

NLRP3 inflammasome inhibitor MCC950 attenuates primary dysmenorrhea in mice via the NF-κB/COX-2/PG pathway

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Research

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Abstract

Background: Primary dysmenorrhea (PD) constitutes a common gynecological disease among young women. The NLRP3 inflammasome may be activated and expressed in PD, but the mechanistic link between NLRP3 inflammasome activation and PD is still unclear.

Methods: To investigate the potential role of NLRP3 inflammasome activation in the pathogenesis of PD, thirty female Kunming mice without pregnancy were used for experiments. The PD mouse model was constructed by 11 days of successive co-treatment with estradiol and oxytocin. MCC950, a potent and specific small-molecule inhibitor of the NLRP3 inflammasome, was used to treat PD mice. The disease level was assessed by the writhing response and hot water tail-flick test. The levels of prostaglandin E₂ (PGE₂) and prostaglandin F₂ alpha (PGF_{2α}) in the uterine tissues of mice were detected by ELISA. The expression levels of protein and cytokines, including NLRP3, cysteine aspartic acid-specific protease 1 (caspase-1), interleukin (IL)-1β, IL-18, nuclear factor kappa B (NF-κB) p65, phospho-NF-κB p65, and cyclooxygenase-2 (COX-2) were revealed by western blot analysis.

Results: MCC950 greatly ameliorated the writhing response induced by the combination of oxytocin and estradiol, with an increasing length of tail-flick latency. MCC950 also significantly decreased the levels of PGF_{2α} and PGE₂, and the expressions of NLRP3, caspase-1, IL-1β, IL-18, phospho-NF-κB p65, NF-κB p65, and COX-2 in the uterus.

Conclusions: MCC950 markedly alleviated the pain and pathological damage in PD mice by inhibiting NLRP3 activation. The underlying mechanism may be related to hypoactive uterine inflammation via suppression of NLRP3 activation and the NF-κB/COX-2/PG pathway in uteruses of PD mice.

Background

Primary dysmenorrhea (PD), a common gynecological disease among young women, constitutes functional dysmenorrhea without a definite gynecological pathological origin and seriously affects every aspect of the lives, work, and study of female patients [1]. Clinically, the main symptom of PD is menstrual pain.

Currently, excessive secretion of prostaglandins (PG) in uterine tissues has been proposed as a trigger for PD, though the mechanisms responsible for PD remain poorly understood [2]. Cyclooxygenase-2 (COX-2), a key enzyme involved in PG production, has its expression dramatically enhanced in the period of pre-menstruation. Subsequently, increased COX-2 converts arachidonic acid to PG, which induces spastic pain [3]. Nuclear factor kappa B (NF-κB) is a crucial factor for the regulation of COX-2 [4]. Increasing studies have shown that the NF-κB/COX-2/PG pathway plays a pathogenic role in PD, which suggests that therapeutic interventions of the NF-κB/COX-2/PG pathway can exert protective effects against PD [5, 6].

It has been reported that inflammation potently mediates the pathological process of PD. Activated macrophages produce a wide array of inflammatory cytokines, such as interleukin-1 β (IL-1 β), IL-6, and tumor necrosis factor α , which stimulate the synthesis and release of PG, leading to excessive contraction of uterine smooth muscles and ischemic injury in PD [7, 8]. As a core part of inflammation, the nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome is involved in a wide array of acute and chronic diseases, such as coronary heart disease, stroke, pneumonia, acute kidney injury, and viral hepatitis [9]. The assembly of the NLRP3 inflammasome as stimulated by NLRP3 irritants triggers proteolytic cleavage of dormant procaspase-1 into active caspase-1, which converts the cytokines pro-IL-1 β and pro-IL-18 into mature and biologically active IL-1 β and IL-18, respectively, leading to a cascade of deleterious inflammation [10]. Moreover, there is increasing evidence indicating that the NLRP3 inflammasome plays a regulatory role in the expression of COX-2 via the NF- κ B pathway [11, 12]. Our previous studies have found that the NLRP3 inflammasome can be activated and expressed in PD, and effective traditional electroacupuncture therapy to treat PD is associated with the inhibition of NLRP3 inflammasome activation [13]. However, the mechanistic link between NLRP3 inflammasome activation and PD is still unclear. Therefore, we constructed a PD mouse model to investigate whether inhibition of NLRP3 inflammasomes through the NF- κ B/COX-2/PG pathway exerts protective effects against PD induced by a combination of estradiol and oxytocin.

