

# Liriodendrin alleviates sciatic endometriosis-associated pain in rats via suppressing the inflammatory response and regulating the signaling pathway of PI3K/Akt/mToR

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**Submitted:** 31 May 2021; **Accepted:** 28 October 2021

**Online publication:** 7 November 2021

Arch Med Sci

DOI: <https://doi.org/10.5114/aoms/143423>

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## Abstract

**Introduction:** LY294002 has been validated as a PI3K pan-inhibitor in the pathogenesis of airway inflammation, which attenuated IL-25-induced asthma-like AHR. Liriodendrin was proved to play essential roles in attenuating endometriosis-associated pain. This work aimed to gain a mechanical insight into the therapeutic efficiency of liriodendrin administration in attenuating endometriosis-associated pain.

**Material and methods:** The von Frey filament test and thermal hyperalgesia test were carried out to evaluate the acute and daily efficiency of drug administration. Western blot was used to analyze the expression of substance P, PI3K/AKT/mTOR, p-PI3K/p-AKT/p-mTOR, and CGRP. ELISA was performed to examine interleukin (IL)-1, IL-2, IL-6, TNF- $\alpha$  and PGE2 levels.

**Results:** As the results showed, liriodendrin significantly attenuated pain in endometriosis rats and restored the up-regulation of p-PI3K/p-AKT/p-mTOR in the endometriosis rats. The increased IL-1, IL-2, IL-6, TNF- $\alpha$ , and PGE2 levels were remarkably restored by liriodendrin in endometriosis rats. Moreover, the activated expression of substance P in the ventral horn of the spinal cord of endometriosis rats was notably restored by liriodendrin in addition to CGRP. Furthermore, liriodendrin effectively restored the lipopolysaccharide (LPS)-induced up-regulation of p-PI3K/p-AKT/p-mTOR protein as well as the LPS activated IL-6, TNF- $\alpha$ , and IL-1 $\beta$  mRNA and protein expression in 12Z cells.

**Conclusions:** We found that compared with LY294200, liriodendrin exerted a more evident therapeutic effect in the treatment of endometriosis-associated pain by suppressing the secretion of pro-inflammatory cytokines.

**Key words:** liriodendrin, endometriosis, pain, inflammation, PI3K, Akt, mToR.

## Introduction

The presence of stromal or glandular cells on the edge of the uterine cavity is a defining characteristic of endometriosis [1]. Chronic pain such as radicular pain, back pain, dyspareunia, and dysmenorrhea affects about 5–10% of young women [2, 3]. Endometriosis affecting the sciatic nerve is a very rare condition [4]. The current treatment options include extracting the ectopic lesions and administration of hormones and non-steroidal anti-inflammatory drugs (NSAIDs) [5]. Nonetheless, the current treatment options have limited efficacy and unwanted side effects [6].

Normal cellular functions such as metabolism, survival and growth are regulated by two signaling pathways: the phosphatidylinositol-3 kinases (PI3Ks) and the mammalian target of rapamycin (mTOR) [7, 8]. Both the pathways are interconnected by a type of serine/threonine kinase-like AKT [9]. Protein kinase B, also known as AKT, plays a role in several cellular functions via PI3K and mTOR [10]. Stimuli from different types of receptors, such as antigen receptors, cytokine receptors, insulin receptors, or the insulin-like growth factor I receptor, activate the PI3K and mTOR pathways. Upon activation, PI3K causes phosphorylation of the phosphorylate phosphatidylinositol 4,5-bisphosphate (PIP2), which produces a second messenger, phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 further stimulates downstream pathways such as AKT. Elevated levels of the PI3K/Akt/mTOR pathway components were observed in the eutopic endometrium of women with endometriosis [11]. The multilevel signaling pathway involving PI3K, Akt and mTOR correlated well with pain and endometriosis [12].

Deying Dai, an expert in Chinese medicine, invented the prescription formula of *Caulis Sargentodoxae* prescription. The formula was found to be effective against EMs (90%), menstrual fever (90.63%) and dysmenorrhea (96.25%) [13]. Extensive research has been performed to assess its effects in ulcerative colitis in humans. *Sargentodoxa cuneata* is very effective for treating several inflammation conditions affecting the appendix, stomach and rheumatic arthritis. However, the mechanism of action of the medicine remains elusive. Another example of traditional medicine called liri dendrin, a type of lignin, has been studied for its therapeutic effects. This disaccharide lignan named liri dendrin, (+)-syringaresinol di-O-b-D-glucopyranoside, is the bioactive component of *Sargentodoxa cuneata* (Oliv.) Rehd. Et Wils, and a plethora of biological effects against inflammation, pain, and arrhythmia have been observed for the medicine [14–16]. This medicine has shown efficacy against disorders such as arrhythmia, myocardial ischemia, inflammation and

oxidative stress [14, 17]. Moreover, according to previous findings, liri dendrin administration restored the elevation of neuropeptides in the ventral horn of the spinal cord of ENDO rat models. Upon oral administration liri dendrin is transformed to syringaresinol, which is attributable to the anti-inflammatory effect [17]. In various hormone-dependent conditions, the phytoestrogen syringaresinol binds selectively to the estrogen receptors [18].

