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Endocannabinoid System and Nitric Oxide are Involved in the Deleterious Effects of Lipopolysaccharide on Murine Decidua

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ABSTRACT

Endocannabinoids are an important family of lipid-signaling molecules that are widely distributed in mammalian tissues and anandamide (AEA) was the first member identified. The uterus contains the highest concentrations of AEA yet discovered in mammalian tissues and this suggests that it might play a role in reproduction. Previous results from our laboratory have shown that AEA modulated NO synthesis in rat placenta. The production of small amounts of nitric oxide regulates various physiological reproductive processes such as implantation, decidualization and myometrial relaxation. But in an inflammatory setting such as sepsis, NO is produced in big amounts and has toxic effects as it is a free radical. The results presented in this study indicate that LPS-induced NO synthesis and tissue damage were mediated by AEA. Decidual LPS-induced NO production was abrogated either by co-incubation with CB1 (AM251) or CB2 (SR144528) antagonists which suggests that both receptors could be mediating this effect. On the other hand, LPS-induced tissue damage and this deleterious effect was partially abrogated by incubating tissue explants with LPS plus CB1 receptor antagonist. Our findings suggest that AEA, probably by increasing NO synthesis, participates in the deleterious effect of LPS in implantation sites. These effects could be involved in pathological reproductive events such as septic abortion.

1. Introduction

Nitric oxide (NO) plays an important role during pregnancy since the production of small amounts of this mediator regulates various physiological events such as implantation, decidualization, vasodilation of decidual, placental and uterine vessels and myometrial relaxation [1–3]. On the other hand, in an inflammatory setting such as sepsis, NO is produced in big amounts and has toxic effects as it is a free radical [4]. Our previous results indicate that lipopolysaccharide (LPS), an integral part of Gram negative bacteria, is capable of inducing embryonic resorption in mice due to NO increased production both in uterus and decidua [5] and that LPS also stimulates protein nitration [6].

Endocannabinoids are lipid mediators isolated from brain and peripheral tissues [7,8]. A major endocannabinoid, anandamide (AEA), is an endogenous agonist of cannabinoid receptors that binds to both brain (CB1) and peripheral (CB2) cannabinoid receptors, thus mimicking some of the psychotropic, hypnotic, tranquilizing and analgesic effects of THC [8,9]. The uterus contains the highest concentrations of AEA yet discovered in mammalian tissues [11] and this suggests that it might play a role in reproduction. Anandamide can be generated from its membrane precursor *N*-arachidonoyl phosphatidylethanolamine (NAPE) through cleavage by a phospholipase D (NAPE–PLD, one of its synthesizing enzymes). After being transported into the cell, AEA is subsequently broken down into arachidonic acid (AA) and ethanolamine by an endoplasmic reticular integral membrane-bound enzyme called fatty acid amide hydrolase (FAAH) [7]. Uterine AEA levels fluctuate with the state of pregnancy: down-regulation of its levels is associated with uterine receptivity, while up-regulation has been shown to impair pregnancy and embryo development in mice [10] and is correlated with uterine refractoriness to embryo implantation [11]. In addition, Liu et al. have shown that LPS induces AEA synthesis in murine macrophages [12] and also increases NAPE–PLD activity in RAW264.7 cells [12]. Previously results from our laboratory have shown that uterine NO production induced by LPS is modulated by AEA in mice [13]. As we have also shown that AEA modulated NO synthesis in rat placenta [14] the aim of the present study was to examine whether *in vitro* NO production induced by LPS is also regulated by AEA in the murine decidua.

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2. Materials and methods

2.1. Reagents

LPS from *Escherichia coli* 05:B55, nitrate reductase, lactate dehydrogenase, secondary horse radish peroxidase (HRP) conjugated antibody, anti-β-actin antibody, *R*-(+)-methanandamide and Hoescht 33342 were purchased from Sigma Chemical Co.(St Louis, MI, USA). AM251 was purchased from Tocris Cookson Inc.(Ellisville, MO, USA). SR144528 was kindly provided by Sanofi–Aventis. [^{3}H]-anandamide (specific activity 172.4 Ci/mmol) was provided by Perkin Elmer (Boston, MA, USA). TLC aluminum Silica Gel plates were purchased from Merk KGaA (Darmstadt, Germany). The Western blotting reagents were obtained from Bio–Rad. Dulbecco's modified eagle media (DMEM), fetal calf serum (FCS) and antibiotics were purchased from GIBCO (Rockville, MD). A total of 1400 W and NAPE–PLD antibody were purchased from Cayman Chemical (Ann Arbor, MI, USA). The anti-FAAH antibody was a gift from Dr. Benjamin Cravatt. The anti-nitrated tyrosine (NT) residues antibody was purchased from Upstate (Billerica, MA, USA). The anti-iNOS antibody was provided by BD Biosciences (Franklin Lakes, NJ, USA). All other chemicals were analytical grade.