Materials And Methods

Animals

Thirty healthy female nonpregnant Kunming mice (18–22 g) were purchased from Hunan Slack Jingda Lab Animal Co., Ltd. [Hunan, China, SYXK (Xiang) 2013-0004] All animals were kept on natural circadian rhythms at optimal temperature (20°C–25°C) and humidity (50%–70%), with free access to water and food. After a week, the 30 mice were divided into three groups (Saline group, PD group, and MCC950 group), with 10 mice in each group. All experimental procedures were approved by the Instructive Notions with Respect to Caring for Laboratory Animals promulgated by the Ministry of Science and Technology of China in 2006.

Pharmaceutical and main reagents

Oxytocin was purchased from Shanghai Hefeng Pharmaceutical Co., Ltd. (No. H31020850, Shanghai, China), and estradiol (No. J20130009, Bayer, Germany) was purchased from Bayer. Both are approved by the State Food and Drug Administration (SFDA). Other reagents and materials included MCC950 (CP-456773, Selleck, USA; CAS number: 210826-40-7; formula: C20H24N2O5S; molecular weight: 404.48 g/mol), mouse PGF_{2 α} kit (production lot number: 516011, Cayman Chemical, USA), mouse PGE₂ kit (production batch number: 514010, Cayman Chemical, USA), BCA protein quantification kit (production lot number: A53225, Thermo, USA), NLRP3 antibody (production batch number: NBP2-12446, Novus Biological, USA), phospho-NF- κ B-p65 antibody (production batch number: #3033, Cell Signaling Technology, USA), NF- κ B-p65 antibody (production lot number: #4746, Cell Signaling Technology, USA),

IL-1 β antibody (production batch number: ab9787, Abcam, UK), IL-18 antibody (production lot number: ab191860, Abcam, UK), caspase-1 antibody (production batch number: NBP1-45433, Novus Biological, USA), beta-actin mouse monoclonal antibody (production lot number: #4211, Cell Signaling Technology, USA), goat anti-rabbit secondary antibody (production batch number: AP132P, Merck Millipore, Germany), and goat anti-mouse secondary antibody (production lot number: AP124P, Merck Millipore, Germany).

Construction of the PD mouse model

Mice in the Saline group were given 0.2 ml saline by gavage at 18:00 for 10 days and intraperitoneally injected with 0.2 ml saline on the 11th day. To mimic PD *in vitro*, mice in the PD group and MCC950 group were given estradiol (0.2 mg/ml, 0.2 ml/day) by gastric gavage at 18:00 for 11 days. Intraperitoneal injection of 0.2 ml MCC950 (2 mg/ml) was given to mice in the MCC950 group. On the 11th day, both groups were given estradiol via intraperitoneal injection and 0.2 ml oxytocin (2 U/ml) within an hour [14]. All mice were fasted for 12 h before gavage. After daily intervention, they had free access to water and food.

Measurement of writhing times

To assess the writhing response, we recorded 20-min writhing times and the writhing latency of mice. (Writhing response: the abdomen of the mouse was contracted and concave with straight hind limbs; writhing latency: from intraperitoneal injection of oxytocin to the first writhing reaction [15].)

Thermal tail-flick test

The thermal tail-flick test of acute pain was performed on the 11th day after pharmaceutical injection. Each experimental mouse was placed in a fixator with the tail exposed. When it was stable, 1/3rd of the tail was placed in hot water at 52°C. The tail-flick latency (from the tail entering the water to exiting) was measured with a stopwatch (accuracy: 0.01 s) for 4 consecutive times each with an interval of 1 min [16]. Each tail was measured in triplicate, and the average value was calculated.

Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of PGF_{2 α} and PGE₂ in uterine tissues were determined with an ELISA. The supernatant including total protein (TP) was obtained by homogenization and centrifugation from the same segment of ipsilateral uteruses of 5 mice randomly selected in each group. Each sample was incubated overnight with diluent and standard solution at 4°C. Subsequently, the plate was washed and Ellman's reagent was added, it was reacted at 4°C in the dark for 60 min, and the absorbance value of each hole at 412 nm was measured. The concentration of PGF_{2 α} was determined by the standard curve according to absorbance value of the standard hole and its corresponding concentration. The TP in collected supernatant was quantified by a BCA protein quantitative kit. Each sample was added with prepared standard compound based on the gradient method and incubated with working solution at 37°C for 30 min, then the absorbance value of each hole at 412 nm was measured. The concentration of TP was determined by the

standard curve according to absorbance value of the standard hole and its corresponding concentration. The ratio between PGF_{2α}/PGE₂ and TP indicated the content of PGF_{2α} in the supernatant of uterine homogenate. All procedures were according to the manufacturer's protocol.