The proposed mechanism of action of liri dendrin involved reduced secretion of inflammation-inducing cytokines such as interleukin (IL)-6, tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and IL-1 $\beta$  in the colon tissues. A similar decrease in proinflammatory cytokines was observed in DSS-induced colon damage models. In the intestine, a link was observed between the nuclear factor  $\kappa$ B (NF- $\kappa$ B) cascade and the AKT signaling at transcription and translation levels [19, 20]. Liri dendrin blocks protein-1 and/or NF- $\kappa$ B in SW982 human synovial sarcoma cells [21].

Based on the above-mentioned evidence, LY294002 may alleviate endometriosis-associated pain via regulating PI3K signaling, and liri dendrin as an extract of *Sargentodoxa cuneata* may also inhibit PI3K signaling, and in addition, liri dendrin may also suppress the inflammatory response which is also involved in endometriosis-associated pain. We aimed to validate our hypothesis that the administration of liri dendrin could exhibit a therapeutic effect in the treatment of endometriosis-associated pain, the efficacy of which is supposed to be better than LY294002 in endometriosis. And the novelty of our study was to compare the efficacy of liri dendrin and LY294002 in the treatment of endometriosis.

## Material and methods

### Animal and treatment

Female SD rats with ages ranging between fourteen and eighteen weeks and weights in the range of 180–220 g were purchased from Charles River Laboratories Portage, MI. The rats were housed under alternating cycles of dark (12 h) and light (12 h) at room temperature and humidity near 70%. The rats were allowed access to food and water. Animals were divided into four groups with 8 rats in each group, i.e.: 1) SHAM group (group of animals received the sham operation), 2) ENDO group (group of animals were subjected to the surgical procedure and established as endometriosis rat model), 3) ENDO + LY294002 group (endometriosis rat models subjected to the surgical procedure to create a rat model of endometriosis and received i.p. injection of LY294002) and 4) ENDO + liri dendrin group (endometriosis

rat models subjected to the surgical procedure to create a rat model of endometriosis and received oral administration of liriodendrin). Models of sciatic nerve endometriosis were created using previously reported procedures [22].

Irrespective of the estrous cycle of the female rats, the autologous uterine tissue was grafted near the site of the sciatic nerve. Initially, the rats were put under anesthesia using isoflurane then a 2 cm long incision was created near the lower abdomen which exposed the right uterine horn. Later, a 5 mm length of uterus tissue was dissected and extracted from the horn region of the uterus. It was kept in a Petri dish which was preloaded with a phosphate buffer solution containing 1% penicillin and streptomycin. Subsequently, the right sciatic nerve was revealed on the rats positioned on the left side and an incision of 1 cm long near the thigh was made. The connective tissues near the desired area were cautiously separated. The longitudinal section of the uterus was opened and tied around the nerve with uterus endometrial tissue next to the nerve. In the case of rats that received the Sham operations, no implant was installed near the exposed right sciatic nerve. After the surgical procedure, 6.0-grade silk thread was used to suture the incision, and to prevent the infection gentamicin (50 mg/ml, 0.2 ml, Thermo Fisher Scientific, Waltham, MA) was injected into the stomach. In group 3, the dose of LY294002 was 15 mg/ml, which was dissolved in DMSO. A dose of 30 mg/kg was administered to the animals via a microsyringe into the i.t. tubing. In group 4, 100 mg/kg liriodendrin was pretreated about 3 days before the surgery. The animal experiment procedures were approved by the institutional ethical committee.

### Behavioral testing

To assess the behavior of the rats, the von Frey filament test was used. Briefly, the von Frey filament test was performed following the standard protocol, which involves applying mechanical stimuli to measure the hypersensitivity to mechanical stress. The key factor to measure was the paw withdrawal threshold. A series of ten von Frey filaments of equal logarithmic bending force was used in a sequence. To perform the test, the rats were allowed to habituate on the wire gauge surface for 15 min. To analyze the thermal hyperalgesia, a heat source was applied to the hind paw of the rats. The heat source was made up of a lamp fitted with a high-intensity bulb of power 50 W, and a voltage of 8 V. The exposure limit was decided to be 20 s so that the rats did not get skin burns. Following these behavior tests, the effects of LY294002 following the sciatic endometriosis procedure were evaluated.

### Procedure for abdominal puncture

The medicine was administered orally to rats for 21 days and after that 10 ml of PBS buffer was filled into the stomach cavity of rats under anesthesia with the help of a syringe. The rats were shaken gently for 10 min and a puncture was made into the abdominal wall of the rats using a needle. The peritoneal fluid was extracted with the help of a needle. Later, the rats were euthanized, and the samples were collected. The upper clear layer of the peritoneal fluid and blood samples were obtained and refrigerated at -80°C.

### Enzyme-linked immunosorbent assay

The peritoneal fluid samples and blood samples were extracted and stored at 2–8°C for a maximum of 5 days. Then, substance P, IL-1, IL-2, IL-6, TNF- $\alpha$ , PGE2, and CGRP levels were analyzed using the protocol provided with the kit in accordance with published methods [23]. The antibody was incubated and procedures for washing were performed based on the published methods. The absorbance of the ELISA experiments was recorded using the absorbance microplate reader (Molecular Devices, San Jose, CA).

