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Non-steroidal anti-inflammatory drugs increase MRP4 expression in an endometriotic epithelial cell line in a PPARα dependent manner

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Abstract. – OBJECTIVE: Endometriosis is a debilitating disease characterized by chronic inflammation. The transporter multidrug resistance-associated protein 4 (MRP4/ABCC4) is expressed in human endometrial tissue; it is over-expressed in ectopic endometrial tissue, and is modulated by the anti-inflammatory lipid Lipoxin A4 (LXA4). Recently, it was demonstrated that aspirin induces platelet MRP4 over-expression, through genomic modulation in megakaryocytes. Since patients with endometriosis frequently use aspirin or other non-aspirin Non-Steroidal Anti-Inflammatory Drugs (NSAIDs), the aim of this study was to verify whether aspirin and other NSAIDs enhance MRP4 expression in 12Z human endometriotic epithelial cells and whether this was peroxisome proliferator-activated receptor alpha (PPARa) dependent.

MATERIALS AND METHODS: MRP4 and PPARa expression was analyzed by Q-RT-PCR using TaqMan® Master Mix and TaqMan® Assay Reagents (Life Technologies, Monza, Italy) and Western blot.

RESULTS: In 12Z cells, aspirin and other NSAIDs enhanced MRP4 mRNA and protein expression; these treatments also induced PPARa expression. Aspirin and diclofenac-induced increases in MRP4 expression were not observed in cells where PPARa was knocked down using siRNA. NSAIDs-induced MRP4 expression was correlated with augmented PGE2 secretion, indicating functional relevance.

CONCLUSIONS: MRP4 expression was increased in cells treated with NSAIDs and the nuclear receptor PPARa is involved. Elevated PGE2 levels in cell supernatants correlate with its increased transport by MRP4 after NSAID treatment. More importantly, we provide evidence that in endometriotic epithelial cells aspirin and non-aspirin NSAIDs treatments alter gene expression.

Key Words:
Endometriosis, MRP4/ABCC4, Pain, Aspirin, NSAID.

Introduction

Endometriosis is a debilitating disease with features of chronic inflammation that affects approximately 10% of women in reproductive age. It is defined as the presence of functional endometrial glands and stroma outside the uterine cavity[1]. As one of the most common benign gynecological conditions, endometriosis is a debilitating disease with detrimental effects on social, occupational and psychological functioning[2]. Despite its prevalence, this disease remains poorly understood and current studies have proved that there is no relationship between the extent of the disease and its symptomatology[1]. The most frequent symptom is a chronic pelvic pain, which may include: dysmenorrhea (painful periods), periovulatory pain, dyspareunia (pain during or after sexual intercourse), dysuria, dyschezia, and leg pain. For this

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reason, patients with endometriosis frequently use either non-aspirin Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) or aspirin. Endometriosis is divided by the classification system of the American Fertility Society into four stages of severity from stage I, that is represented by minimal disease, to stage IV, which means severe disease. Staging does not correspond with degree or severity of symptoms but assesses the extension of disease visible by laparoscopy. In addition, an elevated level of CA125 (epithelial ovarian cancer associated antigen) in patients with endometriosis is associated with the progression of the disease[3]. Women's quality of life, including their careers, everyday activities, sexual and non-sexual relationships and fertility may be really affected by endometriosis. The United Kingdom – Endometriosis UK (www.endometriosis-uk.org/), which represents a patient support organization, confirmed that the 65% of women with endometriosis had negatively influenced their employment, the 10% of women reduced the hours of work, and 30% of them lost the job, which brought them to perceive a feeling of distress and low self-esteem[4]. Moreover, infertility has been identified as one of the most relevant complications related to endometriosis, which has been observed in 15-25% of patients. In 2018, has been demonstrated that the levels of inflammatory cytokines (IL-6, IL-10, IL-13, and TNF) are particularly increased in peritoneal fluid of patients affected by endometriosis complicated with infertility. Therefore, the high level of those cytokines may determinate embryo toxicity and could affect sperm motility, fertilization, and implantation leading to an elevated rate of infertility among patients affected by endometriosis[5,6]. Endometriosis can be suspected in reproductive age women with clinical symptoms. Transvaginal ultrasonography can reliably detect endometriomas but failure to reveal cystic structures does not exclude the diagnosis of endometriosis. Magnetic resonance imaging (MRI) is increasingly used to identify subperitoneal deposits, although retroversion, endometrioma, and bowel structures may mask small nodules[7]. Risk factors generally relate to menstruation: early menarche, late menopause, nulliparity and first pregnancy late in life increase the risk whereas the use of oral contraceptives is associated with a reduced risk of developing the disease. While the etiology of endometriosis still remains unclear, the most widely accepted hypothesis first advanced by Sampson[8] is that viable endometrial tissue fragments are refluxed through the fallopian tubes into the pelvic cavity during retrograde menstruation, and after being migrated adhere and invade other tissues. Different studies[9-11] indicate that growth factors, cytokines, and prostaglandins promote the establishment and maintenance of endometriosis. In endometriosis patients, concentrations of prostaglandin E_2 (PGE_2) in the peritoneal fluid are higher compared to that of endometriosis-free women[9]; PGE_2 plays a pivotal role in endometriosis-associated inflammation and pain[12,13]. COX-2 protein is abundantly expressed in ectopic endometrium and in the eutopic endometrium of women with endometriosis. Banu et al[14], showed that inhibition of COX-2 and of PGE_2 receptors PTGER2 and PTGER4 decrease survival and invasion of human endometriotic epithelial and stromal cells through multiple mechanisms. Their results support that COX-2/PGE_2 promotes the pathophysiology and pathogenesis of endometriosis in humans. The inhibition of PTGER2 and PTGER4 *in vitro*, inhibits adhesion of human endometriotic epithelial cells 12Z and stromal cells 22B to collagen I, vitronectin, fibronectin, collagen IV, and laminin in a substrate- and epithelial-stromal cell-specific manner of the peritoneal extracellular matrix (ECM)[15]. Together, these results suggest the importance of the PTGER2 and PTGER4 associated signaling pathways in the pathogenesis of endometriosis.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most frequently used first-line drugs for pain in women affected by endometriosis. Nevertheless, there is no sufficient evidence that showed whether or not they are really effective in relieving endometriosis-associated pain. Moreover, it has not been demonstrated if there is a NSAID more effective than another[4]. Currently, has been affirmed that the expression of COX-2 in ectopic endometrial cells is higher than in eutopic endometrium. Nonsteroidal anti-inflammatory drugs inhibit the function of the enzyme COX-1 and COX-2, preventing the production of PGs, which are implicated in the genesis of endometriosis-associated pain. Furthermore, have been registered several side effects related with NSAIDs including nausea, diarrhoea, headache, dryness of the mouth[16]. In 2016 the Cochrane Gynecology and Fertility Group Specialized Register of Controlled Trials published a comparison performed from January 2008 to October 2016, by using NSAIDs (naproxen) *vs.* placebo in women with endometriosis-related pain. Data revealed no evidence of a positive effect on pain relief (odds ratio (OR)