2.2. Animals and treatments

BALB/c 8- to 12-week-old virgin female mice were paired with 8- to 12-week-old BALB/c males, and the day of appearance of a coital plug was taken as day 0 of pregnancy. Animals received food and water *ad libitum* and were exposed to a 14 h light:10 h dark cycle. Mice were sacrificed by cervical dislocation. The experimental procedures reported here were approved by the Animal Care Committee of the Center of Pharmacological and Botanical Studies of the National Research Council (CEFYBO-CONICET) and carried out in accordance with the Guide for Care and Use of Laboratory Animals (NIH).

2.3. Culture of decidual explants from implantation sites

On day 7 (09:00–10:00 h) of pregnancy female mice were sacrificed by cervical dislocation. In each implantation site, the uterus and the decidua were separated. Decidual explants were weighed and cultured in wells that contained 500 μl DMEM supplemented with 10% FCS and antibiotics: 20 iu/ml penicillin G, 20 μg/ml streptomycin and 50 ng/ml amphotericin B. Tissues were maintained for 12 h in 5% CO_2 at 37 °C and then culture supernatants were immediately frozen at −70 °C until used.

2.4. Nitrate and nitrite assay

NO produced by decidua was measured as nitrate (NO_3^-) plus nitrite (NO_2^-) in culture supernatants, using the technique described by Grisham et al. [15]. Briefly, culture supernatants, 2 mM NADPH and 10 U/ml *Aspergillus niger* nitrate reductase were allowed to react in flat-bottomed 96-well culture plates with gentle mixing for 30 min at room temperature. Next, 100 mM pyruvic acid and 1000 U/ml lactate dehydrogenase were added and incubated for 10 min. Later, 10 mg/ml sulphanilic acid was added and incubation continued for 10 min. Finally, 1 mg/ml naphthyl–ethylenediamine was added and incubated for 5 min in the dark. The absorbance of the colored product was measured at 540 nm, using 595 nm readings as reference wavelength to compensate for non-specific absorbance. Media supplemented with FCS were cultured without any tissue and used as a blank. The concentration of NO_3^- plus NO_2^- was deduced from a standard nitrite curve. Results were expressed as μM NO_2^-/mg wet weight.

2.5. Western blot analysis

Tissues were homogenized in an Ultra-Turrax homogenizer in a 20 mM Tris buffer (pH 7.4) containing 0.25 mM sucrose, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mg/ml benzamidine, 1 mg/ml caproic acid, 10 μg/ml soybean trypsinogen inhibitor and 1 M EDTA. Next, samples were sonicated. After centrifugation at 7800 × *g* for 10 min, the supernatants were collected and stored at −70 °C until Western blotting was performed. One hundred micrograms of protein was loaded in each lane. Samples were separated by electrophoresis on 12% sodium dodecyl sulphate-polyacrylamide gel for NAPE–PLD, 10% for FAAH and nitrated proteins (NT), 7.5% for iNOS and transferred to a nitrocellulose membrane. Blots were incubated overnight with anti-FAAH (1:100), anti-iNOS (1:200), anti-NAPE–PLD (1:200) or anti-NT (1:400) and 30 min with anti-actin (1:4000). Blots were washed with buffer (10 mM Tris, 100 mM NaCl and 0.1% (v/v) Tween 20, pH 7.5) followed by 1 h incubation with horse radish peroxidase conjugated anti-rabbit secondary antibody and developed using the enhanced chemiluminescence Western blot system. Photographs of the membranes were taken using a digital camera and analysed using the Image J software package.

2.6. 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:acetic acid: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 [16]. Enzyme activity is reported as nmol [^{3}H]-AA/mg protein/h. The optimal reaction conditions were previously determined (data not shown).