### Cell culture

In this study, we established our cell models with 12Z cells (Applied Biological Materials (abm) Inc., Canada), which were an immortalized human endometriotic cell line. The cells were cultured in 37°C and 5% CO<sub>2</sub> in Roswell Park Memorial Institute 1640 medium containing 10 mM HEPES and other antibiotics according to previously published methods [24]. The cells were transferred into 6-well plates and cultured until they were confluent. To these cells, the drugs at the desired concentration were added to the wells. The cells were divided into four groups: 1) negative control group, 2) LPS group; the cells were stimulated with 100 ng/ml of lipopolysaccharide (LPS), 3) LPS + LY294002 group; the cells were treated initially with 10  $\mu$ M LY294002 and 6 h later with 100 ng/ml of LPS, and 4) LPS + liriodendrin group; the cells were pretreated with 50  $\mu$ M liriodendrin and 6 h later treated with 100 ng/ml of LPS. All experimental procedures were done four times using the above-mentioned protocol.

### RNA isolation and real-time PCR

The authors performed a polymerase chain reaction test using the RNA extraction kit obtained from Thermo Fisher Scientific. The genetic material was removed from the samples using the kit. The reverse transcription of the RNA material in the samples into the cDNA was carried out using

the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Later, the quantitative RT-PCR was carried out using the iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA). The polymerase reaction was performed with Applied Biosystems Real-Time PCR Instruments (Thermo Fisher Scientific, Waltham, MA). The relative expression of substance P, IL-6, TNF- $\alpha$ , IL-1 $\beta$ , NF- $\kappa$ B and CGRP was calculated relative to the reference standard genes by the  $2^{-\Delta\Delta Ct}$  method following published protocols [25].

### Western blot analysis

The Western blot analysis was performed according to published protocols [26]. The cultured cells and tissue samples obtained from the decapitated animals were first washed with PBS buffer. Radioimmunoprecipitation buffer was used as a lysed buffer to treat the cells and tissues. This procedure was used to extract the enclosed proteins from the cells and tissue samples. The mixture of proteins obtained from the samples was separated into bands using 10% SDS-polyacrylamide gel. Then, the total protein content isolated from the cells was resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel using an electrophoresis system (VWR, Radnor, PA). Later, the separated bands were expanded on a PVDF membrane and the reaction was blocked by skim milk. Subsequently, antibodies for CGRP (Cat#ab47207, Abcam, Cambridge, UK), PI3K (Cat#ab32089, Abcam, Cambridge, UK), AKT (Cat#ab8805, Abcam, Cambridge, UK), mTOR (Cat#ab134903, Abcam, Cambridge, UK), p-PI3K (Cat#ab278545, Abcam, Cambridge, UK), p-AKT (Cat#ab38449, Abcam, Cambridge, UK), p-mTOR (Cat#ab109268, Abcam, Cambridge, UK), substance P (Cat#ab14184, Abcam, Cambridge, UK), TNF- $\alpha$  (Cat#ab183218, Ab-

cam, Cambridge, UK) and IL-1 $\beta$  (Cat#ab254360, Abcam, Cambridge, UK) were applied to the protein bands followed by the secondary antibodies.

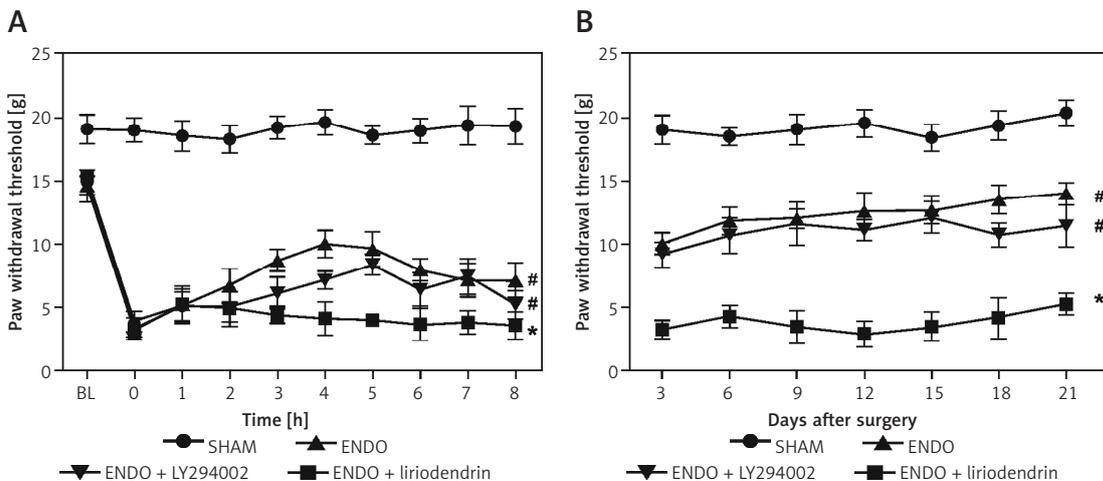
### Statistical analysis

Statistical analysis was carried out by making use of SPSS 19.0 software (IBM, Chicago, IL). One-way ANOVA and Student's *t*-tests were used to compare intergroup differences. Tukey's test was used as the post hoc test following one-way ANOVA. All statistical tests were two-sided, while the *p*-values of < 0.05 indicated statistical significance.

