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Cyclooxygenase-2 prostaglandins mediate anandamide-inhibitory action on nitric oxide synthase activity in the receptive rat uterus

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ABSTRACT

Anandamide, an endocannabinoid, prostaglandins derived from cyclooxygenase-2 and nitric oxide synthesized by nitric oxide synthase (NOS), are relevant mediators of embryo implantation. We adopted a pharmacological approach to investigate if anandamide modulated NOS activity in the receptive rat uterus and if prostaglandins mediated this effect. As we were interested in studying the changes that occur at the maternal side of the fetal–maternal interface, we worked with uteri obtained from pseudopregnant rats. Females were sacrificed on day 5 of pseudopregnancy, the day in which implantation would occur, and the uterus was obtained. Anandamide (2 ng/kg, i.p.) inhibited NOS activity ($P<0.001$) and increased the levels of prostaglandin E_2 ($P<0.001$) and prostaglandin $F_{2\alpha}$ ($P<0.01$). These effects were mediated via cannabinoid receptor type 2, as the pre-treatment with SR144528 (10 mg/kg, i.p.), a selective cannabinoid receptor type 2 antagonist, completely reverted anandamide effect on NOS activity and prostaglandin levels. The pre-treatment with a non-selective cyclooxygenase inhibitor (indomethacin 2.5 mg/kg, i.p.) or with selective cyclooxygenase-2 inhibitors (meloxicam 4 mg/kg, celecoxib 3 mg/kg, i.p.) reverted anandamide inhibition on NOS, suggesting that prostaglandins are derived from cyclooxygenase-2 mediated anandamide effect. Thus, anandamide levels seemed to modulate NOS activity, fundamental for implantation, via cannabinoid receptor type 2 receptors, in the receptive uterus. This modulation depends on the production of cyclooxygenase-2 derivatives. These data establish cannabinoid receptors and cyclooxygenase enzymes as an interesting target for the treatment of implantation deficiencies.

1. Introduction

Implantation is initiated when embryonic development is synchronized with the appropriate preparation of the uterus. The quality of implantation determines the quality of pregnancy and fetal well-being. Failure to achieve 'on-time' implantation risks pregnancy outcome.

Anandamide (N-arachidonoylethanolamine), an endocannabinoid, is a ligand for the cannabinoid receptors cannabinoid receptor type 1 and cannabinoid receptor type 2 (Mechoulam et al., 1998). Down-regulation of uterine anandamide and blastocyst cannabinoid receptor type 1 levels with the onset of implantation suggests a role in modulating the implantation window (Lim et al., 2002). Although low doses of anandamide are stimulatory, high doses inhibit blastocyst growth, implying regulated endocannabinoid signaling during implantation (Lim et al., 2002; Paria et al., 2001). In fact, it has been reported higher anandamide levels in the blood of nonpregnant compared to pregnant women after undergoing in vitro fertilization and embryo transfer (Maccarrone et al., 2002).

Nitric oxide synthase (NOS) activity is present in the uterus (Yallampalli et al., 1993) and seems to be relevant during implantation (Biswas et al., 1998; Novaro et al., 1997). We observed that anandamide modulates nitric oxide levels in the rat placenta (Cella et al., 2008) and in the rodent deciduas treated with lipopolysaccharide (Vercelli et al., 2009). We have recently published that anandamide levels seem to modulate NOS activity in an in vitro model of implantation and that this modulation depends on the presence of the blastocyst (Sordelli et al., 2011). Based on these evidences we became interested in investigating the in vivo crosstalk between anandamide and nitric oxide at the maternal side of the fetal–maternal interface. Thus, we studied if the administration of anandamide modulates NOS activity in the rat uterus at the time of implantation. Prostaglandins play critical roles in female reproduction. The liberation of arachidonic acid followed by the action of cyclooxygenases (COX-1 or COX-2) generates prostaglandins. Prostaglandins derived from COX-2 are the most relevant (Bonventre et al., 1997; Lim et al., 1997). COX-2 is restricted to the implantation site in most species studied and COX-$2^{-/-}$ mice have defective implantation and decidualization (Lim et al., 1997, 2002). We (Aisemberg et al., 2007; Cella et al., 2006; Ribeiro et

