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SELECTIVE CB₂ UP-REGULATION IN WOMEN AFFECTED BY ENDOMETRIAL INFLAMMATION.

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Abstract

Endometritis is defined as an inflammation of the endometrial mucosa of the uterus. In the endometritis a large amounts of toxic mediators, including nitric oxide (NO), are released by inflammatory cells. As a consequence of NO-dependent injury, the cells respond by triggering protective mechanisms, as by changing the endocannabinoid system (ECS) which comprises both CB₁ and CB₂ cannabinoid receptors and their endogenous ligands.

The aim of our study was to seek out evidence for the presence of cannabinoid receptors in inflammatory endometrial tissue as well as for their potential role in endometrial inflammation.

Our results showed a selective up-regulation of both transcription and expression of CB₂ receptors in biopsies from women affected by endometrial inflammation compared to healthy women. The experiments with the NO-donor S-Nitroso-L-Glutathione (GSNO) suggest that such a selective up-regulation may be related to the NO release occurring during endometrial inflammation. In addition, we demonstrated an increase in chymase expression, a marker of mast cells, in biopsies of women affected by endometritis. Therefore our results support the hypothesis that the up-regulation of CB₂ occurs mainly on mast cells and that it might tend to sensitize these cells to the anti-inflammatory effect exerted by endogenous cannabinoids by binding their receptor and thus preventing the mast cell degranulation and the release of pro-inflammatory mediators .

In conclusion, we believe that the selective CB₂ up-regulation might play a role as a novel prognostic factor in endometrial inflammation.

Key words: endometrial inflammation, nitric oxide, mast cell, chymase, CB₁, CB₂.

Introduction

Endometritis is defined as an inflammation of the endometrium usually resulting from an ascending infection from the lower genital tract. Clinically significant acute endometritis is usually associated with pregnancy or abortion as described elsewhere [1,2]. Currently, a precise histological definition of endometritis clearly discerning between acute and chronic disease is hampered by the wide range of different features observed. Indeed, the inflammatory infiltrate may be confined to the surface of epithelium or spread more deeply into the stroma; also, inflammatory cells may include neutrophils and/or plasma cells; furthermore inflammatory mononuclear cells may be hard to differentiate from endometrial stromal cells; finally, the two phases of inflammation may often merge each other and co-exist [3].

An immunological/inflammatory aetiology of endometritis has been demonstrated by the elevated concentrations of leukocytes [4], plasma cells and T cells [5] observed in the inflammatory endometrial infiltrate of affected patients. An increased amount of activated mast cells has been also described in endometrial immune inflammations [6]. All these inflammatory cells locally secrete several pro-inflammatory products including growth factors, cytokines and possibly free oxygen radicals such as nitric oxide (NO), which amplify the inflammatory process.

It has been demonstrated that women with endometrial inflammation have significantly higher levels of inducible NO synthase (iNOS) expression and NO production compared to healthy women [7,8]. High levels of NO are involved in antimicrobial and antitumoral activities of activated macrophages and an increase of NO is likely to exhibit pro-inflammatory effects [7]. Indeed, low levels of NO seem to be important for a number of

essential physiological processes, including maintenance of smooth muscle tone, neurotransmission and modulation of apoptosis [9].

It has been speculated that cannabinoids exert a wide array of effects on the CNS as well as on peripheral sites including the immune, cardiovascular, digestive, reproductive and ocular systems [10]. Currently, it is widely accepted that most of these effects are mediated by the activation of specific G protein-coupled receptors normally bound by a family of endogenous ligands, the endocannabinoids [11]. Two different cannabinoid receptors have so far been characterized and cloned from mammalian tissues: the CB₁ receptor, which is mainly expressed in the brain which is responsible for the psychoactive properties of cannabinoids [12], and the CB₂ receptor, which is primarily present on cells from the immune system and is likely to be unrelated to cannabinoid psychoactivity [13]. Nevertheless, the co-expression of both CB₁ and CB₂ receptors on immune system cells has been extensively documented [14], among them mast cells also express both CB₁ and CB₂ exhibiting differential roles during antigen-driving mast cell response [15]. Recently, it has been demonstrated that 2-arachidonoyl glycerol, the endocannabinoid which preferentially binds to CB₂ receptors, decreases the immunological activation of guinea pig mast cells [16]. Such potentially anti-inflammatory properties of cannabinoids prompted us to seek out further evidence for the presence of CB receptors in human biopsies from women affected by endometrial inflammation, since previous studies have indicated that endocannabinoid receptors expression may be modulated by a number of pro-inflammatory mediators, including NO and cytokines [17, 18].