3.27, 95% confidence interval (CI) 0.61 to 17.69; one trial, 24 women; very low-quality evidence). In addition, the work did not demonstrate improvements concerning the quality of life, effects on daily activities, absence from work or school in patients treated with NSAIDs[17]. Clinical experiences of our center evaluated the impact of NSAIDs, which are used as pain therapy in endometriosis. It showed that 39 (12.8%) out of 305 patients, did not require any therapy, 84 (27.5%) patients did not report if they needed drugs, 18 patients (5.9%) stated they needed treatment but did not specify which therapy. About the remaining patients: 111 (36.4%) take only NSAIDs, 41 (13.4%) take NSAIDs in combination with other drugs, and 12 (3.9%) use drugs different from NSAIDs. In our opinion, these data are very important because, if we sum up all the patients who use NSAIDs, alone and in combination, the total of the patients who take NSAIDs, increases to 152 (49.8%). The transporter multidrug resistance-associated protein 4 (MRP4) is also expressed in human endometrial tissue; it is overexpressed in the ectopic endometrial tissue, and is modulated by the anti-inflammatory lipid lipoxin A_4 (LXA_4)[11]. Increased MRP4 expression in the ectopic endometrium of women suffering from endometriosis was confirmed by a subsequent study[18]. MRP4, an ATP binding cassette (ABCC4) and unidirectional transporter of endogenous molecules including several PGs, plays a key role in cellular communication and signaling. Elevated MRP4 expression may result in increased extracellular PGE_2, which binds its receptors and activates various signaling pathways in endometriosis. MRP4, as well as PGE_2 itself, is implicated in proliferative conditions and could, therefore, serves as a potential biomarker for disease severity, as well as a possible drug target for endometriosis therapy[13]. We demonstrated that aspirin causes MRP4 overexpression in human platelets, through an activation of the nuclear receptor PPARα in megakaryocytes[19]. Recently we reported that non-aspirin Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) can induce MRP4 overexpression in human platelets. Patients with endometriosis frequently suffer from dysmenorrhea, which negatively affects daily activities, and for this reason, they frequently take aspirin or non-aspirin NSAIDs[20]. We can assume that MRP4 can be modulated by the action of aspirin and non-aspirin NSAIDs even in the endometrium, leading to enhancement of PGE_2 secretion. In this study the role of aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) in regulating MRP4 expression in human endometriotic epithelial cells (12Z), the involvement of nuclear receptor PPARα and PGE_2 secretion were investigated.