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al., 2003, 2004) and others (Novaro et al., 1996; Shirasuna et al., 2008; Väisänen-Tommiska et al., 2005) have previously reported that COX derivatives regulate NOS activity and expression in the rodent uterus and other reproductive systems. Besides, some authors have recently shown that anandamide is capable of modulating the production of prostaglandin (PG) E_2, PGD_2 and $PGF_{2\alpha}$ (Mitchell et al., 2008; Navarrete et al., 2009; Van Dross, 2009). Thus, the second aim of this work was to investigate if prostaglandins mediate the effect of anandamide on NOS activity during implantation in the receptive rat uterus. The finding that anandamide regulated NOS activity through COX-2 derived prostaglandins contributes to better understand the significance of these mediators as possible effectors that coordinate the series of events at the maternal side of the fetal–maternal interface during implantation and that finally would lead to a successful pregnancy.

2. Materials and methods

2.1. Ethics statement

The experimental procedures reported here were approved by the Animal Care Committee of the Centro de Estudios Farmacológicos y Botánicos (CEFYBO-CONICET) and by the Institutional Committee for the Care and Use of Laboratory Animals, permit number: 2550/2010 (CICUAL, Comité Institucional para el Cuidado y Uso de Animales de Laboratorio) from the Facultad de Medicina (Universidad de Buenos Aires), and were carried out in accordance with the Guide for Care and Use of Laboratory Animals (NIH). All animals were provided by the animal facility of the Facultad de Odontología (Universidad de Buenos Aires).

2.2. Animals

Female rats of the Wistar strain were housed in group cages under controlled conditions of light (12 h light, 12 h dark) and temperature (23–25 °C). Animals received food and water ad libitum. Where mentioned, animals were sacrificed in a carbon dioxide chamber and all efforts were made to minimize suffering.

We were interested in studying the changes that occur at the maternal side of the fetal–maternal interface without the influences of the embryos. Thus, we decided to work with the uterus obtained from pseudopregnant females because in this model, the uterus undergoes all the normal changes that prepare it for implantation, but no embryos are present in the uterine lumen. Pseudopregnancy could be induced in female rats treated with equine chorionic gonadotrophin (PMSG). Prepuber rats (25–28 days of age) received 50 IU PMSG i.p. (Lahav et al., 1989; Ribeiro et al., 2009). PMSG was dissolved in saline. Day 1 of pseudopregancy was considered 24 h after the injection. Females were sacrificed at day 5 of pseudopregnancy, which mimics the day of implantation, and the uterus was obtained.

Where mentioned, pseudopregnant rats were treated in vivo with different cannabinoid receptor agonists (anandamide) and antagonists (SR141716A or SR144528) and with selective (celecoxib, meloxicam) and non-selective (indomethacin) COX inhibitors. All drugs were administrated i.p. in a final volume of 200 µl. Control animals received 200 µl of vehicle i.p.

2.3. Total NOS enzyme assay

NOS enzyme activity was quantified by the modified method of Bredt and Snyder (1989) which measures the conversion of [^{14}C]-L-arginine into [^{14}C]-L-citrulline. Nitric oxide and L-citrulline are produced in equimolar amounts.

Uterine slices were weighted, homogenized (Ultra Turrax, T25 basic, IKA Labortechnik) and incubated at 37 °C in a HEPES buffer (20 mM HEPES, 25 mM L-valine, 0.45 mM $CaCl_2$, 100 mM DTT) containing 0.6 µCi/ml [^{14}C]-L-arginine and 0.5 mM NADPH.

After 15 min of incubation, samples were centrifuged for 15 min at 12,000 *g*. They were then applied to a 1 ml DOWEX AG500-X column (Na^+-form) and [^{14}C]-L-citrulline was eluted in 2.5 ml of distilled water. The [^{14}C]-L-citrulline radioactivity was measured by liquid scintillation counting. Protein concentration was determined by the Bradford method (Bradford, 1976). Enzyme activity was expressed as pmol L-citrulline/mg protein/h.