Materials and Method

Fifteen patients (Group A) diagnosed as having endometritis by means of targeted biopsies performed during outpatient hysteroscopies were enrolled in the study. Twenty patients (Group B) matched for age, BMI and parity undergoing an office hysteroscopy plus targeted biopsies for benign conditions constituted the control group.

All patients signed an informed consent to participate to the study. The study was approved by our Institutional Review Board.

All hysteroscopies were performed by the same operator (A.D.S.) using a 5 mm continuous-flow operative office hysteroscope with a 2.9 mm rod lens (Bettocchi office hysteroscope, Karl Storz, Tuttlingen, Germany). All the procedures were carried out under an outpatient regimen being neither analgesia nor local anaesthesia administered to the patient. Distension of the uterine cavity was obtained using normal saline solution and the intrauterine pressure was automatically controlled by an electronic irrigation and suction device (Endomat, Karl Storz, Tuttlingen, Germany). The intrauterine pressure was set at 45 mmHg, being the balance of irrigation flow around 200mL/minute and a vacuum of 0.2 bars.

Hysteroscopies were performed during the follicular phase of cycle (premenopausal patients) or within two weeks before the surgical procedure (postmenopausal patients).

None of the patients had received any hormonal or antibiotic treatment in the six months before the hysteroscopy.

Chemicals

All materials for biopsies culture were purchased from Biowittaker (Caravaggio, BG, Italy). GSNO was purchased from Sigma (Milan, Italy). CB₁ and CB₂ receptor antibodies for

immunoblotting were purchased from SpioBio (Freemont CA, USA); iNOS antibody was purchased from BD Biosciences (Pharmingen, Milan, Italy) and anti-chymase from Sigma.

Histological features

Formalin-fixed, paraffin-embedded tissues from endometrial biopsies were retrieved from the pathology files at the Federico II University of Naples – Italy. The tissue was sectioned at 5 μm , and stained with haematoxylin and eosin.

Tissue culture

Endometrial biopsies were placed in 24-well culture plates and cultured in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 5% Foetal Bovine Serum (FBS), 2 mM glutamine, 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin at 37°C in 5% CO₂/95% air for 24 h according to Coeffier [19]. In some experiments, the S-Nitroso-L-Glutathione (GSNO) (100 μM), a NO-generating compound [20], was added to cultured biopsies from healthy women. After 24h of GSNO-stimulation, the biopsies were frozen in liquid nitrogen for further analysis.

MPO activity

Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear (PMN) accumulation, was determined as previously described [21]. Endometrial tissues, collected at the end of the reperfusion period, were homogenised in a solution containing 0.5% (w/v) hexadecyltrimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000g at 4°C. An aliquot of the supernatant was then allowed to react with a solution of tetra-methyl-benzidine (1.6mm) and 0.1mm H₂O₂. The rate of

change in absorbance was spectrophotometrically measured at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 mmol of hydrogen peroxide/min at 37° C and was expressed in units per gram weight of wet tissue.

Nitrite assay

Nitrite production, as the stable metabolites of NO, was measured in 24 h-cultured biopsy supernatants by using the method previously described [17]. Briefly, the medium of cultured biopsies (0.1 ml) was added to an equal amount of Griess reagent (1% sulphanilamide, 0.1% naphthylendiammine, 2.5% H₃PO₄) and kept at room temperature for 10 min. The absorbance of constituted chromophore was determined using a UV/visible spectrophotometer at 550 nm. Nitrite levels were determined using a sodium nitrite standard curve and expressed as μM/tissue protein.

