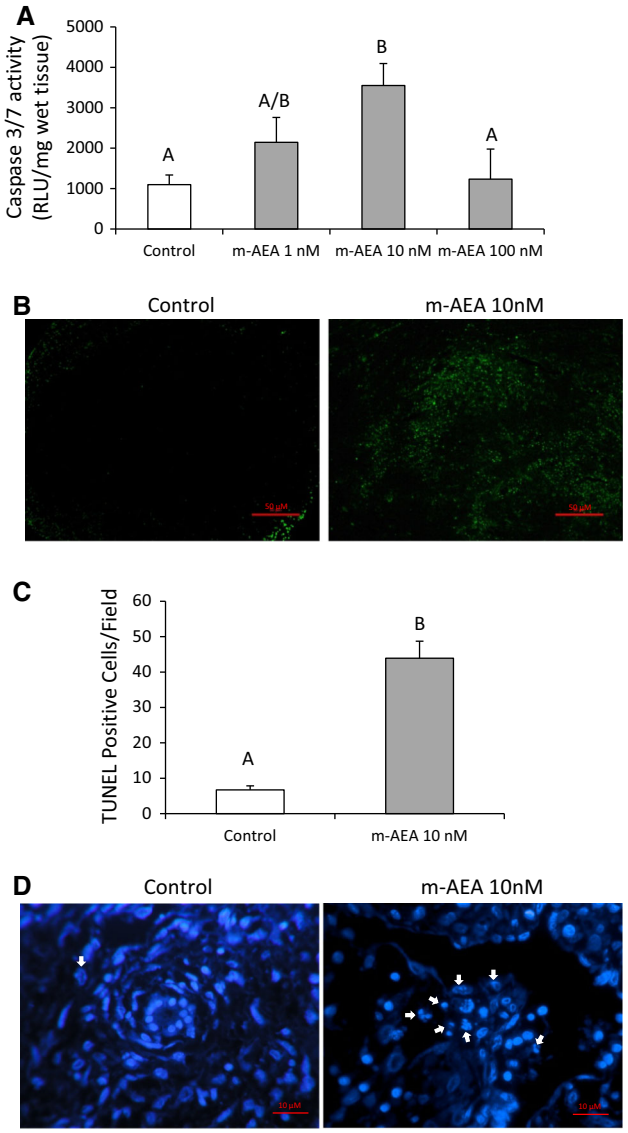

Fig. 2 **a** Dose–response apoptotic effect of R(+)-methanandamide (m-AEA) on caspase 3/7 activity. Uterine explants from 7-days pregnant BALB/c mice were incubated with different concentrations of m-AEA (1–100 nM). After 9 h of treatment, caspase 3/7 was measured using Caspase-Glo® 3/7 Assay kit. Statistical analysis was performed with blocks design. Data are shown as media ± SEM ($n = 8$), A ≠ B, $p < 0.05$. **b** Uterine explants from 7-days pregnant BALB/c mice were incubated with m-AEA (10 nM). After 12 h of treatment, apoptosis in the uterine explants was determined by TUNEL assay. TUNEL positive cells appear as bright green in uterine tissue sections. **c** TUNEL positive cells were quantified counting the number of bright green cells in ten random non-overlapping fields from tissue sections from each treatment. Statistical analysis was performed with blocks design. Data are shown as media ± SEM ($n = 4$), A ≠ B, $p < 0.05$. **d** Uterine explants from 7-days pregnant BALB/c mice were incubated with m-AEA (10 nM). After 12 h of treatment, apoptosis in the uterine explants was determined by condensed chromatin staining with Hoechst 33342. *White arrows* show cells with apoptotic nucleus (condensed and/or fragmented chromatin)

assessed using the TUNEL assay. As shown in Fig. 4a, b, both LPS (1 μg/ml) and m-AEA (10 nM) induced a marked increase in the number of TUNEL positive cells in the uterine explants from 7-days pregnant CD1 *WT* mice but failed to have any effect on uterine explants from 7-days pregnant CD1 CB1-*KO* mice, suggesting that CB1 receptor is involved in LPS pro-apoptotic effects.