2.4. Prostaglandin radioimmunoassay

Uterine horns were incubated in Krebs–Ringer bicarbonate modified solution (Ribeiro et al., 2003) at 37 °C for 1 h in a 95% O_2/5% CO_2 atmosphere. Afterward, medium was acidified to pH = 3 with 1 M HCl in ethyl acetate and extracted twice. Pooled ethyl acetate extracts were dried. $PGF_{2\alpha}$ and PGE_2 concentrations were determined by radioimmunoassay (Campbell and Ojeda, 1987). The $PGF_{2\alpha}$ and PGE_2 antiserums were highly specific and showed low crossreactivity. Sensitivity was 5–10 pg per tube and $ka = 1.5 \times 10^{10}$ l/mol. Protein concentration was determined by the Bradford method (Bradford, 1976). Values were expressed as pg of $PGF_{2\alpha}$ or PGE_2/mg protein/h.

2.5. Determination of fatty acid amide hydrolase (FAAH) activity

FAAH (EC 3.5.1.4) activity was assayed as previously described by Paria et al. (1996) and by our laboratory (Vercelli et al., 2009). Briefly, uterine slices were weighted, homogenized (Ultra Turrax, T25 basic, IKA Labortechnik) and sonicated in buffer Tris–HCl 10 mM and EDTA 1 mM (pH = 7.6). Homogenates (100 µg) were incubated at 37 °C for 15 min in 200 µl of 50 mM Tris buffer (pH = 8.5) containing 100 µM [^{3}H]-anandamide (172.4 Ci/mmol, 100 µCi/ml). The reactions were terminated by the addition of chloroform:methanol (1:1 v/v). The aqueous phase was extracted twice with chloroform and pooled extracts were dried. Dried samples were resuspended in chloroform and seeded in a TLC plate. The hydrolyzed [^{3}H]-arachidonic acid 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 arachidonic acid. 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 arachidonic acid was calculated and expressed as a percentage of the total radioactivity of the plates. Protein concentration was determined by the method of Bradford (1976). Enzyme activity is reported as nmol [^{3}H]-arachidonic acid/mg protein/h. The optimal reaction conditions were previously determined (data not shown).

2.6. Statistical analysis

Statistical analysis was performed using the GraphPad Prism Software (San Diego, CA, USA). Comparisons between values of different groups were performed using one way ANOVA (analyze of variance). Significance was determined using Tukey's multiple comparison tests for unequal replicates. A number of 4–6 animals were used for each treatment. All values presented in this study represent means ± S.E.M. Differences between means were considered significant when P was 0.05 or less.

2.7. Drugs and chemicals

[^{14}C]-L-arginine monohydrochloride (specific activity 317 mCi/mmol), [5, 6, 8, 9, 11, 12, 14, 15(n)-[^{3}H]-$PGF_{2\alpha}$] (160 Ci/mmol) and [5, 6, 8, 9, 11, 12, 14, 15(n)-[^{3}H]-PGE_2] (130 Ci/mmol) were provided by Amersham Corporation (Arlington Heights, IL, USA). [^{3}H]-anandamide (specific activity 172.4 Ci/mmol) and Optiphase-3 scintillation solution were provided by Perkin Elmer (ETC, Buenos Aires, Argentina). TLC

aluminum silica gel plates were purchased from Merck KGaA (Darmstadt, Germany). Anandamide, $PGF_{2\alpha}$ and PGE_2 antiserums, NADPH, HEPES, valine and Dowex AG500-X column (Na^+-form) were purchased from Sigma Chemical Company (Buenos Aires, Argentina) and Bio Rad (Tecnolab, Buenos Aires, Argentina). Meloxicam was purchased from Boehringer Ingelheim (Argentina), celecoxib was purchased from Panalab S.A. (Argentina) and indomethacin was from Montpellier (Argentina). PMSG (equine chorionic gonadotrophin, NOVORMON®) was kindly provided by Syntex S.A. (Buenos Aires, Argentina). Cannabinoid receptor type 1 selective antagonist (SR141716A, N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole carboxamide) (Coutts et al., 2000) and cannabinoid receptor type 2 selective antagonist (SR144528, N-[1(S)-endo-1,3,3-trimethyl-bicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methyl-benzyl)-pyrazole-3-carboxamide) (Portier et al., 1999) were kind gifts from Sanofi-Aventis Recherche (Montpellier, France). All other chemicals were of analytical grade.