2.7. Histological analyses

After 12 h of incubation with or without LPS 1 μg/ml in the presence/absence of AM251 (10^{-8} M) decidual explants were fixed in 4% paraformaldehyde overnight at 4 °C, dehydrated with 70–100% ethyl alcohol and embedded in paraffin. Microtome sections were stained with hematoxylin-eosin (H&E) and mounted with Permount. Sections were evaluated under microscope and photographed at 400×. In another set of experiments, after 24 h of incubation with or without LPS 1 μg/ml decidua were fixed in 4% paraformaldehyde overnight at 4 °C. The tissues were embedded in paraffin, microtome sections were stained with Hoescht 33342 and H&E and

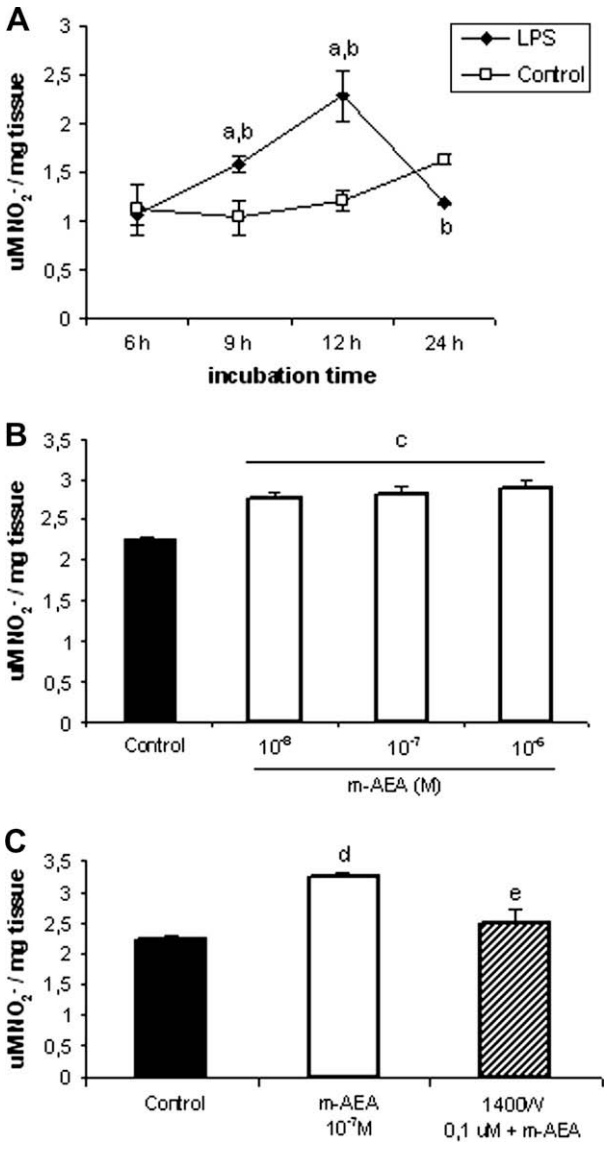

Fig. 1. **(A)** Temporal curve of NO_3^- plus NO_2^- production for decidual explants incubated with LPS 1 μg/ml. ANOVA test. a, $p < 0.05$ vs 6 h; b, $p < 0.05$ vs control. $n = 6$. **(B)** NO_3^- plus NO_2^- production for decidual explants incubated with m-AEA for 12 h. ANOVA test. c, $p < 0.001$ vs control. $n = 5$. **(C)** NO_3^- plus NO_2^- production for decidual explants co-incubated with m-AEA 10^{-7} M and 1400 W 0.1 μM for 12 h. ANOVA test. d, $p < 0.05$ vs control; e, $p < 0.05$ vs m-AEA. $n = 4$.

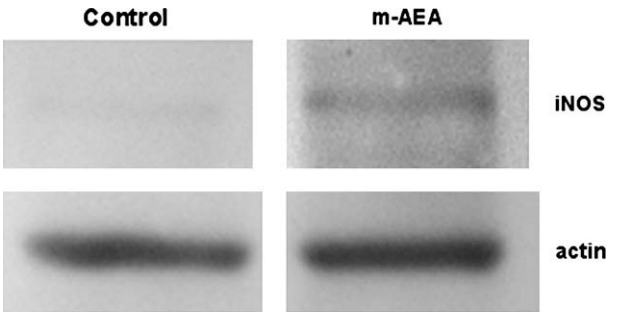

Fig. 2. Protein expression of iNOS in decidual explants incubated with/without m-AEA 10^{-7} M for 12 h. $n = 3$.

mounted with Permount. Sections were evaluated as mentioned above. Scale bar = 100 μm.