Heparin prevents LPS-induced apoptosis in uterine explants from 7-days pregnant BALB/c mice: interaction with the endocannabinoid system

Heparin has been extensively used as an adjunct in assisted reproduction [26] as well as in the prevention of unexplained frequent miscarriage [19, 20]. However, little is known regarding the possible interaction between heparin and the endocannabinoid system. Therefore, we decided to study whether heparin could protect the uterine tissue from LPS-induced apoptosis and whether this effect could be mediated by heparin targeting components of the endocannabinoid system. Firstly, we treated in vitro uterine explants from 7-days pregnant BALB/c mice with LPS (1 μg/ml) in the presence or absence of heparin (100 μg/ml) and 6 h later we proceeded to measure the activity of the fatty acid amide hydrolase (FAAH), the main catabolic enzyme for AEA and other *N*-acylethanolamines. As shown in Fig. 5a, in vitro treatment with LPS (1 μg/ml) induced a reduction of FAAH activity in uterine explants from 7-days pregnant BALB/c mice which was completely reverted with the co-treatment of heparin (100 μg/ml). The same effect was observed when the lower concentration of LPS (0.5 μg/ml) was used (Fig. 5b). Next, we proceeded to study whether heparin may prevent the LPS-induced apoptosis. Uterine explants from 7-days pregnant BALB/c were treated with LPS (1 μg/ml) in the presence or absence of heparin (100 μg/ml) and the level of apoptosis was quantified using the TUNEL assay. Under this condition, heparin failed to protect uterine explants from LPS-induced apoptosis (data not shown). However, when used the lower concentration of LPS (0.5 μg/ml), heparin (100 μg/ml) fully protected the uterine explants from LPS-induced damage. Thus, the in vitro treatment of uterine explants with LPS (0.5 μg/ml) induced an increase of caspase 3/7 activity which was prevented by the co-treatment with heparin (100 μg/ml) (Fig. 6a). Moreover, the number of TUNEL positive cells was highly increased in the uterine explants treated with LPS (0.5 μg/ml) and this effect was completely prevented in the presence of heparin (100 μg/ml) (Fig. 6b, c).

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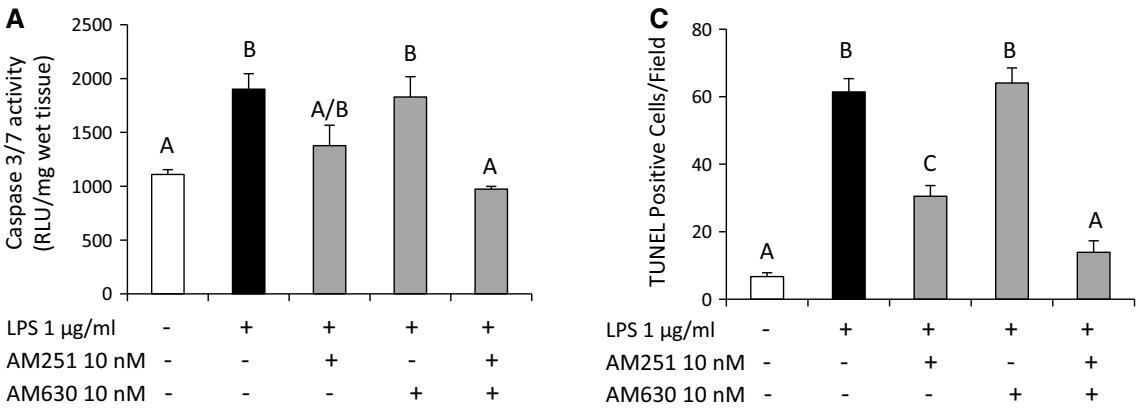