3. Results

First, we were interested in studying if the in vivo administration of anandamide regulated NOS activity in the receptive rat uterus. In order to determine optimal treatment conditions, three doses of anandamide were tested. Day 5 pseudopregnant rats were injected with 2 ng/kg, 0.2 μg/kg and 2 μg/kg of anandamide i.p. and were sacrificed 5 and 15 min later. The time of sacrifice was selected due to anandamide short half life (Willoughby et al., 1997). Treatment with anandamide 2 ng/kg for 15 min significantly inhibited the activity of NOS enzyme (Fig. 1). The administration of 0.2 μg/kg and 2 μg/kg of anandamide for 15 min did not exert any effect. The treatment with anandamide 2 ng/kg, 0.2 μg/kg and 2 μg/kg for 5 min did not modify NOS activity (Fig. 1). Thus, in subsequent experiments day 5 pseudopregnant rats received anandamide 2 ng/kg i.p. and were sacrificed 15 min later.

To study which receptors mediated anandamide effect in the receptive rat uterus, animals were pre-treated with SR141716A (a cannabinoid receptor type 1 selective antagonist) or SR144528 (a cannabinoid receptor type 2 selective antagonist) at three different doses. After 30 min, pseudopregnant rats were injected with anandamide 2 ng/kg i.p. and the animals were sacrificed 15 min later. Based on both binding and functional data, SR141716A and SR144528 at the selected doses are highly potent and selective antagonists for the cannabinoid receptor type 1 and cannabinoid receptor type 2 receptors respectively (Rinaldi-Carmona et al., 1995, 1998). Nor SR141716A neither SR144528 alone at any of the doses tested presented any effect on NOS activity (Table 1). SR141716A 0.3, 3 and 10 mg/kg i.p., the cannabinoid receptor type 1 selective antagonist, did not modify anandamide action (Fig. 2A). However, SR144528 10 mg/kg i.p., the cannabinoid receptor type 2 selective antagonist, completely reverted anandamide inhibitory effect on NOS activity (Fig. 2B). Thus, anandamide inhibited NOS activity through cannabinoid receptor type 2 receptors in the receptive rat uterus at the time of implantation.

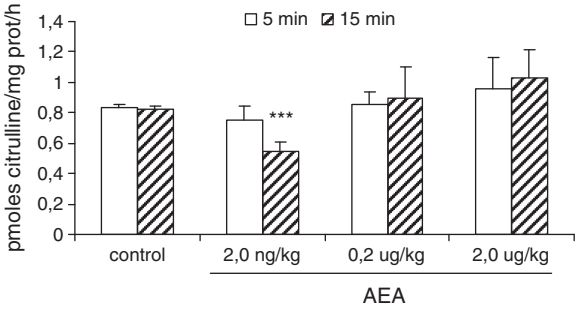

Fig. 1. Anandamide inhibited uterine NOS activity on day 5 of pseudopregnancy. Rats on day 5 of pseudopregnancy were injected i.p. with different doses of anandamide (AEA) and sacrificed 5 and 15 min later. NOS activity is expressed as pmoles citrulline/mg prot/h. ***: P<0.001 vs the rest.

Table 1
Effect of selective cannabinoid receptors antagonists on uterine NOS activity. Rats on day 5 of pseudopregnancy were injected i.p. with SR141716A 10 mg/kg, a selective cannabinoid receptor type 1 antagonist, or with SR144528 10 mg/kg, a selective cannabinoid receptor type 2 antagonist, and sacrificed 30 min later.

	NOS activity pmoles citrulline/mg prot/h
Control	0.84 ± 0.05
SR141716A 10 mg/kg	0.88 ± 0.02
SR144528 10 mg/kg	0.76 ± 0.02

Afterward, we investigated if prostaglandins mediated anandamide-inhibitory effect on NOS activity at the time of implantation. To address this issue, first we analyzed if the administration of anandamide modulated the production of prostaglandins in our model. Day 5 pseudopregnant rats treated with anandamide 2 ng/kg i.p. and sacrifice 15 min later, showed an increase in the production of PGE_2 (Fig. 3A) and $PGF_{2\alpha}$ (Fig. 3B) in the receptive rat uterus. Interestingly, while the pre-treatment with a cannabinoid receptor type 2 selective antagonist SR144528 10 mg/kg i.p. completely reverted anandamide-stimulatory effect on both prostaglandins, the pre-treatment with SR141716A 10 mg/kg i.p., the cannabinoid receptor type 1 selective antagonist, did not exert any effect (Fig. 3A and B).