2.8. Statistical analyses

Statistical analysis was performed using the Graph Pad Prism Software (San Diego, CA, USA). Comparisons between values of different groups were performed using one-way ANOVA. Significance was determined using Tukey's multiple comparison tests for unequal replicates or Student's *t*-test. All values presented in this study represent means ± SEM. Differences between means were considered significant when p was 0.05 or less.

3. Results

3.1. LPS increased NO_3^- plus NO_2^- levels in a time-dependent manner

In order to determine the optimal culture conditions for the following experiments, a temporal curve of NO_3^- plus NO_2^- production induced by LPS was carried out for decidual explants. Our results show that LPS 1 μg/ml [13] caused an increase in NO production in a time-dependent manner. NO levels reached a maximum after 12 h of incubation with LPS (Fig. 1A) and these conditions were used in subsequent experiments.

3.2. R-(+)-methanandamide increased iNOS expression and NO_3^- plus NO_2^- levels

To evaluate the effect of AEA on NO production, decidual explants were incubated for 12 h with or without methanandamide (m-AEA, stable synthetic AEA analog) and NO_3^- plus NO_2^- levels were assessed. Fig. 1B shows that incubation with m-AEA significantly augmented NO levels after 12 h of incubation with all concentrations assessed. We have previously shown that AEA modulated uterine NO production via inducible NO synthase (iNOS) [13]. To confirm that AEA was modulating decidual NO production via iNOS, decidua were incubated for 12 h in the presence of m-AEA plus 1400 W (a selective iNOS inhibitor). Fig. 1C shows that NO production induced by m-AEA was significantly reduced when tissues were incubated with m-AEA (10^{-7} M) plus 1400 W (0.1 μM). Incubation of decidua with 1400 W alone did not increase NO levels compared to control values (data not shown).

We next evaluated the effect of AEA on iNOS protein expression. Fig. 2 shows that m-AEA (10^{-7} M) augmented iNOS expression in decidual explants.

3.3. Effect of LPS on NO production in the presence of cannabinoid receptor antagonists

Previous results from our laboratory showed the presence of CB1 and CB2 receptors in the murine uterus [17] and in rat placenta [14]. Since m-AEA was capable of inducing NO production, we examined whether LPS-induced NO production was mediated by AEA. Thus, decidual explants were incubated in the presence or absence of LPS (1 μg/ml), LPS plus AM251 (CB1 receptor antagonist) or LPS plus SR144528 (CB2 receptor antagonist). We observed that co-incubation with AM251 or with SR144528 significantly diminished LPS-induced NO synthesis (Figs. 3A,B).

3.4. LPS increased FAAH activity and NAPE–PLD and FAAH protein expression

Liu et al. [12] have shown that LPS up-regulates FAAH mRNA expression and FAAH activity. Thus, to determine whether LPS could modulate FAAH in the decidua we assessed FAAH activity and protein levels in decidual explants. Tissues incubated with LPS showed a significant increase in FAAH activity (Fig. 4A).

Western blot analysis showed an immunoreactive band of the molecular size expected for FAAH (approx. 60 Kd), the intensity of which was higher in LPS-treated explants (Fig. 4B).

On the other hand, Guo et al. [18] proposed that NAPE–PLD is an important determinant of AEA uterine levels. So, we decided to evaluate the effect of LPS on NAPE–PLD by assessing NAPE–PLD protein expression in decidual explants incubated with/without LPS 1 μg/ml. Our results showed that LPS-induced a significant increase in NAPE–PLD protein expression (Fig. 4C).

3.5. LPS-induced tissue damage and protein nitration

As shown in Fig. 1, decidual NO production fell after 12 h of incubation with LPS reaching values below those of control explants. This could be due to the fact that at high concentrations NO affects tissue viability probably through peroxinitritate ($ONOO^-$), a powerful oxidant with diverse deleterious effects