Materials and Methods

Cell Culture

The simian virus 40 T-antigen-transformed human ectopic endometriotic epithelial cell line, 12Z (kindly provided by Dr. M. Beste, Massachusetts Institute of Technology), was maintained in Dulbecco's Modified Eagle Medium (DMEM)/Ham's F12 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 20 mM L-glutamine, 100 units/ml of penicillin G sodium, and 100 μg/ml streptomycin sulphate in a humidified atmosphere containing 5% CO_2 at 37°C[21]. For stimulation experiments, cells (250,000/well) were seeded in 12-well plates; the following day, stimulation with various factors was carried out in media for 48 h. Cells were treated with either aspirin (25 μM, 50 μM, and 100 μM), or diclofenac (25 μM, 50 μM, and 100 μM), or nimesulide (25 μM, 50 μM, and 100 μM), or acetaminophen (50 μM), or ketoprofen (50 μM), or ibuprofen (50 μM), or naproxen (50 μM). In a mock culture, an equivalent amount of DMSO, the vehicle, was added. After treatment, cells were processed for RNA and protein extraction. For PGE_2 analysis and measurement of prostaglandin H synthase activity, 12Z cells (250,000/well) were seeded in 12-well plates; the following day, stimulation with various factors was carried out in media for 48 h (50 uM aspirin or diclofenac or nimesulide). After 48 hours of treatment, cells were washed and maintained in medium without any drugs for 24 hours, after which supernatants were collected for PGE_2 and cells collected for the measurement of prostaglandin H synthase activity and MRP4 expression.

Protein Extraction and Western Blot

To analyze MRP4 protein, cells were washed twice with cold phosphate-buffered saline (PBS), collected and centrifuged at 400 g for 10 min. Cell pellets were then resuspended in lysis buffer (RIPA buffer: 10 mM Tris-HCl pH 7.6, 160 mM NaCl, 1 mM EGTA, 1% Deoxycholic acid, 1% Triton, 0.1% SDS), incubated on ice for 30 min and centrifuged at 12,000 g for 30 min, the supernatant was then collected. Protein extracts (30 μg)

were incubated at 37°C for 30 min and separated on 4-12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, transferred onto polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Milano, Italy), and probed with rat anti-MRP4 (Alexis, Plymouth Meeting, PA, USA) and mouse anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) monoclonal antibodies. Immunoreactive bands were visualized by enhanced chemiluminescence (PerkinElmer, Waltham, MA, USA). The densitometric analysis was performed with the National Institutes of Health ImageJ analyzer program.

RNA Preparation and Real-Time Quantitative PCR Analysis

Total RNA from human cell lines was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). For mRNA detection 1 μg of total RNA was transcribed using the GeneAmp Gold RNA PCR Reagent Kit pAW109 (Life Technologies, Monza, Italy) according to the manufacturer's protocol. Gene expression analysis was carried out by means of Q-RT-PCR using TaqMan® Master Mix and TaqMan® Assay Reagents (Life Technologies, Monza, Italy). The amplification program, monitored using ABI Prism 7900 Sequencer Detector (Life Technologies, Monza, Italy), was as follows: 50°C for 2 min, 95°C for 10 min, 95°C for 15 sec, and 60°C for 1 min, the latter two temperatures were repeated for 40 cycles. All amplification reactions were performed in duplicate using 25 ng of cDNA. Changes in MRP4, PPARα and ACTIN mRNA amounts were quantified using the ΔΔCt method for relative quantification of gene expression using SDS software version 2.3 (Life Technologies, Monza, Italy).

RNA Interference

Double strand interfering RNA (siRNA) targeting human PPARα and control non-specific siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were transfected using Lipofectamine RNAi MAX Reagent (Life Technologies, Monza, Italy). 24 hours after siRNA administration, cells were treated with aspirin (50 μM) or diclofenac (50 μM) (Sigma-Aldrich Chemicals Company, St. Louis, MO, USA). 48 hours after treatment, cells were processed for RNA and protein analysis.

PGE2 Quantification

For PGE_2 analysis, 12Z cells (250,000/well) were seeded in 12-well plates and treated as described above. Supernatants were then collected and PGE_2 levels were quantified using a monoclonal PGE_2 EIA kit (Cayman Chemical Co., Ann Arbor, MI, USA) according to the manufacturer's instructions. PGE_2 levels (pg/10^5 cells) are reported as ratio between treated and untreated cells (relative levels).

Measurement of Prostaglandin H Synthase Activity

Prostaglandin H synthase activity was performed as previously described [22]. Briefly, cells were washed with PBS and pre-loaded with 2 μM 5-(and-6)-carboxyl-2',7'-dichlorodihydrofluorescein (CDCF) (Molecular Probes Inc., Eugene, OR, USA), serving as a reducing substrate for the peroxidase activities of COX-1. Platelet COX activity was measured as arachidonic acid induced (8 μM) fluorescence enhancement in a Victor-3 spectrofluorimeter (PerkinElmer, Waltham, MA, USA), thermostatically regulated (37°C). 5 μM Nicotinamide adenine dinucleotide phosphate oxidase inhibitor (diphenyliodonium) was added to avoid reactive oxygen species production-mediated interference. The results are reported as the rise in CDCF fluorescence (Arbitrary Units) recorded for 5 min.