Western blotting

The supernatant containing TP of the collected mouse uterine tissues was separated by homogenization using liquid nitrogen and 1 ml cold lysis buffer for 10 min, followed by centrifugation at 4°C and 12,000 r/min for 10 min. To fully denature TP, the supernatant was added with sample buffer and heated in a boiling water bath for 10 min. The calculation and balance of protein concentration, together with the protein standard curve, was based on protein quantification via a BCA protein quantification kit. Samples were subjected to electrophoresis, membrane transfer, and blocking. Immunoblot analysis was performed with specific primary antibodies followed by secondary antibody: NLRP3 (1:1000), caspase-1 (1:500), IL-1β (1:1000), IL-18 (1:1000), phospho-NF-κB p65 (1:1000), NF-κB p65 (1:1000), COX-2 (1:1000), or β-actin (1:5000). The membrane was then stripped and incubated with β-actin as an internal control. The intensity of chemiluminescence exposed by ECL was measured by Quantity One gray analysis software.

Statistical analysis

All data were analyzed using the SPSS version 22.0 statistical analysis package. All data are expressed as means ± standard deviation (S.D.) and were analyzed by two-tailed, unpaired, Student's *t*-test or ANOVA if appropriate. A value of *p* < 0.05 was considered statistically significant.

Results

MCC950 inhibited the activation of NLRP3 inflammasomes in uterine tissues of mice with PD

Uterine tissues were examined by western blotting to detect the levels of NLRP3, caspase-1, IL-1β, and IL-18. Western blotting analyses of the uterine tissues of saline-injected mice showed low levels of NLRP3, caspase-1, IL-1β, and IL-18. In contrast, within 20 min after oxytocin injection, the expressions of NLRP3, caspase-1, IL-1β, and IL-18 in uterine tissues of the PD group increased significantly (Fig. 1, *P* < 0.01), but they did not increase in the MCC950 group (Fig. 1, *P* < 0.01).

MCC950 reduced writhing times and prolonged writhing latency and tail-flick latency in mice with PD

To investigate whether NLRP3 inflammasome activation promotes PD pain, we first examined the effect of an NLRP3 inhibitor on PD induced by estradiol and oxytocin in mice. There was no writhing response in the Saline group, while mice in the PD group and MCC950 group showed alert writhing responses. Within 20 min after oxytocin injection, the writhing response numbers of the PD group significantly increased (Fig. 2A, *P* < 0.01) with abbreviated tail-flick latency (Fig. 2C, *P* < 0.01) compared with the Saline group. Compared with the PD control group, the writhing numbers of the MCC950 group were

significantly decreased (Fig. 2A, $P < 0.01$), and the writhing latency and tail-flick latency were increased (Fig. 2B and Fig. 2C, $P < 0.01$) within 20 min (Fig. 2).

MCC950 inhibited production of PG in uterine tissues of mice with PD

PG is known to play a role in PD as a mediator of inflammation. We next tested whether an NLRP3 inhibitor can regulate PG in the presence of PD. Compared with the Saline group, PGF_{2α} and PGE₂ in the PD group were significantly increased (Fig. 3A, $P < 0.05$ or $P < 0.01$). Compared with the PD group, the levels of PGF_{2α} and PGE₂ in the MCC950 group were decreased (Fig. 3B, $P < 0.05$).

MCC950 decreased the level of COX-2 in uterine tissues of mice with PD

Next, we determined the effect of NLRP3 inflammasome inhibition on COX-2 as an upstream regulatory protein of PG in PD mice. As expected, the protein level of COX-2 in the uterine tissues of mice in the Saline group was very low and could not be detected by western blotting. Compared with the Saline group, the COX-2 protein level in the uterine tissues of the PD group was significantly increased (Fig. 4, $P < 0.01$). Subsequently, we confirmed that the levels of COX-2 were decreased significantly in the MCC950 group compared with those of PD group (Fig. 4, $P < 0.01$).