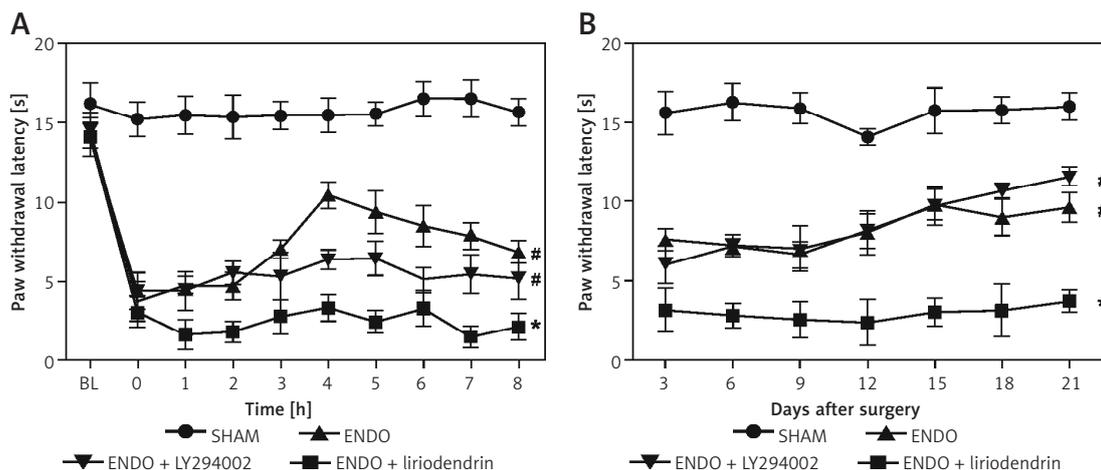
## Results

### LY294002 or lirioidendrin administration attenuated the increased pain in endometriosis rat models

The paw withdrawal threshold was significantly lower in ENDO rats when compared with the control. LY294002 and lirioidendrin administration remarkably maintained the paw withdrawal threshold in ENDO treated rats, indicating the efficiency of acute drug administration (Figure 1 A). Moreover, daily drug administration efficiency was evaluated every three days using the von Frey filament test. LY294002 and lirioidendrin administration showed considerable efficiency in restoring the reduction in the paw withdrawal threshold in the ENDO group (Figure 1 B). Additionally, a thermal hyperalgesia test was used to examine the efficiency of acute and chronic drug administration. LY294002 and lirioidendrin administration showed considerable efficiency in restoring the paw withdrawal latency loss in rats from the ENDO group in both acute drug administration (Figure 2 A) and daily drug administration (Figure 2 B) efficiency evaluation.



**Figure 1.** Chemical structure of lirioidendrin and the efficiency of acute/daily drug administration evaluated by von Frey filament test (\**p* < 0.05 vs. SHAM group; #*p* < 0.05 vs. ENDO group). **A** – Acute LY294002 and lirioidendrin administration remarkably maintained the paw withdrawal threshold in ENDO-treated rats. **B** – Daily LY294002 and lirioidendrin administration remarkably maintained the paw withdrawal threshold in ENDO-treated rats



**Figure 2.** Acute/daily drug administration efficiency evaluated using thermal hyperalgesia test (\* $p < 0.05$  vs. SHAM group; # $p < 0.05$  vs. ENDO group). **A** – Acute LY294002 and liriodendrin administration remarkably maintained the paw withdrawal latency in ENDO-treated rats. **B** – Daily LY294002 and liriodendrin administration remarkably maintained the paw withdrawal latency in ENDO-treated rats

#### LY294002 and liriodendrin administration restored the up-regulation of p-PI3K/p-AKT/p-mTOR in the endometrium of rats with endometriosis

The expression of PI3K/AKT/mTOR and p-PI3K/p-AKT/p-mTOR in the endometrium of rat groups was investigated (Figure 3 A). No obvious difference was observed for the expression of PI3K (Figure 3 B), AKT (Figure 3 C) and mTOR (Figure 3 D) in the endometrium of rats under distinct treatments. However, the expression levels of p-PI3K (Figure 3 E), p-AKT (Figure 3 F) and p-mTOR (Figure 3 G) were remarkably higher in the endometrium of ENDO rats when compared with the control. LY294002 and liriodendrin administration notably decreased the up-regulation of p-PI3K (Figure 3 E), p-AKT (Figure 3 F) and p-mTOR (Figure 3 G) in the endometrium of rats with endometriosis.