Statistical Analysis

Data are presented as mean ±SD. The level of significance was determined employing unpaired, 2-tailed Student's *t*-test (KaleidaGraph software 3.6). Results were considered statistically significant if a *p*-value of less than 0.05 was reached.

Results

MRP4 Expression in NSAID Treated 12Z Cells

The effect of aspirin and other NSAIDs on MRP4 over-expression has recently been demonstrated in a human megacaryoblastic cell line (DAMI)[19,20]. In order to examine whether these drugs also induced MRP4 expression in endometriotic cells, we treated 12Z cells with aspirin, diclofenac, nimesulide, acetaminophen, ketoprofen, ibuprofen, and naproxen for 48 hours. Among the NSAIDs used, aspirin (1.6 fold increase), diclofenac (1.6 fold increase), acetaminophen (1.6 fold increase), ketoprofen (1.9 fold increase) and nimesulide (2.3 fold increase) induced a significant increase in MRP4 mRNA expression in 12Z cells compared to the control culture (all $p<0.05$) (Figure 1). In contrast, in 12Z cells treated with naproxen and ibuprofen, no increase in MRP4

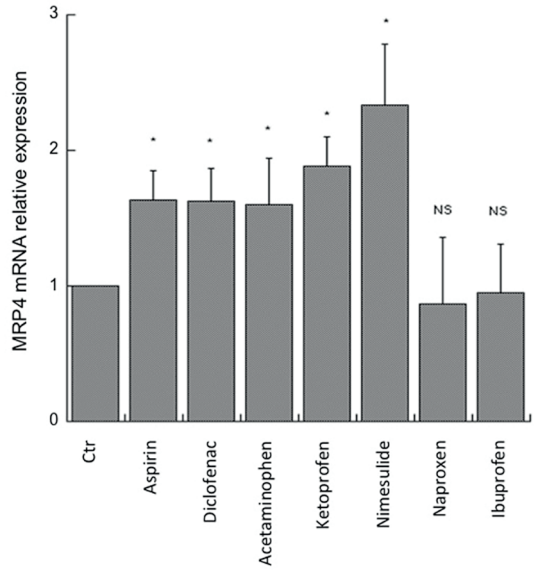

Figure 1. Aspirin and NSAIDs induce MRP4 mRNA expression in 12Z cells. Q-RT-PCR analysis of endogenous MRP4 expression in 12Z cells treated with aspirin, diclofenac, acetaminophen, ketoprofen, nimesulide, naproxen and ibuprofen (50 μM for 48 h) compared to vehicle control (Ctr). Data were normalized with ACTB expression and reported as mean ±SD of 3 experiments (*p<0.05; NS: not significant; t-test).

expression was observed (Figure 1). In the same experimental conditions, Western Blot analysis revealed augmented MRP4 protein expression in 12Z cells treated with aspirin, diclofenac, acetaminophen, ketoprofen, and nimesulide (50 μM for 48 h) (Figure 2A). Densitometric analysis showed a statistically significant increase in MRP4 protein expression in 12Z cells treated with aspirin (1.6 fold increase), diclofenac, acetaminophen, ketoprofen, and nimesulide (1.4 fold increase) in comparison with untreated cells (p<0.05) (Figure 2B). We recently demonstrated that the nuclear receptor PPARα is involved in the aspirin-induced MRP4 overexpression in megakaryocytes[19]. To explore whether PPARα was responsible for the MRP4 expression increases in NSAID treated 12Z cells, we analyzed PPARα mRNA expression. The results obtained show that treatment with all NSAIDs used, resulted in a significant increase in PPARα mRNA expression compared with untreated cells (1.6 fold increase) (p<0.05), except for naproxen and ibuprofen (Figure 3). Figure 4 (A-F) shows a correlation between MRP4 and PPARα expression and scalar increasing doses of aspirin, diclofenac, and nimesulide (from 25 μM to 100 μM). MRP4 expression was strongly induced in a dose-dependent manner after aspirin (Figure 4A), diclofenac (Figure 4B) and nimesulide (Figure 4C) treatment. Indeed, the highest MRP4 mRNA increase is evident after treatment with 100 μM of these drugs. These increases are also evident for PPARα expression levels after a different dose of drug treatment, for aspirin (Figure 4D), diclofenac (Figure 4E) and nimesulide (Figure 4F).