MCC950 suppressed activation of the NF-κB pathway in uterine tissues of mice with PD

To confirm whether the effect of NLRP3 inflammasomes on PD-induced pain was related to NF-κB p65, we investigated NF-κB p65 phosphorylation by western blotting. Compared with the Saline group, the protein levels of phospho-NF-κB p65 and NF-κB p65 in the PD group were significantly increased (Fig. 5, $P < 0.01$). Compared with the PD group, the protein levels of phospho-NF-κB p65 and NF-κB p65 in the MCC950 group were decreased significantly (Fig. 5, $P < 0.01$).

Discussion

It has become clear that the NLRP3 inflammasome is involved in a broad spectrum of gynecological diseases such as cervical cancer, preterm labor, postpartum inflammation, and mycoplasma and chlamydia infection [17, 18]. Our study found that NLRP3, caspase-1, IL-1β, and IL-18 were dramatically increased in mice with PD, which could be reversed by intervention with MCC950, a specific inhibitor of NLRP3. These results suggested that the NLRP3 inflammasome is expressed and activated in the uteruses of PD mice, which may offer substantial promise in developing new therapeutics for PD. Emerging evidence suggests that NLRP3 inflammasome activation occurs in endometritis cells, while inhibiting NLRP3 inflammasomes generates a significant ameliorative role in the pathological process of endometritis [19]. Moreover, the activation of the NLRP3 inflammasome aggravates the degree of inflammatory infiltration in gynecological diseases such as endometritis and pelvic inflammatory disease, which indicates that therapeutic interventions via the NLRP3 inflammasome may be a novel strategy for treating gynecological diseases [20, 21]. However, the role of the NLRP3 inflammasome in PD is still obscure, so further investigation is warranted.

In our study, mice in the PD group presented obvious writhing responses via administration of oxytocin. In contrast, writhing times were significantly decreased and tail-flick latency was noticeably elongated in the MCC950 group. The writhing response measures pain in a direct way, and the tail-flick latency indirectly evaluates pain threshold changes [22, 23]. The results suggested that NLRP3 suppression exerted an important role in both the anti-inflammatory and analgesic response against PD. Further, we concentrated on the NF- κ B/COX-2/PG pathway to elucidate the potential mechanism of PD.

The PGF_{2 α} and PGE₂ levels of mice uteruses in the PD group robustly increased, while they significantly decreased through MCC950 intervention. It has been reported that both PGF_{2 α} and PGE₂ are the primary members of prostaglandins that play a critical role in the inflammatory process. The levels of PGF_{2 α} and PGE₂ in the uterine tissues and peripheral blood of PD patients are higher than normal [24]. Additionally, numerous studies have illustrated that high levels of PGF_{2 α} lead to pathological pain responsible for uterine contraction, hematological alterations, and the accumulation of acidic metabolites [25]. In this study, we found that PGF_{2 α} and PGE₂ in uterine tissues of PD mice markedly decreased by inhibiting NLRP3 inflammasomes.

COX-2, an important limiting rate enzyme participating in PG synthesis, exhibits a tight relationship with uterine inflammation [26], while upstream, NF- κ B mediates the expression and activation of COX-2. We measured the levels of NF- κ B and COX-2 in the uterine tissues of mice after MCC950 intervention. The results showed that COX-2 in the Saline group was scarce, while it significantly increased in the uterine tissues of PD mice. Furthermore, MCC950 intervention reversed the potent increase of COX-2 protein levels in PD mice. Under physiological conditions, COX-2 exists at a very low level, but it is elevated by inflammatory stimuli. Studies have shown that the expression of COX-2 goes up before menstruation, which catalyzes arachidonic acid into active PG, resulting in spasmodic pain. Nonsteroidal anti-inflammatory drugs, such as ibuprofen, play a profound role in treating the symptoms of PD via COX-2 inhibition with a subsequent decrease of PG [27].