#### Liriodendrin administration restored the elevation of IL-1, IL-2, IL-6, TNF- $\alpha$ and PGE2 in rats with endometriosis

The IL-1, IL-2, IL-6, TNF- $\alpha$  and PGE2 levels in the peritoneal fluid of rats with endometriosis were significantly higher when compared with the control. LY294002 and liriodendrin administration notably decreased the elevation of IL-1, IL-2, IL-6, TNF- $\alpha$  and PGE2 in the peritoneal fluid of rats with endometriosis. Moreover, the therapeutic efficiency of liriodendrin administration at the dose investigated was stronger than LY294002 administration at the dose investigated in restoring the levels of IL-1 (Figure 4 A), IL-2 (Figure 4 B), IL-6 (Figure 4 C), TNF- $\alpha$  (Figure 4 D) and PGE2 (Figure 4 E) in the peritoneal fluid of rats. Furthermore, the PGE2 levels in the blood of rats were examined under distinct conditions. The serum

level of PGE2 in ENDO rats was significantly higher when compared with the control. Liriodendrin administration notably decreased the elevation of PGE2 in the serum of the ENDO group, but no therapeutic efficiency was observed for LY294002 on restoring the PGE2 levels in the blood of rats from the ENDO group (Figure 4 F).

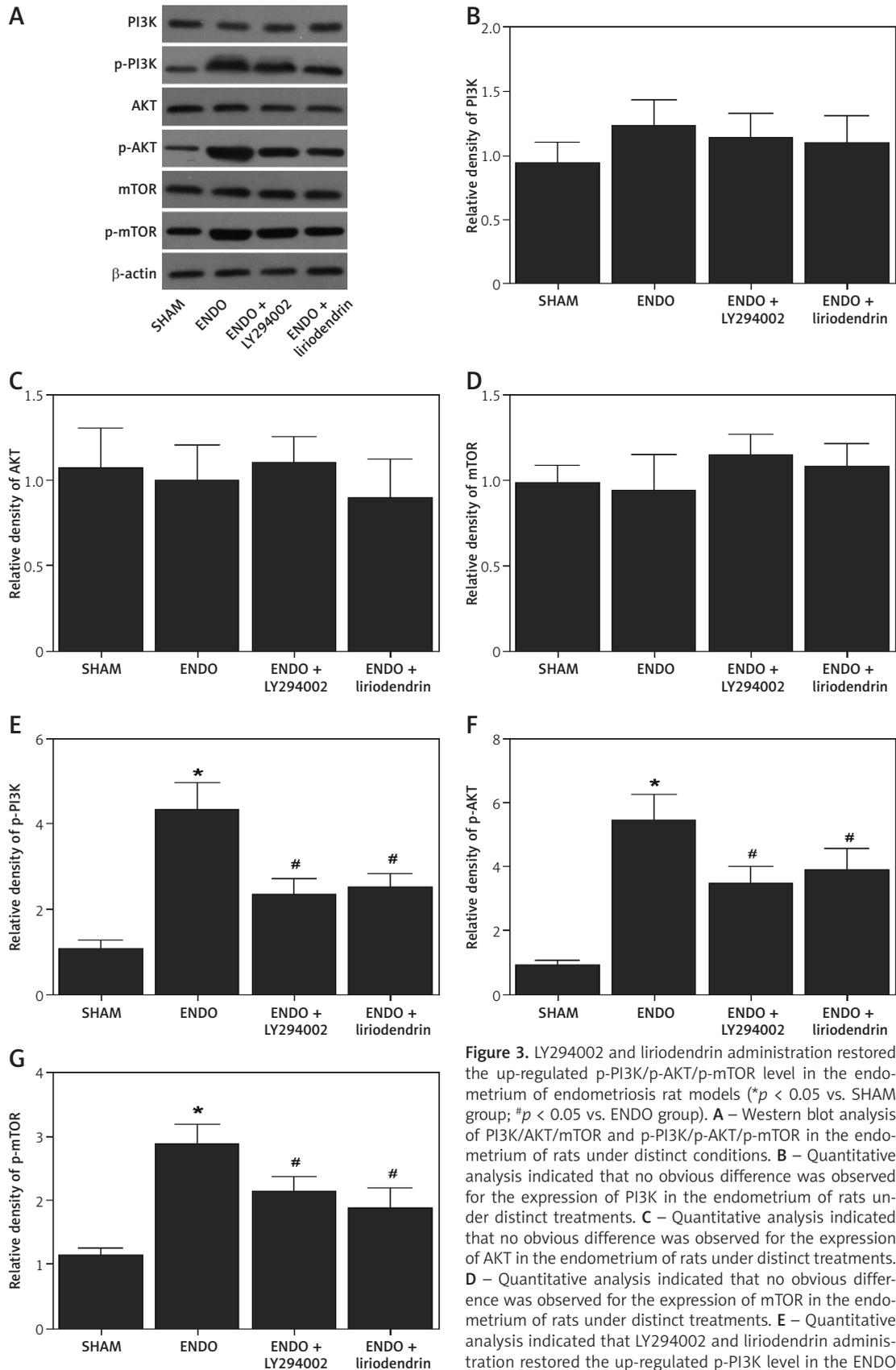
#### Liriodendrin administration restored the elevation of CGRP and substance P in the ventral horn of the spinal cord of rats with endometriosis

LY294002 and liriodendrin administration notably decreased the up-regulated substance P (Figure 5 A) and CGRP levels (Figure 5 B) in the ENDO group. Moreover, LY294002 and liriodendrin administration notably decreased the up-regulated substance P (Figures 5 C, F, G) and CGRP (Figures 5 D, F, H) mRNA and protein in the ventral horn of the spinal cord in the ENDO rats.