In order to investigate if prostaglandins mediated the effect of anandamide on NOS activity, day 5 pseudopregnant rats were pre-treated with a non-selective COX inhibitor (indomethacin 2.5 mg/kg, i.p.) or selective COX-2 inhibitors (celecoxib 3 mg/kg, meloxicam 4 mg/kg, i.p.). After 2 h animals received anandamide 2 ng/kg i.p. and were sacrificed 15 min later. Both the non-selective COX inhibitor (indomethacin) and selective COX-2 inhibitors (celecoxib or meloxicam) completely reverted

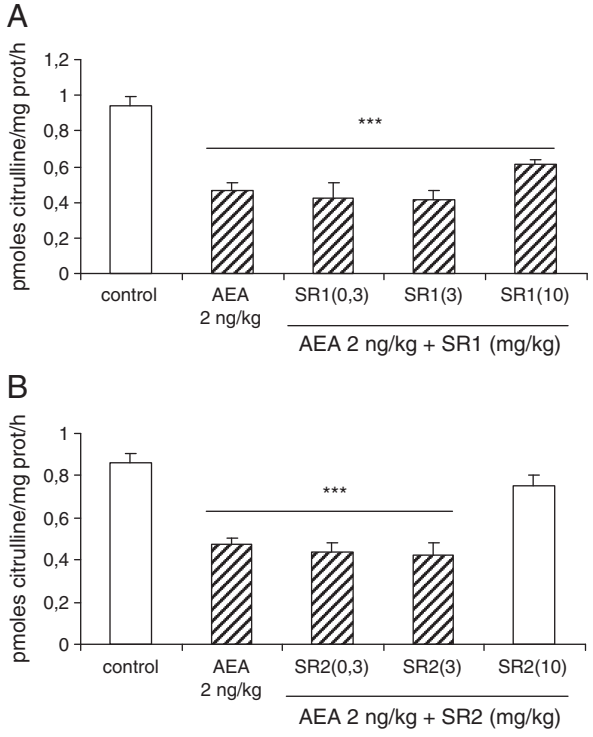

Fig. 2. Cannabinoid receptor type 2 mediated anandamide-inhibitory action on uterine NOS activity. Rats on day 5 of pseudopregnancy were pre-treated i.p. with SR141716A (SR1), a selective cannabinoid receptor type 1 antagonist (A, ***P<0.001 vs the rest), or with SR144528 (SR2), a selective cannabinoid receptor type 2 antagonist (B, ***P<0.001 vs the rest), in different doses. After 30 min, animals were injected i.p. with anandamide (AEA) 2 ng/kg and sacrificed 15 min later. NOS activity is expressed as pmoles citrulline/mg prot/h.