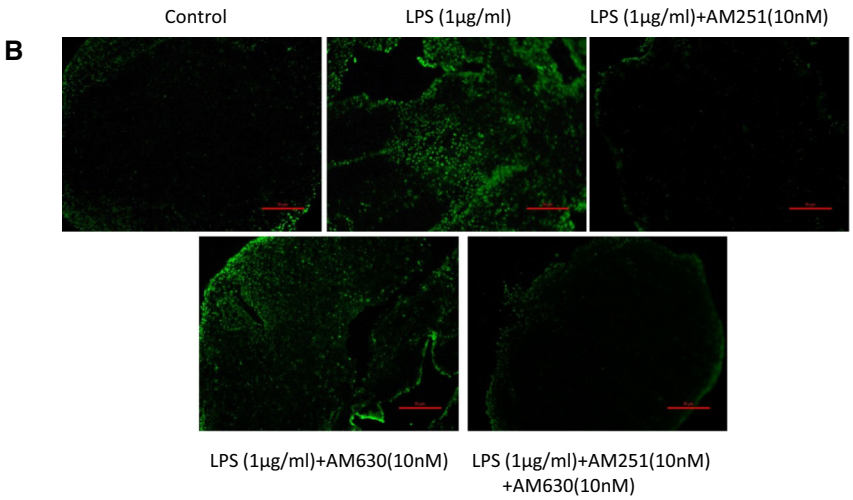

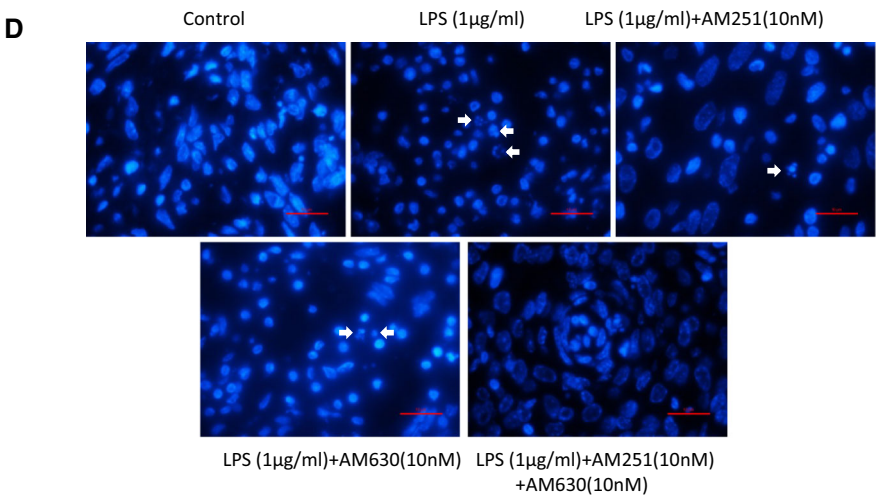

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◀**Fig. 3** **a** Effect of cannabinoid receptors antagonists on LPS-induced activation of caspase 3/7 in uterine explants. Uterine explants from 7-days pregnant BALB/c mice were treated with LPS (1 μg/ml) in the presence or absence of CB1 receptor antagonist, AM251 (10 nM) and/or CB2 antagonist, AM630 (10 nM). After 9 h, caspase 3/7 activity was measured using the Caspase-Glo® 3/7 Assay kit. Statistical analysis was performed with blocks design. Data are shown as media ± SEM (n = 9), A ≠ B, $p < 0.05$. **b** Effect of cannabinoid receptors antagonists on LPS-induced apoptosis in uterine explants. Uterine explants from 7-days pregnant BALB/c mice were treated with LPS (1 μg/ml) in the presence or absence of CB1 receptor antagonist, AM251 (10 nM) and/or CB2 antagonist, AM630 (10 nM). After 12 h of treatment, apoptosis in the uterine explants was determined by TUNEL assay. **c** TUNEL positive cells were quantified counting the number of bright green cells in ten random non-overlapping fields from tissue sections from each treatment. Statistical analysis was performed with blocks design. Data are shown as media ± SEM (n = 4), A ≠ B ≠ C, $p < 0.05$. **d** Uterine explants from 7-days pregnant BALB/c mice were incubated with treated with LPS (1 μg/ml) in the presence or absence of CB1 receptor antagonist, AM251 (10 nM) and/or CB2 antagonist, AM630 (10 nM). After 12 h of treatment, apoptosis in the uterine explants was determined by condensed chromatin staining with Hoechst 33342. *White arrows* show cells with apoptotic nucleus (condensed and/or fragmented chromatin)

Since AEA mediates the deleterious effects of LPS on reproductive tissues [7, 8], our next aim was to evaluate whether heparin could interfere with the pro-apoptotic effects of AEA on uterine explants. Consequently, we treated uterine explants from 7-days pregnant BALB/c with AEA (10 nM) and the presence or absence of heparin (100 μg/ml) and proceeded to determine the degree of apoptosis by quantifying the number of TUNEL positive cells. As expected, AEA (10 nM) highly increased the number of TUNEL positive cells (Fig. 7a), and the co-treatment with heparin (100 μg/ml) completely reversed the effects of the endocannabinoid. Analogously, when the enzyme FAAH was inhibited with URB-597 (1 μM) and therefore the degradation of AEA and other *N*-acylethanolamines are impaired, the number of TUNEL positive cells was increased (Fig. 7b). However, in this case, co-treatment with heparin (100 μg/ml) failed to restore the degree of apoptosis to control levels (Fig. 7b), suggesting that the anti-apoptotic effects of heparin in uterine explants are mediated by FAAH.