As an important transcriptional regulator, NF- κ B plays a crucial role in PD via regulation of COX-2 [28]. As detected by western blot analysis, the levels of phospho-NF- κ B p65 and NF- κ B p65 significantly increased in the uterine tissues of mice with PD. MCC950 intervention strongly attenuated the activation of phospho-NF- κ B p65 and NF- κ B p65 in PD mice, which indicated that the NF- κ B pathway plays a pathogenic role in PD, and that inhibiting NLRP3 inflammasomes could downregulate NF- κ B activation. It is well known that the NF- κ B/COX-2/PG pathway mediates PD [29], and some studies have shown that the NLRP3 inflammasome upregulates NF- κ B activation and promotes COX-2 expression [30]. Therefore, the NLRP3 inflammasome might be involved in the pathological progression of PD through the NF- κ B/COX-2/PG pathway.

Conclusions

In summary, activation of NLRP3 inflammasomes induced deleterious effects in PD mice, and therapeutic interventions targeting NLRP3 inflammasomes via the NF- κ B/COX-2/PG pathway mitigated the

agonizing pain responsible for PD. This novel finding of an inflammatory effect of NLRP3 inflammasomes in PD contributes to our understanding of the disease and provides a promising new target for PD intervention.

Abbreviations

PD: Primary dysmenorrhea;

NLRP3: Nucleotide-binding oligomerization domain-like receptor protein 3;

Caspase-1: Cysteinyl aspartate-specific protease 1;

IL-1 β : Interleukin-1 β ;

IL-18: Interleukin-18;

NF- κ B: Nuclear factor kappa B;

COX-2: Cyclooxygenase-2;

PGE₂: Prostaglandin E₂;

PGF_{2 α} : Prostaglandin F₂ alpha;

ELISA: Enzyme-Linked Immunosorbent Assay;

TP: Total protein;

Declarations

Acknowledgments

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Author contributions

Biao Tang: designed and conducted the experiments, analyzed and interpreted the data, and wrote the main manuscript;

Dan Liu: conceived and wrote the main manuscript;

Lingyu Chen: conducted the experiments and analyzed and interpreted the data;

Yu Liu: conducted the statistical analyses.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon request.

Ethics approval and consent to participate

All procedures were approved by the Institutional Animal Care and Use Committee at Hunan University of Chinese Medicine.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflicts of interest.