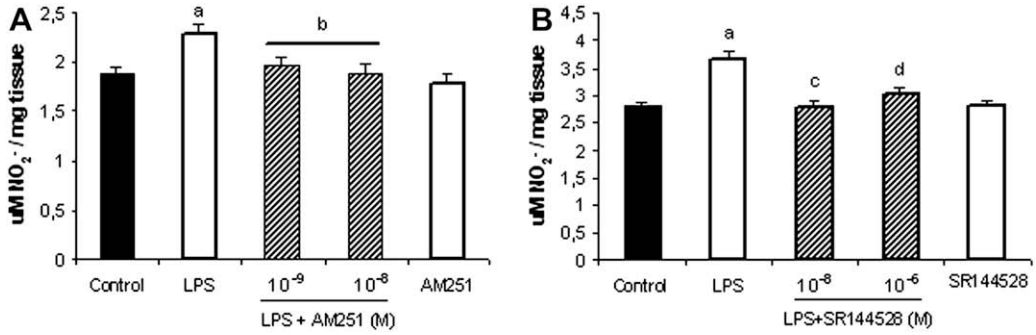

Fig. 3. (A) NO_3^- plus NO_2^- production for decidual explants co-incubated with LPS 1 μg/ml and AM251 for 12 h. ANOVA test. a, $p < 0.01$ vs control; b, $p < 0.05$ vs LPS. $n = 5$. **(B)** NO_3^- plus NO_2^- production for decidual explants co-incubated with LPS and SR144528 for 12 h. ANOVA test. a, $p < 0.01$ vs control; c, $p < 0.01$ vs LPS; d, $p < 0.001$ vs LPS. $n = 5$.

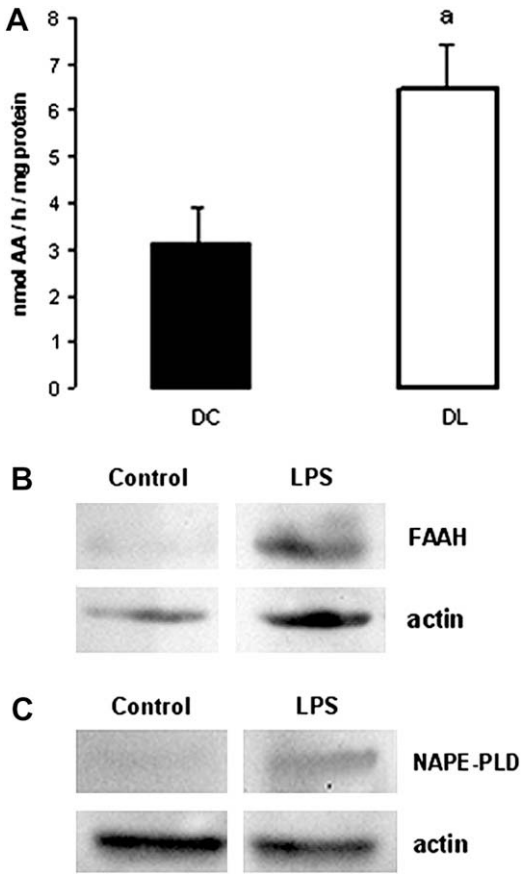

Fig. 4. **(A)** FAAH activity in decidual explants after incubation without (C)/with (LPS) LPS 1 μg/ml. Student's *t*-test. a, $p < 0.05$ vs DC. $n = 5$. **(B)** Protein expression of FAAH in decidual explants incubated without/with LPS 1 μg/ml for 12 h. **(C)** Protein expression of NAPE–PLD in decidual explants incubated without/with LPS 1 μg/ml for 12 h.

including nitration of tyrosine (Tyr) residues on proteins thus altering their function. Then, we analyzed histological differences between treatments at 24 h of incubation with/without LPS by staining decidual explants with Hoescht 33342 and H&E. Decidua treated with LPS revealed an increase of cellular density (nuclear condensation), fragmentation, chromatin condensation as well as small nuclear apoptotic bodies (Figs. 5A.b,A.d). We also analyzed by immunoblotting the nitration of Tyr residues on decidual proteins at 6, 12 and 24 h culture in the presence of LPS 1 μg/ml. Figs. 5B,C illustrates NO_2Tyr immunoreactivity in decidual explants which increased in the presence of LPS in a time-dependent manner.

3.6. AEA mediated LPS-induced tissue damage

As we have shown that LPS-induced protein nitration and tissue damage in decidua, we evaluated if the CB1 antagonist (AM251) was able to block the deleterious effect of LPS on this tissue. Thus, we analyzed histological differences between treatments at 12 h of incubation with/without LPS in the presence/absence of AM251 by staining decidual explants with hematoxylin-eosin. Control decidua after 12 h of incubation showed normal intact cell outline with preserved nuclear and cytoplasmic structure (Fig. 6A). Decidua treated with LPS (Fig. 6B) showed abnormal tissue spaces, cellular disruption and cell shrinking. Fig. 6C showed that LPS-induced tissue damage was partially reversed by AM251 in decidual explants.