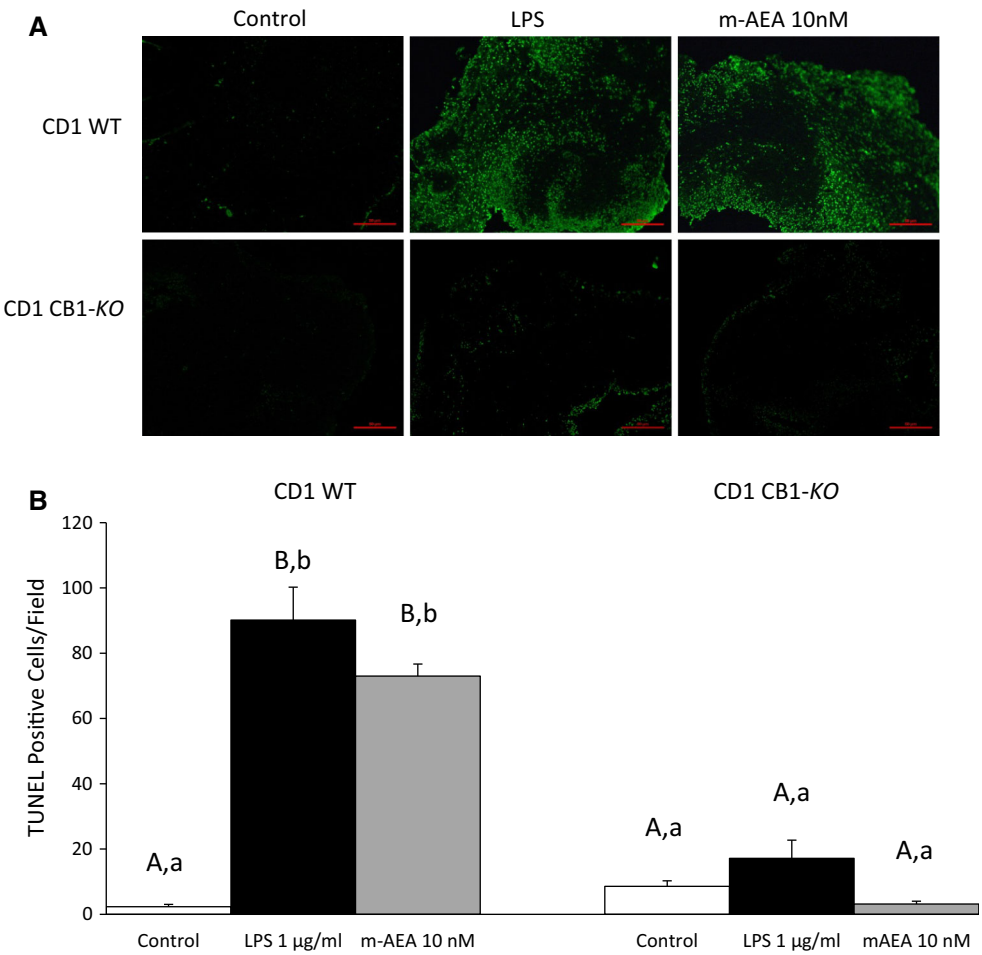

Fig. 4 LPS and m-AEA apoptotic effects are diminished in CD1 CB1-*KO* mice compared to CD1 *wild-type* (WT) mice. **a** Uterine explants from 7-days pregnant CD1 WT or CB1-*KO* mice were treated respectively with LPS (1 μg/ml) or m-AEA (10 nM). After 12 h of treatment, apoptosis in the uterine explants was determined by TUNEL assay. **b** TUNEL positive cells were quantified counting the number of bright green cells in ten random non-overlapping fields from tissue sections from each treatment and each genotype. Statistical analysis was performed with blocks design. *Upper-case letters* represent differences among different treatments. A ≠ B ≠ C, $p < 0.05$. *Lower-case letters* represent differences between genotypes. a ≠ b ≠ c, $p < 0.05$. Data are shown as mean ± SEM (n = 4)