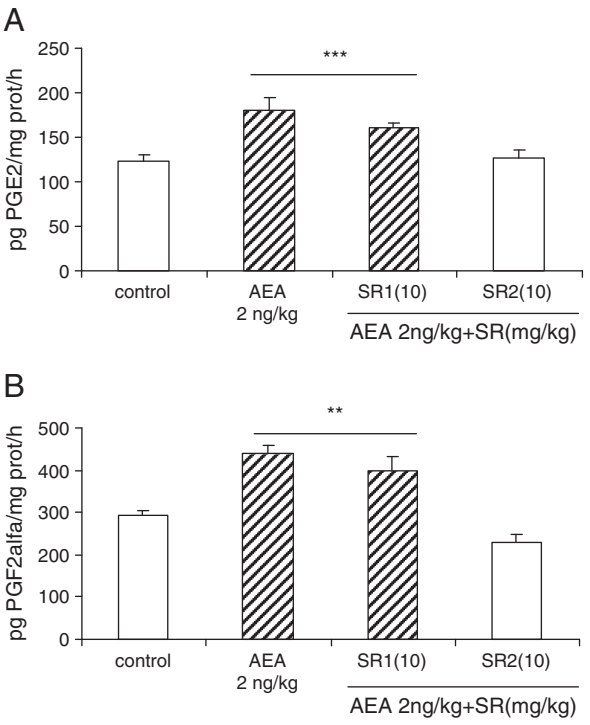

Fig. 3. Anandamide increased uterine levels of PGE_2 and $PGF_{2\alpha}$ through cannabinoid receptor type 2 on day 5 of pseudopregnancy. Rats on day 5 of pseudopregnancy were pre-treated i.p. with SR141716A 10 mg/kg (SR1), a selective cannabinoid receptor type 1 antagonist, or with SR144528 10 mg/kg (SR2), a selective cannabinoid receptor type 2 antagonist. After 30 min, animals were injected i.p. with anandamide (AEA) 2 ng/kg and sacrificed 15 min later. The level of prostaglandin E_2 (PGE_2, A) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, B) was determined by radioimmnunoassay and expressed as pg PGE_2 or $PGF_{2\alpha}$/mg prot/h. ***: $P<0.001$ vs the rest, **: $P<0.01$ vs the rest.

anandamide-inhibitory effect on NOS activity (Fig. 4). This result suggests that COX-2 derived prostaglandins mediated the inhibitory effect of anandamide on the activity of NOS enzyme.

The doses and times of administration of COX inhibitors were selected based on a previously published paper from our laboratory (Aisemberg et al., 2007). To corroborate that these drugs were effective in inhibiting prostaglandins production in our system, pseudopregnant rats were injected with the non-selective COX inhibitor (indomethacin 2.5 mg/kg, i.p.) or selective COX-2 inhibitors (celecoxib 3 mg/kg, meloxicam 4 mg/kg, i.p.) and 2 h later received anandamide 2 ng/kg i.p. Animals were sacrificed 15 min later and PGE_2 and $PGF_{2\alpha}$ were measured. As shown in Table 2, the pre-treatment with indomethacin, celecoxib or meloxicam significantly inhibited anandamide-stimulated

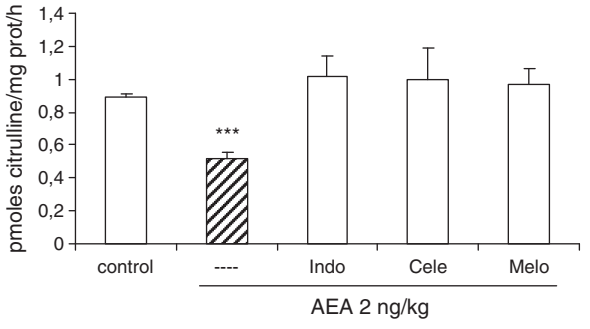

Fig. 4. Cyclooxygenase-2 prostaglandins mediated anandamide-inhibitory action on uterine NOS activity. Rats on day 5 of pseudopregnancy were pre-treated i.p. with a non selective cyclooxygenase inhibitor, indomethacin (Indo) 2.5 mg/kg, or with selective cyclooxygenase-2 inhibitors, celecoxib (Cele) 3 mg/kg or meloxicam (Melo) 4 mg/kg. After 2 h, animals were injected i.p. with anandamide (AEA) 2 ng/kg and sacrificed 15 min later. NOS activity is expressed as pmoles citrulline/mg prot/h. ***: $P<0.001$ vs the rest.

Table 2
Effect of cyclooxygenase inhibitors on anandamide-stimulatory effect on uterine prostaglandin production. Rats on day 5 of pseudopregnancy were pre-treated i.p. with a non selective cyclooxygenase inhibitor, indomethacin (Indo), or with selective cyclooxygenase-2 inhibitors, celecoxib (Cele) or meloxicam (Melo). After 2 h, animals were injected i.p. with anandamide (AEA) and sacrificed 15 min later. The level of prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was determined by radioimmunoassay.

	pg PGE_2/mg prot/h	pg $PGF_{2\alpha}$/mg prot/h
Control	122 ± 8	294 ± 10
AEA 2 ng/kg	$181 \pm 14^{*}$	$440 \pm 20^{**}$
AEA 2 ng/kg + Indo 2.5 mg/kg	100 ± 11	200 ± 30
AEA 2 ng/kg + Cele 3 mg/kg	97 ± 15	220 ± 40
AEA 2 ng/kg + Melo 4 mg/kg	110 ± 20	250 ± 50

* $P<0.001$ vs control.
** $P<0.01$ vs control.