4. Discussion

The results presented in this study indicate that AEA modulated NO production induced by LPS in decidual explants cultured *in vitro*. In accordance with our previous results [5], LPS-induced NO production in murine decidua. NO levels reached a maximum after 12 h of incubation with LPS (Fig. 1A). However, decidual NO production fell after 24 h of incubation with the endotoxin reaching lower values than control explants. Previous results of our laboratory showed that LPS receptor (CD14) was abundant in the decidua

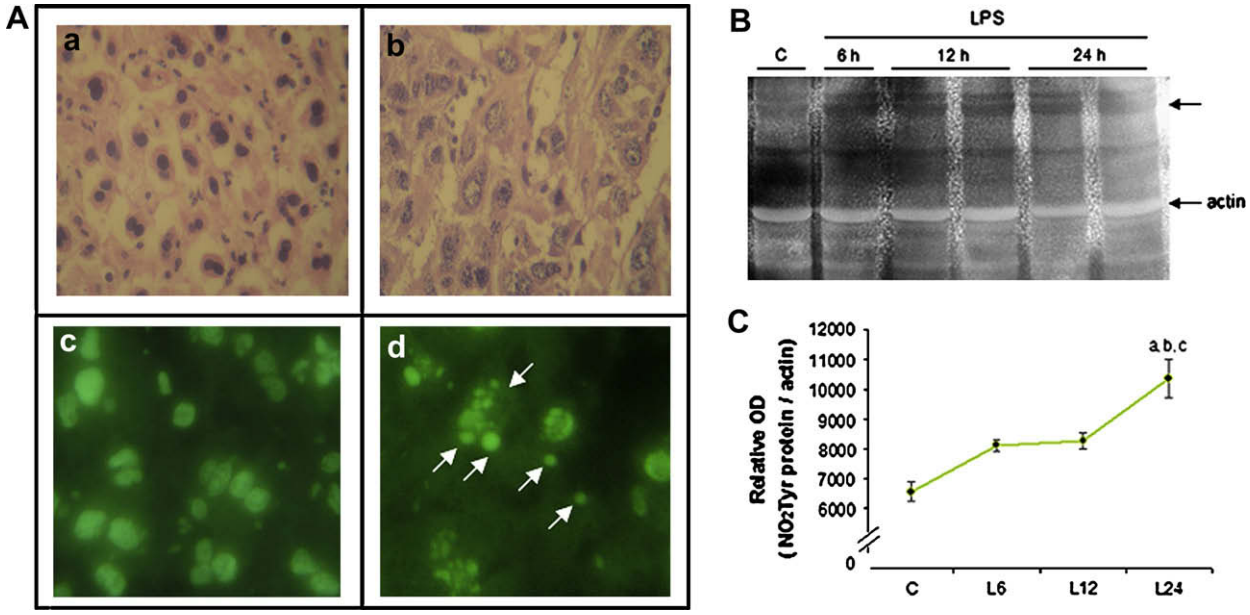

Fig. 5. **(A)** Histological analysis of decidual explants. *Hematoxylin-eosin staining:* A, control and B, LPS (1 μg/ml, 24 h of incubation). *Hoescht staining:* C, control and D, LPS (1 μg/ml, 24 h of incubation). Sections were evaluated under optical microscope and photographed at 400×. Arrows indicate apoptotic bodies. **(B)** Western blot analysis of nitrated proteins. **(C)** Densitometric analysis of bands obtained was carried out using Image J. Arbitrary density units expressed as percentages relative to actin values were plotted. ANOVA test. a, $p < 0.001$ vs control; b, $p < 0.05$ vs L6; c, $p < 0.05$ vs L12. $n = 3$.