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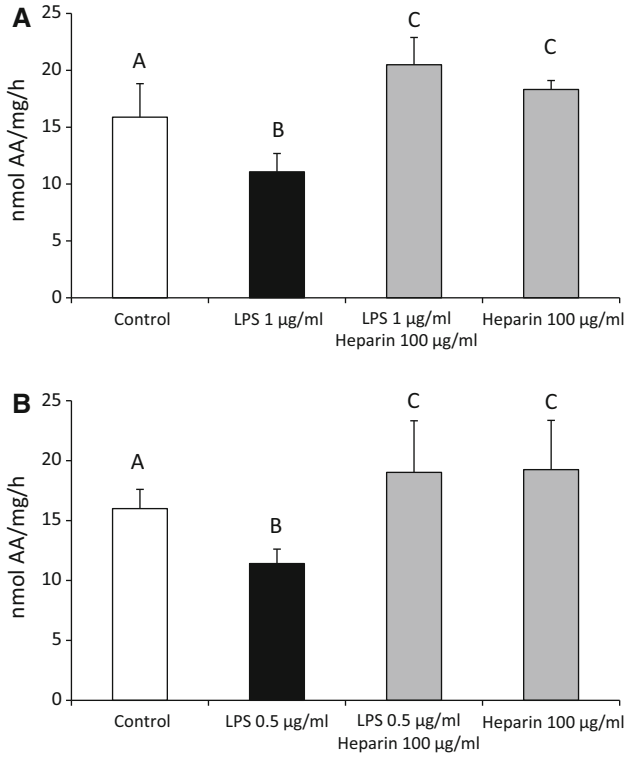

Fig. 5 LPS and heparin effects on uterine fatty acid amide hydrolase (FAAH) activity. **a** Uterine explants from 7-days pregnant BALB/c mice were treated with LPS (1 µg/ml) in the presence or absence of heparin (100 µg/ml). After 6 h of treatment, FAAH activity was measured in tissue explants for each treatment. Statistical analysis was performed with blocks design. Data are shown as media ± SEM (n = 8), A ≠ B ≠ C, $p < 0.05$. **b** Uterine explants from 7-days pregnant BALB/c mice were treated with LPS (0.5 µg/ml) in the presence or absence of heparin (100 µg/ml). After 6 h of treatment, FAAH activity was measured in tissue explants for each treatment. Statistical analysis was performed with blocks design. Data are shown as media ± SEM (n = 6), A ≠ B ≠ C, $p < 0.05$