PPARα siRNA Inhibits MRP4 Expression Induced by NSAIDs

To confirm the involvement of PPARα in MRP4 up-regulation, 12Z cells were transfected with PPARα specific siRNA (PPAR-si); cells transfected with PPAR-si didn't exhibit any significant increase in PPARα mRNA expression after aspirin treatment; while cells transfected with control, non specific siRNA (CTR-si), exhibited the same aspirin-induced expression changes as untransfected cells (1.8 fold increase; p<0.05) (Figure 5A). Similarly, no change in aspirin dependent MRP4 expression was observed in cells treated with PPAR-si, unlike those found in CTR-si transfected cells (1.8 fold increase; p<0.05) (Fi-

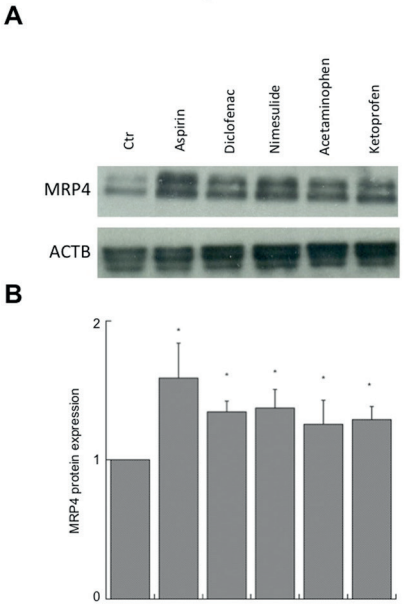

Figure 2. Aspirin and NSAIDs increase MRP4 protein expression in 12Z cells. ***(A)*** A representative Western Blot, of 3 performed, of MRP4 expression in 12Z cells treated with aspirin, diclofenac, acetaminophen, ketoprofen and nimesulide (50 μM for 48 h). ***(B)*** Densitometric analysis reported as the ratio between treated (aspirin, diclofenac, acetaminophen, ketoprofen and nimesulide) and untreated cells (Ctr). Data are reported as mean ±SD; statistical significance was evaluated using Student's t-test for unpaired samples (*p<0.05).

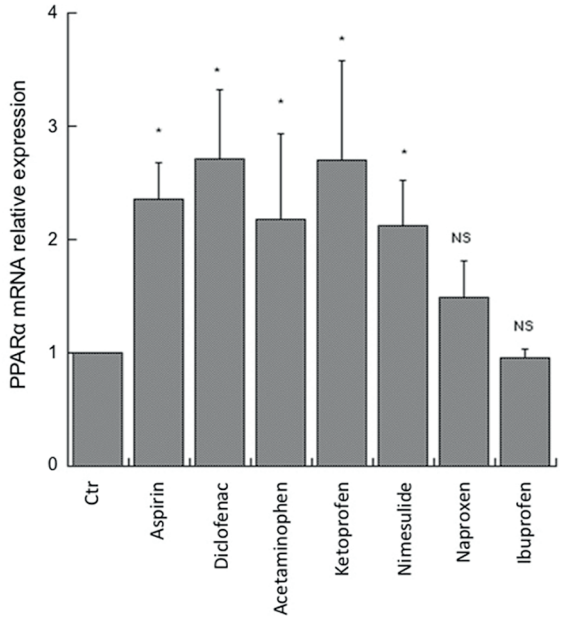

Figure 3. Aspirin and NSAIDs also augment endogenous PPARα mRNA expression in 12Z cells. Q-RT-PCR analysis of endogenous PPAR-alpha expression in 12Z cells treated with aspirin, diclofenac, acetaminophen, ketoprofen, nimesulide, naproxen and ibuprofen (50 μM for 48 h) compared to control culture (Ctr). Data were normalized with ACTB expression and reported as mean ±SD of 3 experiments (*p<0.05; NS: not significant; t-test).

gure 5B). To confirm that aspirin induces PPARα activation through COX1 inhibition, we studied diclofenac-induced PPARα-MRP4 up-regulation in siRNA transfected cells. Among all non-aspirin NSAIDs previously reported, diclofenac was chosen because it induces a similar increase in MRP4 expression to that caused by aspirin. Also in this case, no significant difference in PPARα mRNA expression in cells transfected with PPAR-si was observed after diclofenac treatment; while cells transfected with control, non specific siRNA (CTR-si), exhibited the same diclofenac induced expression changes as untransfected cells (2.1 fold increase; p<0.05) (Figure 5A). Similarly, no increase in diclofenac dependent MRP4 expression was detected in cells treated with PPAR-si, unlike those found in CTR-si transfected cells (1.8 fold increase; p<0.05) (Figure 5B).

NSAID-Induced MRP4 Expression Correlates with Increased PGE2 Secretion by Endometriotic Epithelial Cells

MRP4 may be relevant to endometriosis pathophysiology as increases extracellular secretion of PGE_2, which binds its receptors and activates various signaling pathways[13]. PGE_2 is long known to promote inflammation and pain, and it is considered to be a pivotal mediator for endometriosis development and progression[13,14]. To determine whether NSAIDs induced MRP4 up-regulation was functionally relevant in endometriotic cells, we measured PGE_2 in 12Z cell supernatants after 24 hours suspension of NSAID treatment (50 μM for 48 hours). In order to avoid a possible role of NSAID cell treatment in the inhibition of PGE_2 formation, PGE_2 levels were evaluated 48 hours after the last drug treatment, when the MRP4 expression is still high and prostaglandin H synthase activity is not affected by drug treatment. In fact, in treated cells, MRP4 protein expression after 48h was still up-regulated and prostaglandin H synthase activity was equal to untreated cells (data not shown). As shown in Figure 6, PGE_2 levels were significantly elevated after treatment with aspirin (1.75 fold increase; p<0.01), diclofenac (1.20 fold increase; p<0.05) and nimesulide (1.48 fold increase; p<0.05) compared to control cells (3.7± 1.1 pg/10^5 cells). These data demonstrate that NSAID-induced MRP4 enhancement is correlated with enhanced PGE_2 secretion.