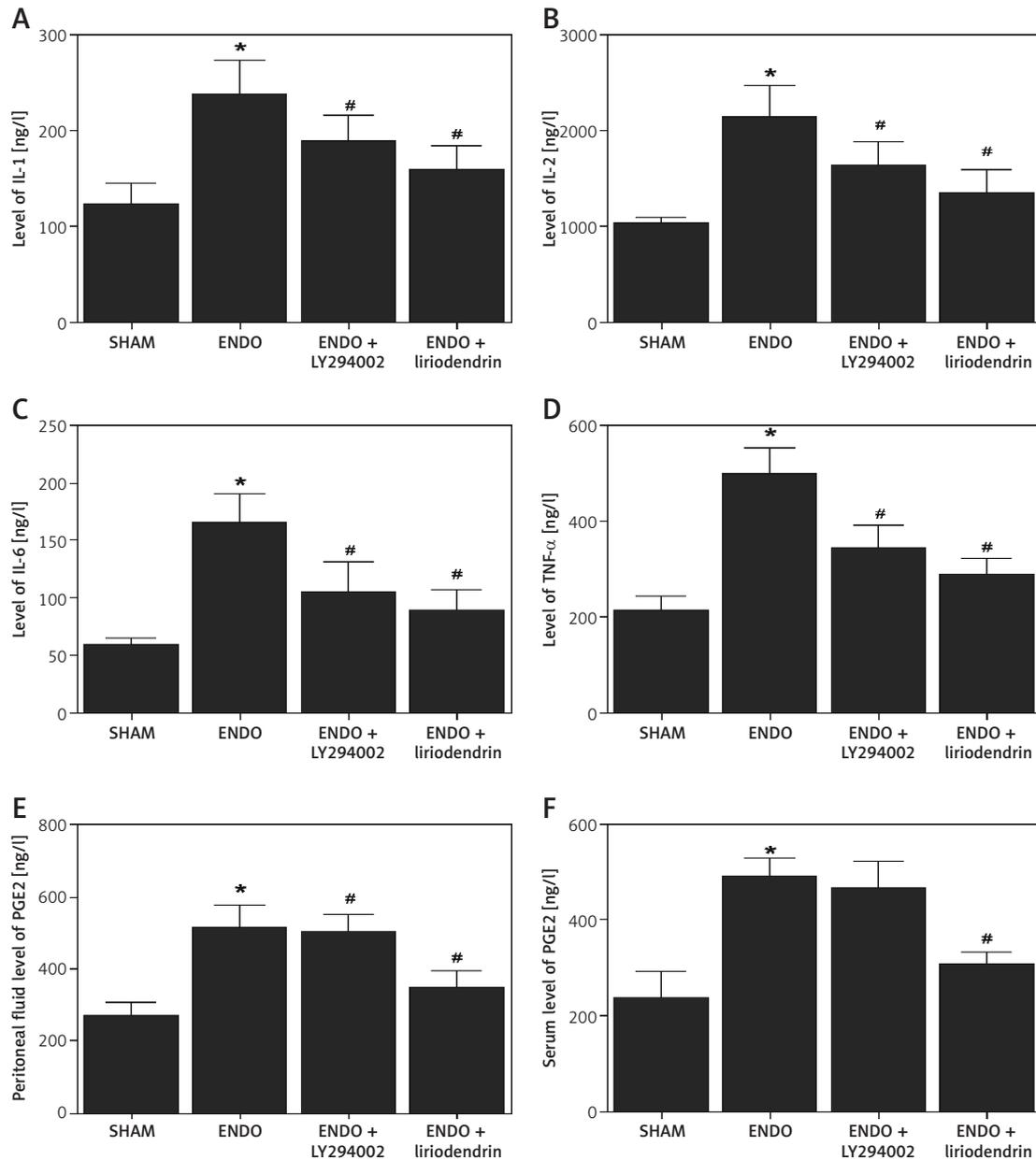
It is worth noting that the efficiency of liriodendrin administration at the dose investigated was more apparent than LY294002 administration at the dose investigated for maintaining the expression of CGRP and substance P in the ventral horn of the spinal cord. Also NF- $\kappa$ B mRNA level (Figure 5 E) showed the same tendency as the gene expression level of CGRP and substance P.