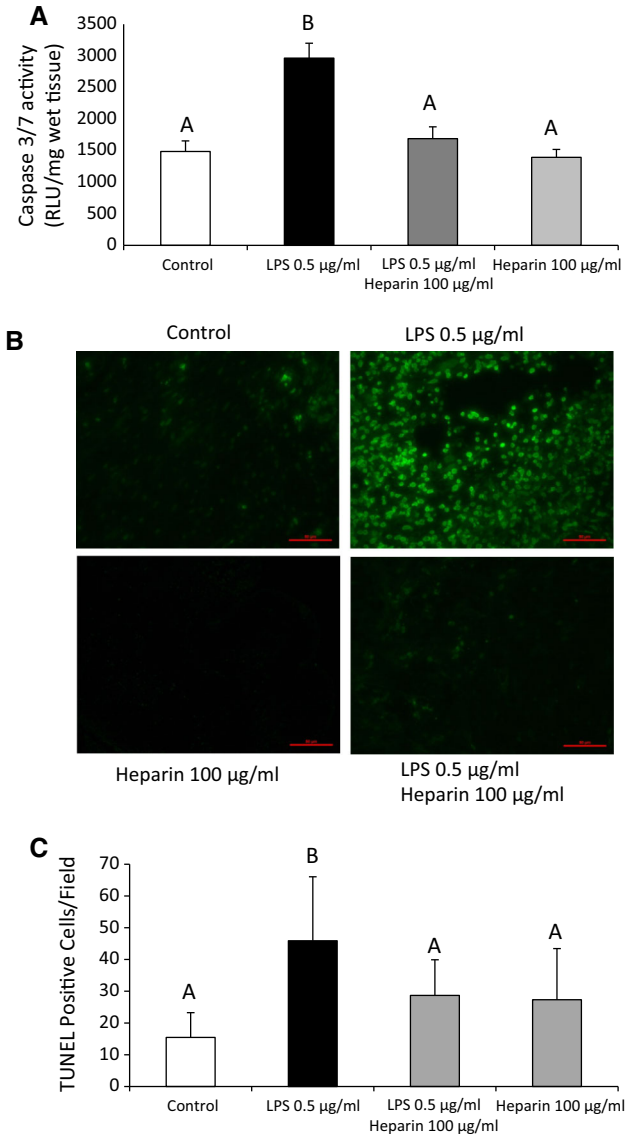

Fig. 6 **a** Effect of heparin on LPS-induced activation of caspase 3/7 in uterine explants. Uterine explants from 7-days pregnant BALB/c mice were treated with LPS (0.5 µg/ml) in the presence or absence of heparin (100 µg/ml). After 9 h, and caspase 3/7 activity was measured using the Caspase-Glo® 3/7 Assay kit. Data are shown as media ± SEM (n = 8), A ≠ B, $p < 0.05$. **b** Effect of heparin on LPS-induced apoptosis in uterine explants. Uterine explants from 7-days pregnant BALB/c mice were treated with LPS (0.5 µg/ml) in the presence or absence of heparin (100 µg/ml). After 12 h of treatment, apoptosis in the uterine explants was determined by TUNEL assay. **c** TUNEL positive cells were quantified counting the number of bright green cells in ten random non-overlapping fields from tissue sections from each treatment. Statistical analysis was performed with blocks design. Data are shown as media ± SEM (n = 4), A ≠ B, $p < 0.05$

Discussion

The aim of this work is to study the participation of the eCS on LPS-induced apoptosis in uterine explants from 7-days pregnant mice and protective effects of heparin. We have previously developed a murine model of LPS-induced early pregnancy loss where LPS administration to 7-days pregnant mice induces embryo resorption after 24 h [6]. This process was associated with a strong inflammation [27, 28] and an increase in the plasma levels of endocannabinoids [8] together with a lower activity and expression of the major endocannabinoid catabolic enzyme, the fatty acid amide hydrolase (FAAH) [17]. Similarly, Maccarrone et al. [13] reported an association between FAAH activity in peripheral lymphocytes and miscarriage in humans whereas Habayeb et al. [14] showed a correlation between high plasma levels of AEA and early pregnancy loss in humans.

We have previously shown the participation of the eCS in the molecular mechanisms involved in LPS-triggered uterine tissue damage [7]. Here, we show that LPS induced apoptosis in uterine explants of 7-days pregnant mice in a

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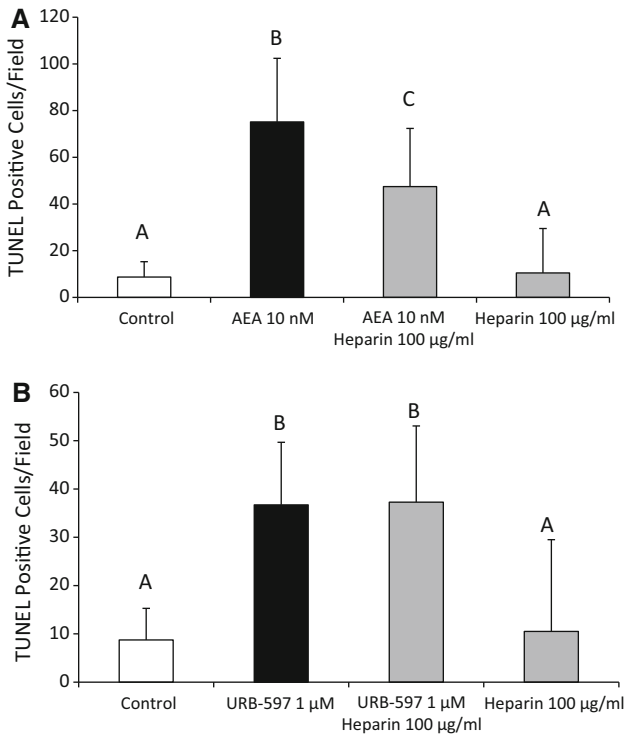