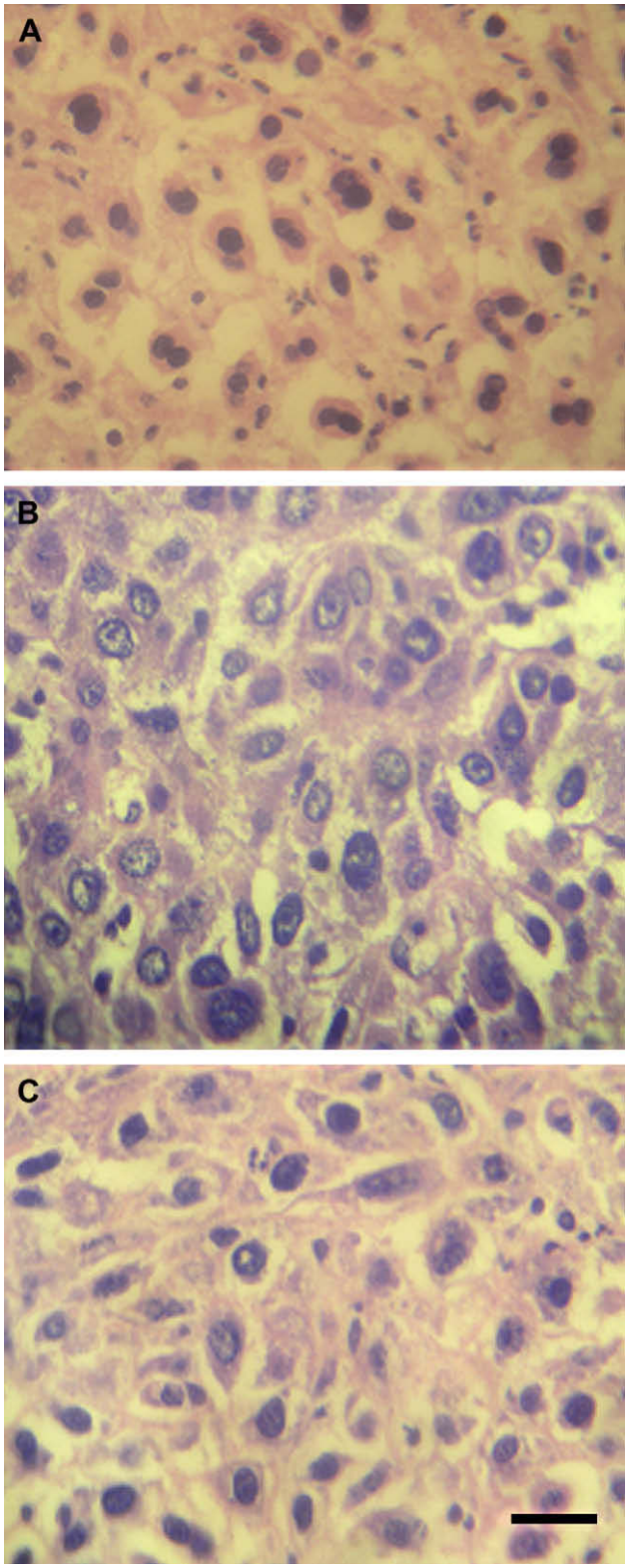

Fig. 6. Histological analysis of decidual explants. *Hematoxylin-Eosin staining:* A, control; B, LPS (1 μg/ml, 12 h of incubation); C, LPS 1 μg/ml + AM251 (10^{-6} M, 12 h of incubation). Sections were evaluated under optical microscope and photographed at 400×. Scale bar = 100 μm.

and endometrial glands [5]. This high expression may explain the increased sensitivity of the decidua to LPS when compared to the uterus [13]; thus, production of NO 24 h after incubation with LPS is low possibly as a result of decidua necrosis. In fact, Fig. 5A not only showed that decidua treated with LPS were necrotized but also showed the presence of apoptotic bodies. Increased NO production may have pathological effects presumably through the generation of peroxinitrite radicals. Furthermore, the •NO-dependent nitration of protein tyrosine (Tyr) residues to 3-nitrotyrosine (NO_2Tyr) increases during oxidative inflammatory conditions [10]; in this work we observed that decidual proteins are nitrated and this fact could be partially responsible for the diminished production of NO at 24 h of incubation with the endotoxin (Figs. 5B,C).

On the other hand, when decidual explants were incubated in the presence of m-AEA, this stable synthetic AEA analog was able to induce NO production (Fig. 1B) and iNOS protein expression (Fig. 2). To confirm that AEA was modulating NO levels via iNOS, decidua were incubated in the presence of m-AEA plus 1400 W (a selective iNOS inhibitor). Fig. 1C shows that NO production induced by m-AEA was totally reduced by co-incubation of m-AEA with 1400 W.