mRNA analysis

The mRNA level of CB₂ in endometrial tissue was determined using the semiquantitative RT-PCR method. Total mRNA was extracted using an ultrapure TRIzol reagent (Gibco BRL, Milan, Italy) as directed by the manufacturer. The concentration and purity of total RNA were determined from the A260/A280 ratio using a UV spectrophotometer DU 40 (Beckman, Fullerton, CA, USA). The primer sequences used for PCR amplification were sense 5'-TA(C/T)CC(G/A) CCT(A/T)CCTACAAAGCTC-3', and antisense 5'-CC(A/T)GGCACCTGCCTGTCCTGGTG-3' for CB₂ receptor and sense: 5'-ATGAAGATCCTGACCGCGCGT-3', antisense: 5'-AACGCAGCTCAGTAACAGTCCG-3' for β-actin. 1μg of total RNA from each specimen was subjected to RT-PCR. RT-PCR was carried out by using a SuperScript™ One-Step RT-PCR with Platinum Taq Kit in a

total reaction volume of 25 μ l, containing 2X reaction mix 12.5 μ l, 25 μ mol/L sense primer 0.5 μ l, 25 μ mol/L antisense primer 0.5 μ l, RT-PCR platinum Taq mix 0.5 μ l, and autoclaved distilled water. The β -actin and CB₂ PCR products were electrophoresed on 1% agarose gel and visualized by staining with ethidium bromide. The integrated density values of the bands representing amplified products were acquired and analyzed by GS 700 Imaging Densitometer (Bio-Rad) and a computer program (Molecular Analyst IBM).

Isolation of Mast Cells from endometrial tissues

Human mast cells (MC) were isolated from endometrial biopsies by means of enzymatic digestion as previously described [22]. Briefly, endometrial tissues were placed into Ca/Mg-free Tyrode's buffer. Thereafter, tissue was chopped into small pieces, and the fragments were extensively washed in Tyrode's buffer. Incubation was carried out at 37° C for 120 min by addition of collagenase (30 U/mL). The dispersed cells were recovered by filtration through Nytex cloth. After washing twice in 0.9% NaCl, the cell suspension was incubated with DNase (0.5 mg/mL), hyaluronidase (0.5 mg/mL), and pronase-E (2 mg/mL) for 15 min at 37°C . Cells were washed three times in NaCl at room temperature. The percentage of MC was determined by toluidine blue staining. Isolated MC were cultured in RPMI-1640 medium supplemented with 10% foetal calf serum, glutamine, and antibiotics at 37°C for at least 24 h before the analysis.

β -Hexosaminidase analysis

Mast cells were stimulated with the calcium ionophore, A23187 (500nM) (Sigma Aldrich), in presence or absence of CB₂ selective agonist, JWH-015 (10^{-8} – 10^{-6} M).

Mast cell degranulation was detected by measuring the release of the granular enzyme β -hexosaminidase using a method first described by Hernandez-Hansen V et al. [23].

Aliquots (50 μ l) of cell medium were collected from each well and transferred into a 96-well plate. Substrate, p-nitrophenyl Nacetyl-*b*-D-glucosaminide (50 μ l of a 2 mM solution diluted in 0.2 M citrate buffer, pH 4.5), was added to each well. The samples were then incubated with the substrate for 2h at 37°C. All incubations were carried out at this temperature because all the reactions are sensitive to temperature changes. Medium and chemicals were also kept at 37° C throughout the experiment to avoid an undesired impact on the result caused by any temperature changes. After 2h, reactions were terminated by adding 150 μ l of 1M Tris-buffer pH 9.0. The absorbances were measured in a microplate reader at 405 nm. The total β -hexosaminidase cell content was determined in parallel wells where the cells were dissolved in 0.01% Triton X-100. The background release from unstimulated cells was also determined. Mast cells degranulation was expressed as percentage of β -hexosaminidase release.

Western blot analysis

Depending upon the experiments, fresh or GSNO-stimulated biopsies were weighted and rapidly homogenized in 60 μ L of ice-cold hypotonic lysis buffer [10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride, 1.5 μ g/mL soybean trypsin inhibitor, pepstatin A 7 μ g/mL, leupeptin 5 μ g/mL, 0.1 mM benzamidine, 0.5 mM dithiothreitol (DTT)] and incubated in ice for 45 min. The cytoplasmic fractions were then obtained by centrifugation at 13000 g for 1 min and protein concentration in the samples was determined using a Bio-Rad assay kit according to the manufacturer's instructions. Immunoblotting analysis of iNOS, CB₁, CB₂ receptors and chymase were performed on a