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Figures

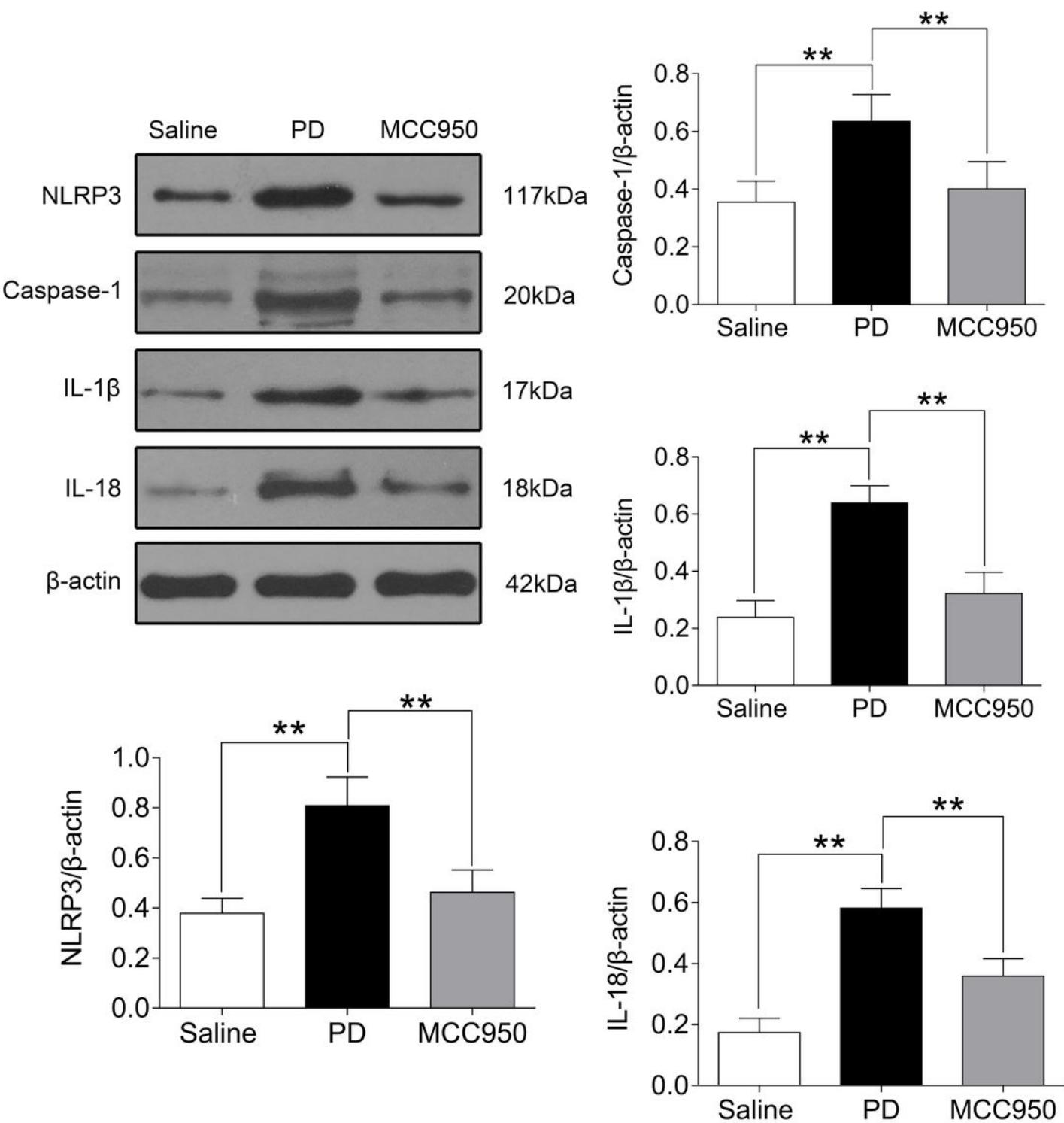


Figure 1

Comparison of NLRP3, caspase-1, IL-1 β , and IL-18 proteins in mouse uteruses among groups ($x \pm s$, $n = 5$). ** $P < 0.01$ compared to the PD group.

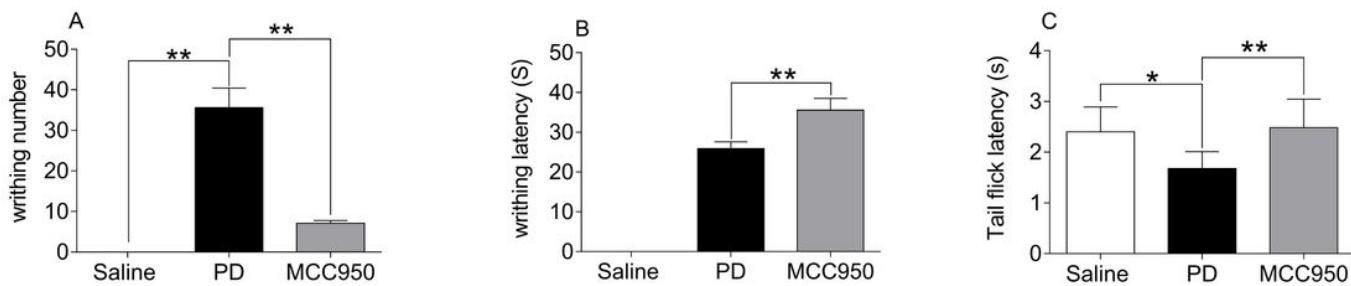


Figure 2

Comparison of writhing number in 20 min, writhing latency, and tail-flick latency of mice among groups ($x \pm s$, $n = 10$). A: writhing number, B: writhing latency, C: tail-flick latency. * $P < 0.05$, ** $P < 0.01$ compared to the PD group.