production of PGE_2 and $PGF_{2\alpha}$ in the receptive rat uterus. It has been published that COX inhibitors could modify the activity of fatty acid amide hydrolase (FAAH), the enzyme that degrades anandamide, in certain biological systems (Bishay et al., 2010; Holt et al., 2007). In order to discard that selective and non-selective COX inhibitors were exerting their effect also on FAAH activity and thus modifying the level of anandamide in our system, day 5 pseudopregnant rats were injected with indomethacin 2.5 mg/kg i.p. or meloxicam 4 mg/kg i.p. alone, and the activity of FAAH was measured in the receptive uterus. We observed that the treatment with COX inhibitors did not affect FAAH activity compared to the control (Table 3), thus corroborating that COX inhibitors in the doses used in this work only affected the production of prostaglandins in our system.

4. Discussion

Although several studies have provided considerable insight into implantation biology, very few information exists about how the uterus spontaneously transits from a non receptive to a receptive phase prior to implantation. There is a growing need to unravel the complexities of uterine receptivity to address two contrasting global issues: infertility and a lack of novel contraceptives. In the present work we analyzed the crosstalk between three mediators involved in the process of implantation in a model of receptive uterus as we were interested in elucidating maternal uterine contributions at the time of implantation.

We observed that the administration of anandamide in vivo inhibits NOS activity in the receptive rat uterus and that this effect is mediated through cannabinoid receptor type 2. This result is in accordance with a previous work from our own, in which we described that the incubation with anandamide inhibited NOS activity in vitro via cannabinoid receptor type 2 receptors (Sordelli et al., 2011).

It is interesting to note that the mouse uterus contains by far the highest levels of anandamide detected in any mammalian tissue (Guo et al., 2005; Paria et al., 2001) and that changing levels of anandamide with changing pregnancy status are consistent with a possible role for this lipid molecule in the receptive uterus during early pregnancy (Guo et al., 2005; Ribeiro et al., 2009; Schmid et al.,

Table 3
Effect of cyclooxygenase inhibitors on uterine fatty acid amide hydrolase (FAAH) activity. Rats on day 5 of pseudopregnancy were treated i.p. with a non selective cyclooxygenase inhibitor, indomethacin (Indo), or with a selective cyclooxygenase-2 inhibitor, meloxicam (Melo). After 2 h, animals were sacrificed and the activity of FAAH was determined by radioconversion.

	FAAH activity nmol [^{3}H]-arachidonic acid/mg protein/h
Control	3.0 ± 0.5
Indo 2.5 mg/kg	4.0 ± 0.3
Melo 4 mg/kg	5.0 ± 0.9

1997). Two processes are fundamental during the establishment of pregnancy: the increase in vascular permeability and decidualization. Nitric oxide is a potent vasodilator and a well known mediator of vascular permeability (Furchgott and Zawadzki, 1980). Besides, nitric oxide augments the expression of some specific matrix metalloproteinases (Novaro et al., 2001) which are known to participate in tissue remodeling. Therefore, our results reinforce the notion that changing levels of anandamide in the receptive uterus would finally modify the known effects of NOS enzyme specifically at implantation sites where it contributes to neovascularization and tissue remodeling during trophoblast invasion.

An interesting review has been published, in which the author discussed the different signaling pathways triggered by cannabinoid receptor type 1 and cannabinoid receptor type 2 in reproduction, especially those controlling the intracellular tone of nitric oxide (Maccarrone, 2008). Furthermore, it has been described that cannabinoid receptor type 1 activates NOS, while cannabinoid receptor type 2 inhibits it in different systems (Demuth and Molleman, 2006; Howlett et al., 2004). The effects of cannabinoid receptors on nitric oxide release might be relevant for the in vivo control of reproduction, because nitric oxide plays several roles in female fertility (Stewart et al., 1992). As we have recently published, cannabinoid receptor type 1 and cannabinoid type 2 are expressed in the endometrium of pregnant rats (Sordelli et al., 2011) and critical changes in these receptors would occur during implantation in the maternal side of the fetal–maternal interface that finally influences the outcome of gestation.