cytosolic fraction of cultured specimens. Cytosolic fraction proteins were mixed with gel loading buffer (50 mM Tris, 10% SDS, 10% glycerol 2-mercaptoethanol, 2 mg bromophenol/ml) in a ratio of 1:1 v/v, boiled for 5 min and centrifuged at 10000 g for 10 min. Protein concentration was determined and equivalent amounts (50 µg) of each sample were separated under reducing conditions in 12% SDS-polyacrylamide minigel. The proteins were transferred onto nitrocellulose membrane according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA). Depending upon the experiments, the membranes were blocked by incubation at 4° C overnight in high salt buffer (50 mM Trizma base, 500 mM NaCl, 0.05% Tween-20) containing 5% bovine serum albumin; they were then incubated for 1 h with anti-iNOS (1:2000 v/v) (BD Biosciences, Pharmingen, Italy), anti-CB₁ (1:250 v/v), anti-CB₂ (1:250 v/v) anti-Chymase (1:250 v/v) (NeoMarkers, Fremont, CA) or anti-tubulin (1:1000 v/v) (Sigma-Aldrich, Milano, Italy) for 2 h at room temperature, followed by incubation with specific horseradish peroxidase (HRP)-conjugate secondary antibody (Dako, Golstrup, DK). The immune complexes were developed using enhanced chemiluminescence detection reagents (Amersham, Italy), according to the manufacturer's instructions and exposed to Kodak X-Omat film. The bands of proteins on X-ray film were scanned and analysed with a GS-700 imaging densitometer (Bio-Rad Laboratories, CA, USA).

RESULTS

Histological features of endometrial biopsies

Endometrial biopsies obtained from patients enrolled in Group A showed a significant increase in both mononuclear inflammatory cells and granulocytes which filled and

destroyed the endometrial glands. The diagnosis of endometritis was done according to the established criteria [24].

Endometrial biopsies obtained from patients enrolled in Group B did not show any feature suggestive of endometrial inflammation (Fig. 1A and 1B).

MPO activity

Biopsies obtained from patients affected by endometritis were characterized by an increase in MPO activity, an indicator of PMN infiltration, compared to healthy patients (Fig. 1C), thus confirming the occurrence of an inflammatory reaction in the endometrial tissue.

Expression of mast-cell chymase in biopsies of patients with endometritis

Western blot analysis of biopsies obtained from patients affected by endometritis revealed an increase in chymase expression, a specific pre-formed mediator released by mast-cells, compared to healthy patients (Fig. 2), thus confirming the presence of mast cells in the inflammatory endometrial tissue.

Increased nitrite levels and iNOS protein expression in cultured biopsies

When biopsies from patients harbouring endometritis were cultured for 24 h at 37° C, nitrite levels, the stable metabolite of NO, were significantly increased compared to biopsies from healthy patients (Fig. 3C). Immunoblotting analysis of the tissues revealed that iNOS protein expression was significantly increased in endometrial biopsies from patients affected by endometritis versus healthy patients (Fig. 3A and 3B).

Selective up-regulation of CB₂ receptor in biopsies of patients with endometritis

Immunoblotting analysis revealed that CB₂ receptor protein expression was significantly increased in endometrial biopsies from patients affected by endometritis compared to healthy patients. Interestingly, no significant differences in CB₁ receptor protein expression were observed in the biopsies deriving from the two groups of patients (Fig.4A, B and Fig. 5A, B).

Moreover RT-PCR analysis revealed that CB₂ receptor mRNA expression was significantly increased in endometrial biopsies deriving from patients affected by endometritis compared to healthy patients (Fig. 6).

Selective up-regulation of CB₂ receptor by GSNO

Western blot analysis of biopsies stimulated for 24 h with the NO donor, GSNO (100μM), showed a significant and selective increase of CB₂ receptor protein expression compared to un-stimulated biopsies. On the contrary, no differences in CB₁ receptor expression were observed between GSNO-stimulated and un-stimulated endometrial biopsies (Fig. 4 C, D and Fig. 5 C, D).

Effect of JWH-015 on A23187-induced mast cell degranulation.

The mast cell secretagogue calcium ionophore A23187 (500nM) [25] induced a significant increase of human mast cells degranulation. Treatment of endometrial mast cells with the selective CB₂ agonist JWH-015 (10⁻⁸-10⁻⁶ M) reduced A23187-induced degranulation in a concentration-dependent manner (Fig. 7).