#### LY294002 and liriodendrin administration restored the LPS-induced up-regulation of p-PI3K/p-AKT/p-mTOR in 12Z cells

The expression of PI3K/AKT/mTOR and p-PI3K/p-AKT/p-mTOR in 12Z cells treated with LPS followed by LY294002 and liriodendrin administration was investigated (Figure 6 A). No obvious difference was observed for the expression of PI3K (Figure 6 B),



**Figure 3.** LY294002 and liriiodendrin administration restored the up-regulated p-PI3K/p-AKT/p-mTOR level in the endometrium of endometriosis rat models (\* $p < 0.05$  vs. SHAM group; # $p < 0.05$  vs. ENDO group). **A** – Western blot analysis of PI3K/AKT/mTOR and p-PI3K/p-AKT/p-mTOR in the endometrium of rats under distinct conditions. **B** – Quantitative analysis indicated that no obvious difference was observed for the expression of PI3K in the endometrium of rats under distinct treatments. **C** – Quantitative analysis indicated that no obvious difference was observed for the expression of AKT in the endometrium of rats under distinct treatments. **D** – Quantitative analysis indicated that no obvious difference was observed for the expression of mTOR in the endometrium of rats under distinct treatments. **E** – Quantitative analysis indicated that LY294002 and liriiodendrin administration restored the up-regulated p-PI3K level in the ENDO group. **F** – Quantitative analysis indicated that LY294002 and liriiodendrin administration restored the up-regulated p-AKT level in the ENDO group. **G** – Quantitative analysis indicated that LY294002 and liriiodendrin administration restored the up-regulated p-mTOR level in the ENDO group

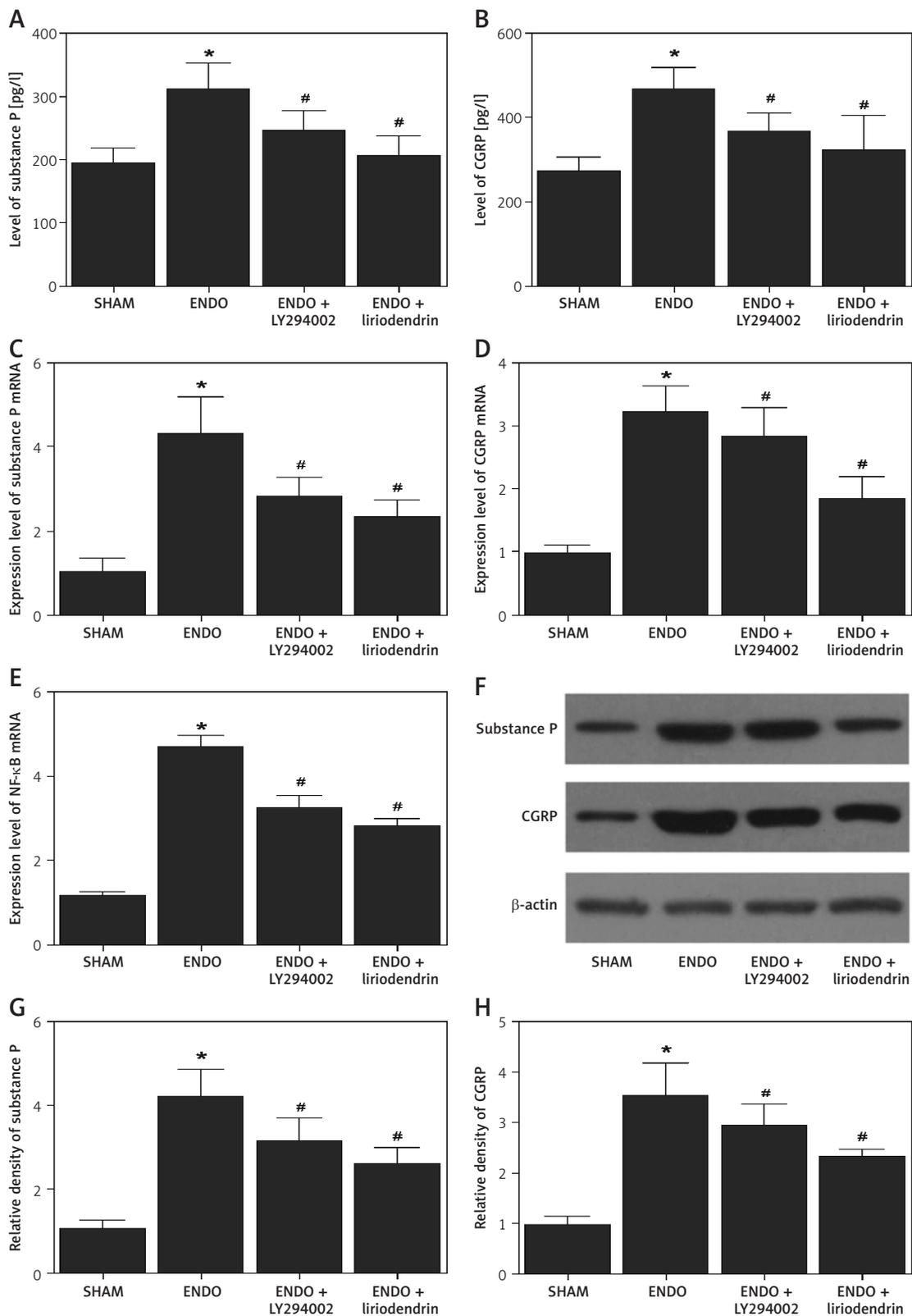


**Figure 4.** Liriodendrin administration restored the elevation of IL-1, IL-2, IL-6, TNF- $\alpha$  and PGE2 in endometriosis rat models (\* $p < 0.05$  vs. SHAM group; # $p < 0.05$  vs. ENDO group). **A** – Liriodendrin administration restored the elevated peritoneal fluid IL-1 level in the ENDO group. **B** – Liriodendrin administration restored the elevated peritoneal fluid IL-2 level in the ENDO group. **C** – Liriodendrin administration restored the elevated peritoneal fluid IL-6 level in the ENDO group. **D** – Liriodendrin administration restored the elevated peritoneal fluid TNF- $\alpha$  level in the ENDO group. **E** – Liriodendrin administration restored the elevated peritoneal fluid PGE2 level in the ENDO group. **F** – Liriodendrin administration restored the elevated serum PGE2 level in the ENDO group