We became interested in studying if prostaglandins mediate the inhibitory action of anandamide on NOS activity as these lipid molecules are well known mediators of crucial events in the receptive uterus at the time of implantation. In this sense, anandamide modulates prostaglandins production in different systems (Mitchell et al., 2008; Navarrete et al., 2009; Van Dross, 2009) and we have previously observed that prostaglandins could modulate the activity of NOS enzyme in the mouse and rat uterus (Aisemberg et al., 2007; Cella et al., 2006; Ribeiro et al., 2003, 2004).

Here we observed that anandamide increased PGE_2 and $PGF_{2\alpha}$ production via cannabinoid receptor type 2 and that COX-2 derived prostaglandins mediated anandamide-inhibitory effect on NOS activity in the receptive rat uterus. These results suggest that anandamide regulated both NOS activity and prostaglandins production through cannabinoid receptor type 2 in the receptive rat uterus at the time of implantation and that prostaglandins derived from COX-2, which is expressed at the sites of implantation (Lim et al., 1997, 2002), participate in anandamide–NOS crosstalk.

Others have also described an effect of anandamide on the production of prostaglandins. D-type prostaglandins were predominantly formed in anandamide-exposed JWF2 cells although significant increases in E- and F-type prostaglandins were also seen (Van Dross, 2009). Activation of glial cells produced a dramatic increase in the production of PGE_2 after 24 h of incubation with lipopolysaccharide and pre-treatment with different concentrations of anandamide led to a significant increase in the formation of PGE_2 and PGD_2 (Navarrete et al., 2009). In the amnion, anandamide caused a significant increase in PGE_2 production through cannabinoid receptor type 1 agonism and an increase in the expression of COX-2 isoform (Mitchell et al., 2008). Previously, we found that while $PGF_{2\alpha}$ and PGD_2 are capable of reversing lipopolysaccharide stimulation on nitric oxide synthesis in the rat uterus, PGE_2 potentiates lipopolysaccharide effect (Ribeiro et al., 2003). The administration of indomethacion or meloxicam to estrogenized rats augments the uterine production of nitric oxide (Ribeiro et al., 2004). Our group also observed that in the estrogenized rat uterus challenged with lipopolysaccharide, the early stimulation in the production of prostaglandins inhibits NOS activity, until the expression of the NOS isoforms is sufficient to overpass the inhibitory effect of the prostaglandins, suggesting that the interaction between NOS and COX might be important in the regulation of physiopathologic events during pregnancy (Cella et al., 2006). In this sense, in a model of septic abortion, meloxicam administration inhibits the lipopolysaccharide effect on uterine NOS activity, whereas celecoxib diminished it in the decidua (Aisemberg et al., 2007). Taking into account our results, it is interesting to mention that genetic and molecular studies with LIF, HB-EGF and Homeobox A-10 (Lim et al., 2002; Stewart et al., 1992) suggest that COX-2 functions as a common downstream pathway at the time of implantation.

One facet of endocannabinoid biology which is now receiving increased attention is the COX-2 derived oxidation products. Anandamide is oxidized to a range of prostamides that closely approach the prostaglandins formed from arachidonic acid (for details see review Woodward et al., 2008). As we reported here that selective COX-2 inhibitors prevented anandamide-inhibitory effect on NOS enzyme, we could not discard the possibility that prostamides were participating in the effect described above. New experiments are being carried out in order to address this issue.

In the present work we described that anandamide inhibits NOS activity through cannabinoid receptor type 2 in the receptive uterus at the time of implantation and that this regulation depends on the production of COX-2 derivatives. We chose the pseudopregnant model as a tool to understand the relative roles that play these mediators at the maternal side of the fetal–maternal interface without the interference of the embryo. During implantation, the uterus and the blastocyst communicate in order to orchestrate the series of events that lead to apposition, adhesion and invasion of the embryo towards the endometrium. In this sense, pseudopregnancy becomes a tool to investigate which of the physiological changes and responses during implantation could be elicited by the uterus itself in the absence of trophoblast signaling. This is of physiological relevance as it should be recalled that maternal triggered specific signaling pathways may exist, which could impact reproductive events in yet-unknown ways. In summary, anandamide levels in the receptive uterus modulate NOS activity, fundamental for implantation, via cannabinoid receptor type 2, and this depends on COX-2 derivatives, establishing cannabinoid receptors and COX-2 dependent pathways as interesting and novel targets for the treatment of implantation deficiencies.

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