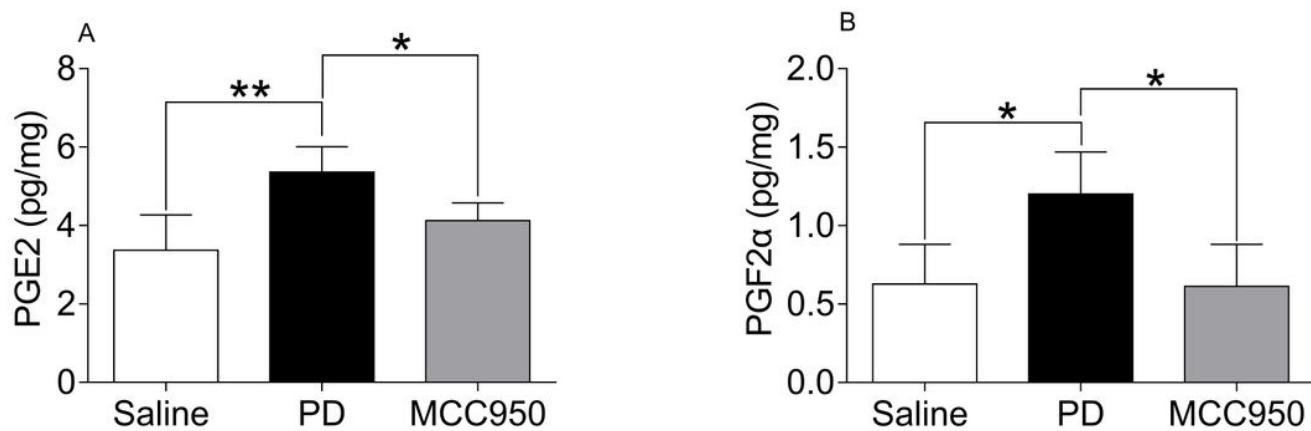


Figure 3

Comparison of PGE2 and PGF2 α in mouse uteruses among each group ($x \pm s$, $n = 5$). A: PGE2, B: PGF2 α . * $P < 0.05$, ** $P < 0.01$ compared to the PD group.

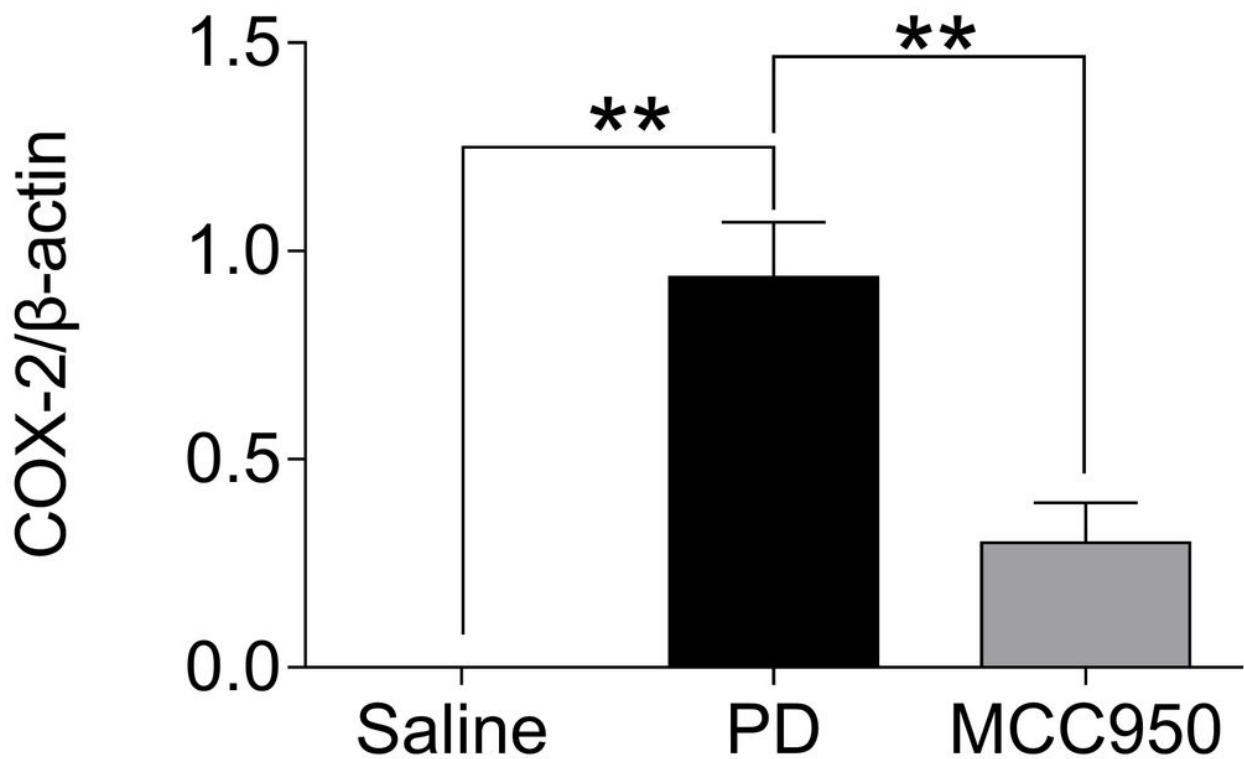
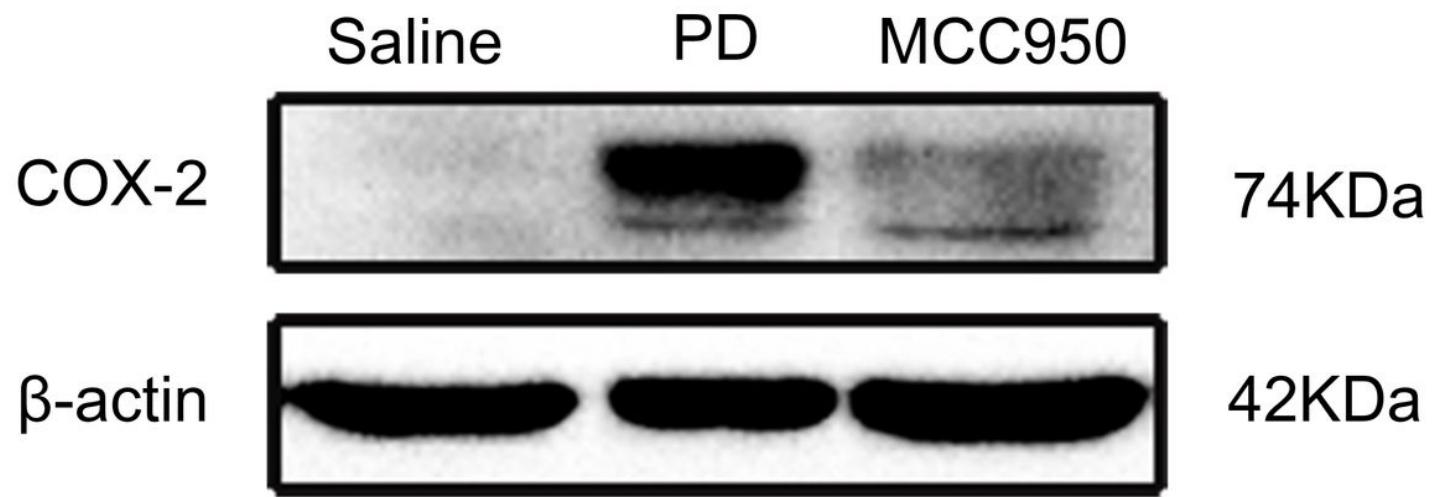