Fig. 7 **a** Heparin (100 µg/ml) reverts AEA-induced apoptosis in uterine explants. Uterine explants from 7-days pregnant BALB/c mice were treated with AEA (10 nM) in the presence or absence of heparin (100 µg/ml). After 12 h of treatment, apoptosis in the uterine explants was determined by TUNEL assay. TUNEL positive cells were quantified counting the number of bright green cells in ten random non-overlapping fields from tissue sections from each treatment. Statistical analysis was performed with blocks design. Data are shown as media ± SEM ($n = 4$), $A \neq B \neq C$, $p < 0.05$. **b** Heparin (100 µg/ml) fails to prevent apoptosis in uterine explants when FAAH enzyme is inhibited with URB-597 (1 µM). Uterine explants from 7-days pregnant BALB/c mice were treated with URB-597 (1 µM) in the presence or absence of heparin (100 µg/ml). After 12 h of treatment, apoptosis in the uterine explants was determined by TUNEL assay. TUNEL positive cells were quantified counting the number of bright green cells in ten random non-overlapping fields from tissue sections from each treatment. Statistical analysis was performed with blocks design. Data are shown as media ± SEM ($n = 4$), $A \neq B$, $p < 0.05$

time-dependent fashion with the eCS being involved in this process. Our results are in agreement with other reports suggesting a pro-apoptotic action of AEA [reviewed in 29]. Furthermore, it has been reported that AEA induced apoptosis in uterine cervix cancer cells [16], primary rat decidual cells [15] and human breast cancer [30, 31]. Likewise, we found that treatment with R(+)-methanandamide to uterine explants mimicked the actions of LPS and AEA on inducing apoptosis. Similarly, the non-hydrolysable AEA analog, R(+)-methanandamide has been shown to induced apoptosis and/or arrest cell proliferation in human cervical carcinoma cells [32], prostate cancer cells [33] and mantle cell lymphoma [34]. Interestingly, we found that R(+)-methanandamide exerted its pro-apoptotic effect in a narrow window of concentration, resulting in a classic inverted U-shaped dose–effect curve. Similarly, others CB1 agonists have been shown to produce a similar dose–effect curve [35–37], suggesting that at high concentrations of ligands, CB1 receptors undergo desensitization. Our results suggest that activation of cannabinoid receptor type 1 (CB1) is directly involved in LPS-induced apoptosis whereas CB2 *per se* seemed not to participate, or rather have a marginal participation, in this effect. However, the effect of LPS-induced apoptosis was reversed when both cannabinoid receptors were blocked by their respective antagonist in comparison to when only CB1 was antagonized. Similarly, Costa et al. [38] reported that AEA also increased the activities of caspase 3/7 and 9, induced loss of inner mitochondrial membrane potential and production of oxygen and nitrogen reactive species in primary human cytotrophoblast cells. These effects were reversed by CB1 or CB2 blockade, except for caspase 3/7 activity which was only reversed by CB2 antagonism [38]. It has been also shown that targeting both CB1 and CB2 abrogated caspase 3 activity in mantle cell lymphoma [34] as well as in other non-Hodgkin lymphomas [39]. In contrast, Fonseca et al. [40] showed that AEA induced cell death in primary rat decidual cell cultures by increasing caspase 3/7 activity and that this effect was mediated only via CB1 activation. Although further research is warranted to establish the precise role of CB1 and CB2 receptors in LPS-induced caspase 3/7 activity, recent studies have suggested that there is a cross-talk between both receptors and that they might even form heteromers [41, 42]. A specific characteristic of a CB1–CB2 heteromer would be the ability to modify the downstream signaling in contrast to the single constituent receptors [41]. Whether there is a cross-talk between CB1 and CB2 or whether these receptors even form CB1–CB2 heteromers in uterine tissues, it needs to be further explored.

The hypothesis that CB1 mediates the LPS deleterious effects of LPS on reproductive tissues was confirmed by the observation that uterine explants from CB1-*KO* mice were resistant to LPS- and AEA-induced apoptosis. Accordingly, Wolfson et al. [8] reported that LPS induced a lower embryo resorption rate in CD1 CB1-*KO* mice when compared to CD1 *WT* mice. Therefore, CB1 activation seems to participate in abortogenic processes. In fact, it has been shown that first trimester placenta from women with spontaneous miscarriage express higher levels of CB1 and lower levels of FAAH when matched with placentas from women who underwent pregnancy termination [43]. The role of CB2 in spontaneous early pregnancy loss, however, seems to be less clear and warrants further investigation.