Discussion

Patients with endometriosis frequently suffer from dysmenorrhea, periovulatory pain, dyspareunia, dyspareunia, chronic pelvic pain, dysuria, dyschezia, and leg pain. Schlincke in 1946 illustrated for the first time a case of endometriosis-induced sciatica, which is considered one of the most common causes in women affected by sciatica. Leg pain has been reported by 4% of women with chronic pelvic pain and it is described that 40% of women affected by endometriosis have leg pain[23,24]. Furthermore, leg pain is a common and disabling symptom related to endometriosis but it is still difficult to determine its cause. We recognize two main causes of endometriosis related leg pain: referred pain and neuropathic pain. Our studies proved that leg pain in patients with endometriosis may be caused to nerve injury or it may be a referred dysmenorrheal pain. The most commonly implicated nerve is the sciatic nerve. In 2013, we showed in a case-control study that pain generally affects the left leg (70% of patients versus 30% of patients in which pain interests the right leg) and it has a usual distribution in the crural and the lateral region of the thigh. In our study, 30% of women

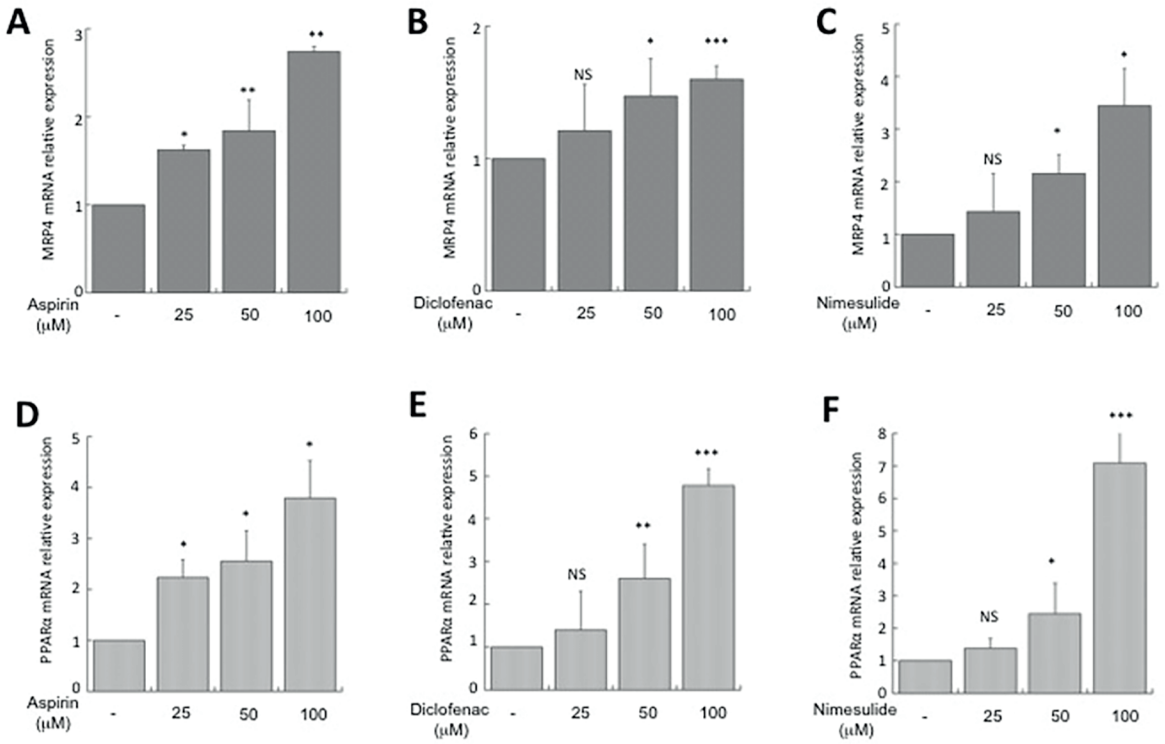

Figure 4. Scalar doses of Aspirin and NSAIDs stimulate endogenous MRP4 and PPARα mRNA expression in 12Z cells. **(A-C)** Q-RT-PCR analysis of endogenous MRP4 expression in 12Z cells treated with different doses of aspirin (A), diclofenac (B) and nimesulide (C) (25 μM, 50 μM and 100 μM for 48 h) compared to control culture (Ctr); data were normalized with ACTB expression and reported as mean ±SD of 2 experiments (*p<0.05, **p<0.01, ***p<0.0001, NS: Not Significant; t-test). **(D-F)** Q-RT-PCR analysis of endogenous PPARα expression in 12Z cells treated with different doses of aspirin (D), diclofenac (E) and nimesulide (F) (25 μM, 50 μM and 100 μM for 48h) compared to control culture (Ctr); data were normalized with ACTB expression and reported as mean ±SD of 2 experiments; (*p<0.05, **p<0.01, ***p<0.0001, NS: Not Significant; t-test).