DISCUSSION

In the present study we have observed, for the first time, a significant and selective increase in both CB₂ receptor transcription and expression in endometrial biopsies obtained from women affected by endometrial inflammation compared to healthy women.

Histological analysis of such biopsies have revealed an increased amount of inflammatory cells; in addition, we have observed an increase in both chymase, a marker of mast cell [26], and mieloperoxydase, a marker of infiltrating leukocytes [27], compared to healthy patients' biopsies. All these infiltrating cells sustain the inflammatory environment by releasing a number of pro-inflammatory mediators, including cytokines, growth factors and free radical species [28]. Cultured biopsies obtained from women affected by endometrial inflammation, released an increase amount of NO produced by the inducible isoform of NOS. Physiologically, consequently to NO-sustained inflammation, the immune cells respond by triggering protective mechanisms, including the capture of free radicals and the bio-transformation of xenobiotics [29]. Recently it has been shown that inflammatory stimuli, such as LPS, may affect endocannabinoid system by increasing AEA production in rat macrophages and 2-AG in rat platelets [30]. Our findings indicate that NO, one of the most important mediator of inflammatory environment, modulates cannabinoid receptor expression. Indeed we have found that the stimulation with GSNO, a compound able to mimicking the inflammatory scenario, by releasing NO, selectively increased CB₂ receptor expression when added to biopsies of healthy patients. The mechanism at the basis of this effect has not been further elucidated but the occurrence of this effect is very likely, since the endocannabinoid system is known to control inflammatory processes [31].

Our findings support the hypothesis that the selective up-regulation of CB₂ receptor may be a responsive mechanism triggered by injured endometrial tissue to control the NO-sustained

inflammatory reaction. In fact, several studies have shown that cannabinoids exhibit anti-inflammatory properties, both in laboratory animals [32] and cell cultures [33], by modulating key pathways of the inflammatory process, thereby directly inhibiting pro-inflammatory mediator release. Such anti-inflammatory activity can be explained by the property of cannabinoids to bind to specific receptors expressed on the surface membrane of several inflammatory cells. In addition, our findings seem to support the hypothesis that the up-regulation of cannabinoid receptors occurs mainly on mast cells. Indeed, mast cells were highly expressed in biopsies from women affected by endometritis in agreement with previous findings describing mast-cell infiltration and accumulation in endometrial inflammation [6]. Moreover, the CB₂ receptor activation on isolated endometrial mast cells, leads to the inhibition of A23187-induced degranulation. During inflammatory process, mast cells release their stored and newly synthesized mediators either *via* the classical immunological IgE-dependent activation or in response to a variety of stimuli [34]. Mast cell mediators include cytokines (TNF- α), histamine and pro-inflammatory mediators such as NO, IL-1 and IL-6 [35]. Therefore, it is conceivable to hypothesize we can speculate that the up-regulation of CB₂ might occur to sensitize mast cells to the anti-inflammatory effect exerted by endogenous endocannabinoids, by binding to the CB₂ receptor, and thus preventing both the degranulation and the release of pro-inflammatory mediators from mast cells.

In conclusion, in view of the fact that nowadays ongoing studies using techniques of molecular biology and immunohistochemistry are trying to identify new prognostic factors to be correlated to the endometrial inflammatory process to support the still controversial histological diagnosis of endometritis [36], we believe that the selective CB₂ up-regulation might play a role as a novel prognostic factor in endometrial inflammation.

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Legends to figures

Figure 1. Inflammatory environment: representative histological analysis of endometrial biopsies from healthy women (A) and women suffering of endometritis (B). . Polymorphonuclear cell infiltration in biopsies from healthy women and women suffering of endometritis, detected by myeloperoxidase activity assay (C). Each bar shows the mean \pm SEM of 6 experiments. *** $p < 0.001$ versus healthy patients.

Figure 2 Representative Western blot analysis (A) and relative densitometric analysis (B) of chymase (M.W. 45 kDa), a marker of mast cells, in biopsies from healthy women and women suffering of endometritis. Tubulin expression is shown as control. Data are representative of six separated experiments. Each bar in the graph shows the mean \pm SEM of 6 experiments. *** $p < 0.001$ versus healthy patients.