Figure 4

Comparison of COX-2 protein expression in mouse uteruses among groups ($x \pm s$, $n = 5$). ** $P < 0.01$ compared to the PD group.

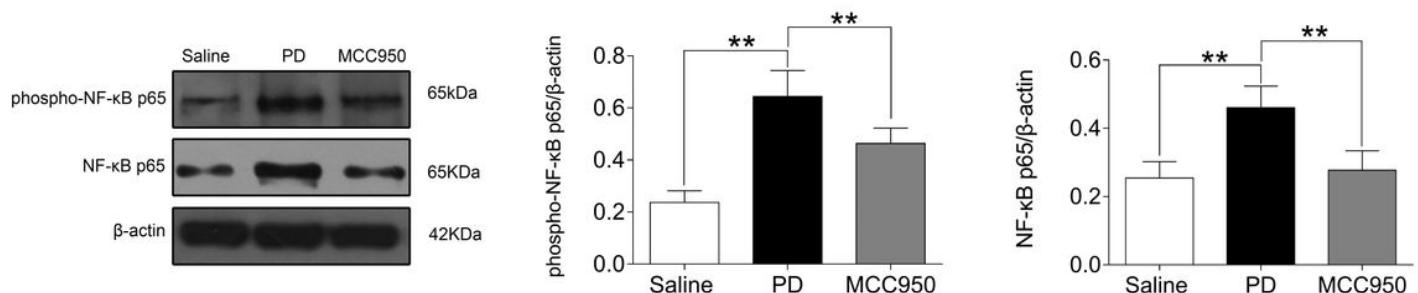


Figure 5

Comparison of phospho-NF-κB p65 and NF-κB p65 protein expression in mouse uteruses among groups ($\bar{x} \pm s$, $n = 5$). ** $P < 0.01$ compared to the PD group.