demonstrated a decreased nociceptive and tactile sensibility in the crural region of the thigh and a positive Wasserman test, suggesting the hypothesis of femoral nerve involvement[25]. Therefore, endometriosis may be considered a debilitating disease characterized by chronic pain. This is the reason why patients with endometriosis often use non-aspirin Non-Steroidal Anti-Inflammatory Drugs (NSAIDs). Furthermore, it has been confirmed that Thrombin-activated fibrinolytic inhibitor (TAFI) is largely expressed in endometriosis and causes epithelial mesenchymal transition (EMT), which increases the cell proliferation and invasion[26]. MRP4 is expressed in the human endometrium and its expression can be up-regulated in ectopic endometrium of women suffering from endometriosis and modulated by the anti-inflammatory lipid Lipoxin A_4 (LXA_4)[13,18]. This transporter likely plays an important role in the pathogenesis of endometriosis, because it facilitates PGE_2 secretion. We recently demonstrated that aspirin and non-aspirin NSAIDs drugs induce platelet MRP4 over-expression through the activation of the nuclear receptor PPARα in megakaryocytes[18,19]. In this work we report for the first time that aspirin and other non-aspirin NSAIDs induce an enhancement of MRP4 expression in a human ectopic endometrial epithelial cell line (12Z cells). In addition we demonstrate that the nuclear receptor involved is PPARα. In fact, MRP4 and PPARα mRNA expression are enhanced in 12Z cells treated with aspirin. The involvement of PPARα in MRP4 up-regulation was confirmed by the fact that 12Z cells transfected with PPARα specific siRNA didn't show a significant increase of aspirin dependent on both PPARα and MRP4 mRNA up-regulation; while cells transfected with control non specific siRNA (CTR-si) show the same aspirin-induced MRP4 up-regulation. Arachidonic acid is metabolized through three different enzymatic pathways: cyclooxygenase, lipoxygenase and cytochrome P450 dependent. Both lipoxygenase and cytochrome P450 enzymes are insensitive to aspirin and non-aspirin NSAIDs action and they produce leukotriene B4 and 20-HETE metabolites

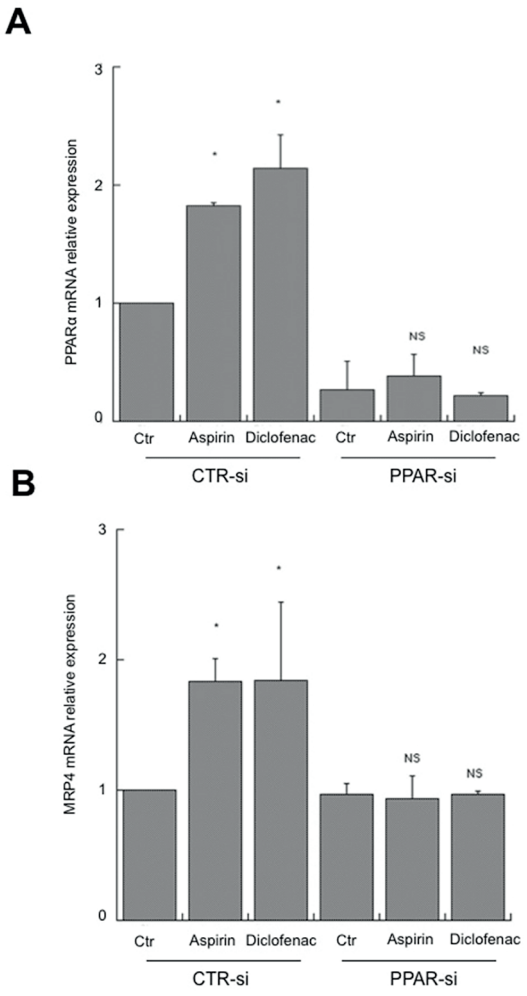

Figure 5. Aspirin and NSAIDs increase MRP4 via a PPARα dependent mechanism in 12Z cells. Control non-specific siRNA (CTR-si) and PPARα specific siRNA (PPAR-si) transfected 12Z cells were treated with either aspirin or diclofenac (50 μM for 48 h). ***(A)*** Q-RT-PCR analysis of endogenous PPARα expression; data were normalized with ACTB expression and reported as mean ±SD of 3 experiments (**p*<0.05, NS: Not Significant; *t*-test). ***(B)*** Q-RT-PCR analysis of endogenous MRP4 expression; data were normalized with ACTB expression and reported as mean ±SD of 3 experiments (**p*<0.05, NS: Not Significant; *t*-test).