Heparin is a highly sulfated glycosaminoglycan with anticoagulant properties widely used in obstetric practice

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as an antithrombotic agent in pregnant women with antiphospholipid syndrome [20, 44], as well as in preventing pregnancy loss in women with frequent miscarriages without a history of thrombophilia [19]. Beside its anticoagulant effects, heparin has also been used in assisted reproduction since it modulates several physiological processes such as blastocyst apposition, adherence and implantation and as well as trophoblast differentiation and invasion [reviewed in 26]. Interestingly, it has been suggested that heparin possesses anti-inflammatory and immunomodulatory properties [21]. Thus, it has been shown to prevent LPS-induced production of the inflammatory cytokines TNF-α, IL-6, IL-8 and IL-1β in murine peritoneal macrophages [45], to inhibit TNF-α-induced activation of NF-κB in human endometrial stromal cells [46] and to forestall complement activation [47]. Similarly, low molecular weight heparins (LMWH) derivatives have been shown to inhibit the carrageenan-induced expression of iNOS and COX-2 in a rat model of lung tissue injury [48] and to modulate fetoplacental IL-6 levels in a model of murine miscarriage [49].

In addition, it has been shown that heparin also exerts anti-apoptotic effects. Thus, Hills et al. [22] reported that heparin prevented TNF-α- and INF-γ-induced apoptosis of primary first trimester villous trophoblast. Similarly, Bose et al. [23] showed that heparin protected the trophoblast cell line BeWo from programmed cell death and Di Simone et al. [24] reported that LMWH rescued decidual cells from undergoing apoptosis. In accordance to these studies, we observed that heparin reduced the number of TUNEL positive cells as well as the activation of caspase 3/7 in LPS-treated uterine explants from 7-days pregnant mice. However, the molecular mechanisms by which heparin exerts anti-apoptotic effects remain unclear. It has been proposed that heparin binds to growth factors such as hepatocyte growth factor, epidermal growth factor (EGF), heparin-binding EGF-like growth factor (HB-EGF) and fibroblast growth factor family (FGFs), consequently enhancing their ability to activate their respective receptors [25]. Hence, Hills et al. [22] reported that heparin triggered the phosphorylation of EGF receptor and the activation of Akt and ERK1/2, JNK and p38 MAPK signaling pathways in first trimester villous trophoblast cultures. Here we propose an alternative mechanism by which heparin prevents apoptosis in uterine explants from 7-days pregnant mice. Our results suggest that heparin enhances FAAH activity, therefore controlling the local AEA levels which have been shown to be involved in the apoptotic process [29]. Indeed, LPS downregulates FAAH expression in human lymphocytes [50]. Similarly, LPS treatment has been proved to decrease FAAH activity in the peripheral blood mononuclear cells (PBMC) of mice [17] which was associated with higher plasma levels of endocannabinoids [8]. Remarkably, FAAH protein expression and activity was restored to control levels when PBMC from LPS mice were co-treated with progesterone [17]. Accordingly, we report here that LPS treatment induced a decreased FAAH activity in the uterine explants from 7-days pregnant mice which was reverted to control levels in the presence of heparin. Likewise, AEA-induced apoptosis on uterine explants was completely prevented in the presence of heparin, suggesting that an enhanced FAAH activity exerts protective effects. Indeed, when FAAH activity was inhibited with URB-597, the anti-apoptotic effect of heparin was completely lost. Noteworthy, heparin was able to prevent LPS-induced apoptosis only when the lower concentration of the endotoxin was used. This observation suggests that other molecular mechanisms, besides activation of the eCS, are involved in LPS-triggered programmed cell death at higher concentrations of LPS. We are, to the best of our knowledge, the first group to report that heparin may decrease the endogenous AEA tone in the murine uterus by increasing uterine FAAH activity. Even though the efficacy of heparin and/or LMWH treatment to women with unexplained recurrent miscarriages has been recently called into question [51], the fact that heparin enhances FAAH activity provides with a new potential therapeutic target for the treatment of this emotionally draining condition.

Collectively, the results presented here provide evidence for a new mechanism involved in heparin anti-apoptotic effect as well as highlight the interest of finding drugs that could enhance FAAH activity and therefore prevent the embryotoxicity associated with high local AEA levels. Therefore, further research on this topic is necessary.

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