It has been reported that AEA is likely to play an important role in the control of vascular tone, as supported by the observation that both rat endothelial cells and macrophages can release it [19,20]. A paracrine mechanism of vasodilation in LPS-induced endotoxic shock has been documented in rats [21] where macrophage-derived AEA seems to be responsible for the activation of vascular CB1 receptors. Maccarrone et al. [22] have also reported that activation of CB1 cannabinoid receptors by AEA causes a stimulation of human umbilical vein endothelial cells (HUVEC) inducible NO synthase activity and expression. All this evidence led us to hypothesize that a similar mechanism may be involved in decidual LPS-induced NO production.

Previous results from our laboratory showed the presence of CB1 and CB2 receptors in the murine uterus [17] and in rat placenta [14]. Since m-AEA was also capable of inducing NO production, we examined whether LPS-induced NO production was mediated by AEA. Our results showed that decidual LPS-induced NO production was abrogated either by co-incubation with CB1 (AM251) or CB2 (SR144528) antagonists (Fig. 3), which suggests that both receptors could be mediating this effect.

It is known that high levels of AEA impair pregnancy and embryo development in mice [10] and are correlated with uterine refractoriness to embryo implantation [11]. Also, Maccarrone et al. [23] have demonstrated that LPS enhances the levels of AEA in human peripheral lymphocytes. Liu et al. [12] have shown that LPS not only induces AEA synthesis in RAW264.7 cells but also increases NAPE–PLD activity. Guo et al. [18] proposed that NAPE–PLD is an important determinant of AEA uterine levels. So, we evaluated the contribution of LPS to AEA production by assessing the protein expression of NAPE–PLD and we found that the endotoxin was able to induce a significant increase in NAPE–PLD expression in decidual explants (Fig. 4C).

Liu et al. [12] have also shown that LPS up-regulates FAAH mRNA expression and FAAH activity. Regarding this data, we analyzed whether LPS could modulate FAAH activity in decidual explants. Here, we described for the first time the *in vitro* degradation of AEA by the decidua where LPS treatment increased FAAH activity and protein expression at 12 h of incubation (Figs. 4A,B). These data suggest that LPS could be modulating not only AEA synthesis but also its degradation. This is different from previous results from our laboratory where uterine explants treated with LPS decrease rather than increase FAAH activity and expression [13]. This has also been reported in human lymphocytes, where much higher concentrations of LPS (10–100 μg/ml) also decreased FAAH activity and expression [23]. Liu et al. [12] have shown that LPS-induced an increase of AEA levels and also up-regulated FAAH protein expression and activity. And this fact was in good agreement with the potentiation of the LPS-induced increase of AEA in FAAH −/− macrophages that the authors showed in that work [12]. In fact, although FAAH activity was increased he showed that AEA was still

capable of inducing a significant hypotensive response [12], maybe because he also reported an increase in the conversion of NAPE to AEA. The mechanism of up-regulation of FAAH expression and activity must be investigated in further studies.

In mice the endometrial stroma undergoes dramatic cytologic changes to form a decidua in response to the implanting embryo. After attachment, the embryo becomes embedded in an enlarging mass of decidual tissue which remains very close to uterine tissue. Such proximity allows molecules to flow freely from one tissue to the other. We have previously seen that LPS inhibited FAAH activity and increase NAPE–PLD expression in uterine explants [13]. In this sense, we proposed that LPS could be increasing AEA levels by inhibiting FAAH activity and also augmenting NAPE–PLD expression in this tissue. If there is a free flow of AEA between uterus and decidua we hypothesize that, even though LPS increased decidual FAAH activity, remaining AEA levels could still be exerting some effect on NO production.

It is worth notice that LPS-induced tissue damage and this deleterious effect was partially abrogated by incubating tissue explants with LPS plus CB1 receptor antagonist (Fig. 6). Since decidual LPS-induced NO production was abrogated by co-incubation with AM251 or SR144528 CB receptor antagonists, the contribution of CB2 receptor to LPS-induced tissue damage should be assessed. These findings suggest that AEA, probably by increasing NO synthesis, participates in the deleterious effect of LPS in implantation sites. The fact that LPS increased protein nitration could contribute to this hypothesis.

Contrary to the known anti-inflammatory, immunomodulatory and protective effects of AEA against inflammation [24,25], in this study AEA seems to act as a pro-inflammatory molecule modulating the production of NO induced by LPS. Little is known about the mechanisms by which AEA affects this process and thus further investigation should be made.

In conclusion, we showed that LPS-induced NO synthesis and tissue damage involve the participation of AEA. This pro-inflammatory effect of AEA may be implicated in pathological reproductive events such as septic abortion.

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