respectively, whose production is higher in aspirinated cells[27,28]. These arachidonic acid metabolites, LTB4[29] and 20-HETE[30] are PPARα ligands. Thus we can hypothesize that, in 12Z cells, when COX-1 is inhibited PPARα is activated by lipoxygenase and cytochrome P 450 metabolites, leading to MRP4 up-regulation. This hypothesis is supported by the fact that in 12Z cells other anti-inflammatory inhibitors of COX-1 and COX-2 (diclofenac, nimesulide, acetaminophen and ketoprofen) caused increased MRP4 expression in comparison with untreated cells. In the same conditions the expression of the nuclear receptor PPARα is enhanced in treated cells compared to untreated cells. The involvement of PPARα in MRP4 up-regulation is confirmed in PPARα specific siRNA transfected 12Z cells. PPAR-si treated cells didn't show any significant increase in PPARα and MRP4 mRNA expression after diclofenac treatment; while cells transfected with control non specific siRNA (CTR-si) exhibited the same diclofenac-induced expression changes as untransfected cells. Among the anti COX-1 and COX-2 drugs analyzed, naproxen and ibuprofen did not increase MRP4 and PPARα expression in human ectopic endometrial epithelial cells, probably due to the fact that these drugs do not inhibit COX enzymes in an efficient manner[31]. Thus, we can affirm that *in vitro* treatment with aspirin and non-aspirin NSAIDs, except for naproxen and ibuprofen, caused a statistically significant increase of MRP4 expression and this modulation is PPARα dependent. With our results we can hypothesize that, in 12Z cells, when COX-1 and/or COX-2 are inhibited PPARα is activated by lipoxygenase and cytochrome P 450 metabolites, resulting in MRP4 up-regulation. It was previously suggested that MRP4 overexpression in ectopic tis-

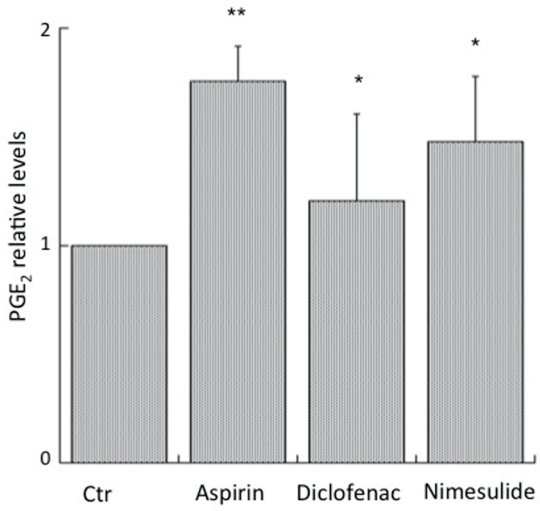

Figure 6. NSAIDs induce PGE_2 release by endometriotic epithelial cells. Prostaglandin (PG) E2 and its transporter multidrug resistance-associated protein 4 (MRP4) are upregulated in NSAID-treated cells in comparison to control cells. After treatment, supernatants were collected and PGE_2 content quantified using an EIA kit according to the manufacturer's instructions. PGE_2 levels (pg/10^5 cells) are reported as a ratio between treated and untreated cells (CTR: 3.7±1.1 pg/10^5 cells). Data represent the mean ± SD from 4 independent experiments (**p*<0.05, ***p*<0.01; *t*-test).

sue plays a pivotal role in endometriosis, because it facilitates PGE_2 secretion[13], as it was transported with high affinity by MRP4[32]. Prostaglandin E_2 is a pro-inflammatory lipid mediator derived from arachidonic acid metabolism which plays a pivotal role in endometriosis-associated inflammation and pain[33], and its production is augmented in lesions and in the peritoneal cavity[34,35]. NSAIDs are often the first-line treatment for endometriosis[36]. As the NSAID dependent MRP4 over-expression persists for a longer period than its capability to reduce COX-1 and/or COX-2 activity, we can suggest that after a high dose of NSAIDs there is a period in which the ratio between MRP4 expression and PGs production is unbalanced, leading to augmented PGs levels in peritoneal fluid and consequently increasing the risk of developing endometriosis. Lipoxin A_4 may reduce MRP4 over-expression by reducing PPAR-activation, and our results further support its capability to inhibit endometriosis progression in a mouse model[37]. Our suggestion was corroborated by the fact that 24 hours after drug removal from the medium, when MRP4 levels are still high, NSAIDs do not affect COX-1 and COX-2 activity and PGE_2 secretion is enhanced. These results indicate that the NSAID-induced MRP4 over-expression leads to enhancement of PGE_2 secretion. These PGE_2 increase may be important to promote endometriosis progression by affecting cell proliferation, angiogenesis and the immune response.

Conclusions

Many women use NSAIDs; treatment with NSAIDs was investigated because they are beneficial in women with primary dysmenorrhea and are relatively safe[36], but we showed that these drugs modify gene expression in endometrial cells. Our data further support the consideration of PPARα or MRP4 as potential targets for endometriosis therapy.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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