Figure 3. Representative Western blot analysis (A) and relative densitometric analysis (B) of iNOS (130 kDa) protein expression in endometrial biopsies from healthy women and women suffering of endometritis. (C) NO production from cultured biopsies from inflamed

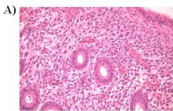
and healthy patients. Each bar shows the mean \pm SEM of 8 experiments. ***P<0.001 versus healthy patients

Figure 4. Western blot analysis of CB₁ (M.W. 60 kDa) expression in: (A) endometrial biopsies from healthy women and women suffering of endometritis; (C) endometrial biopsies stimulated with GSNO; (B and D) relative densitometric analysis. Each bar shows the mean \pm of SEM of 6 experiments.

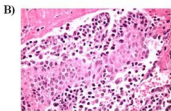
Figure 5. Western blot analysis of CB₂ (M.W. 45 kDa) expression in: (A) endometrial biopsies from healthy women and women suffering of endometritis; (C) endometrial biopsies stimulated with GSNO. (B and D) relative densitometric analysis. Each bar shows the mean \pm of SEM of 6 experiments. ***P<0.001 versus healthy patients.

Figure 6. CB₂ mRNA expression in endometrial biopsies from healthy women and women suffering of endometritis (A) and relative densitometric analysis (B). the expression of β -actin was used as a control. Each bar shows the mean \pm of SEM of 6 experiments. ***P<0.001 versus healthy patients.

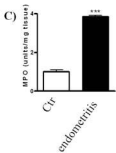
Figure 7. Effect of JWH-015 on A23187-induced mast cell degranulation. Mast cells were isolated from inflamed women's biopsies. Each bar shows the mean \pm of SEM of 3 separated experiments ***P<0.001 versus CTR; °P<0,05, °°P<0,01, °°°P<0,001 vs A23187.



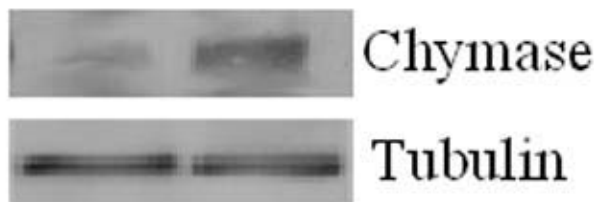
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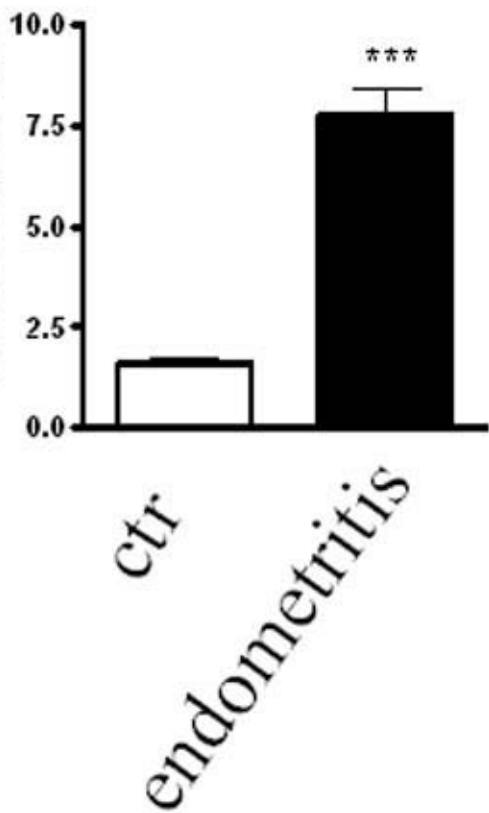
endometritis

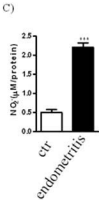
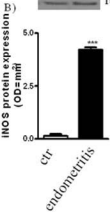


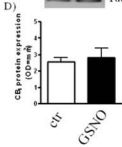
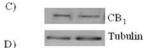
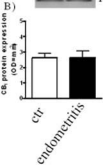
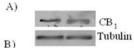
A)

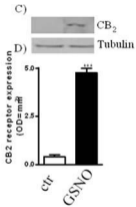
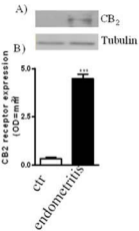


B)
Mast-cell chymase protein
expression (OD = \bar{x}) m









A)



CB₂



β -actin

B)