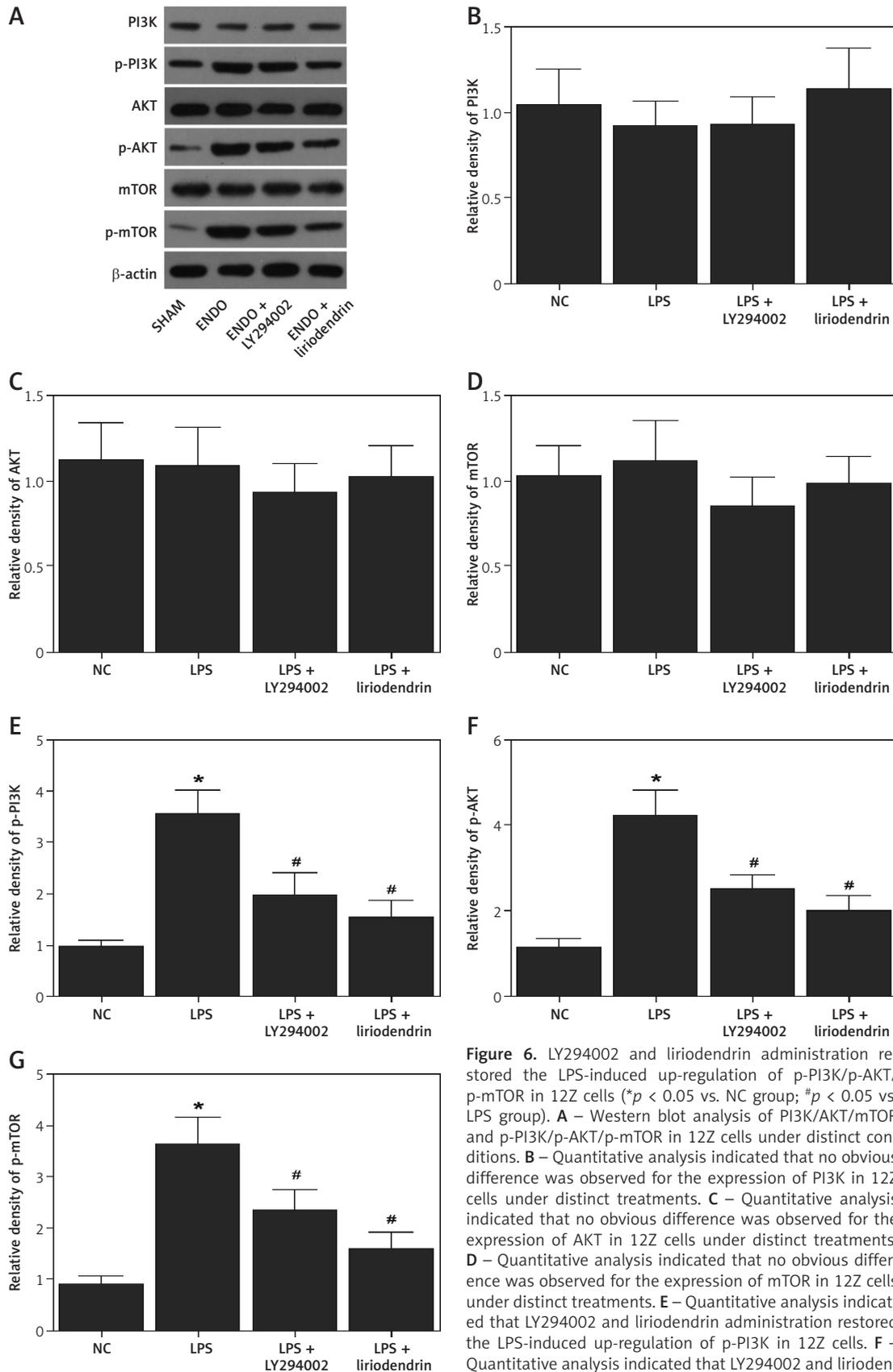
AKT (Figure 6 C) and mTOR (Figure 6 D) in 12Z cells under differential conditions. However, the expression levels of p-PI3K (Figure 6 E), p-AKT (Figure 6 F) and p-mTOR (Figure 6 G) were remarkably higher in LPS-treated 12Z cells when compared with the control. LY294002 and liriodendrin administration notably decreased the LPS-induced up-regulation of p-PI3K (Figure 6 E), p-AKT (Figure 6 F) and p-mTOR (Figure 6 G) in LPS-treated 12Z cells.

**Liriodendrin administration restored the LPS-induced up-regulation of IL-6, TNF- $\alpha$ , and IL-1 $\beta$  mRNA and protein expression in 12Z cells**

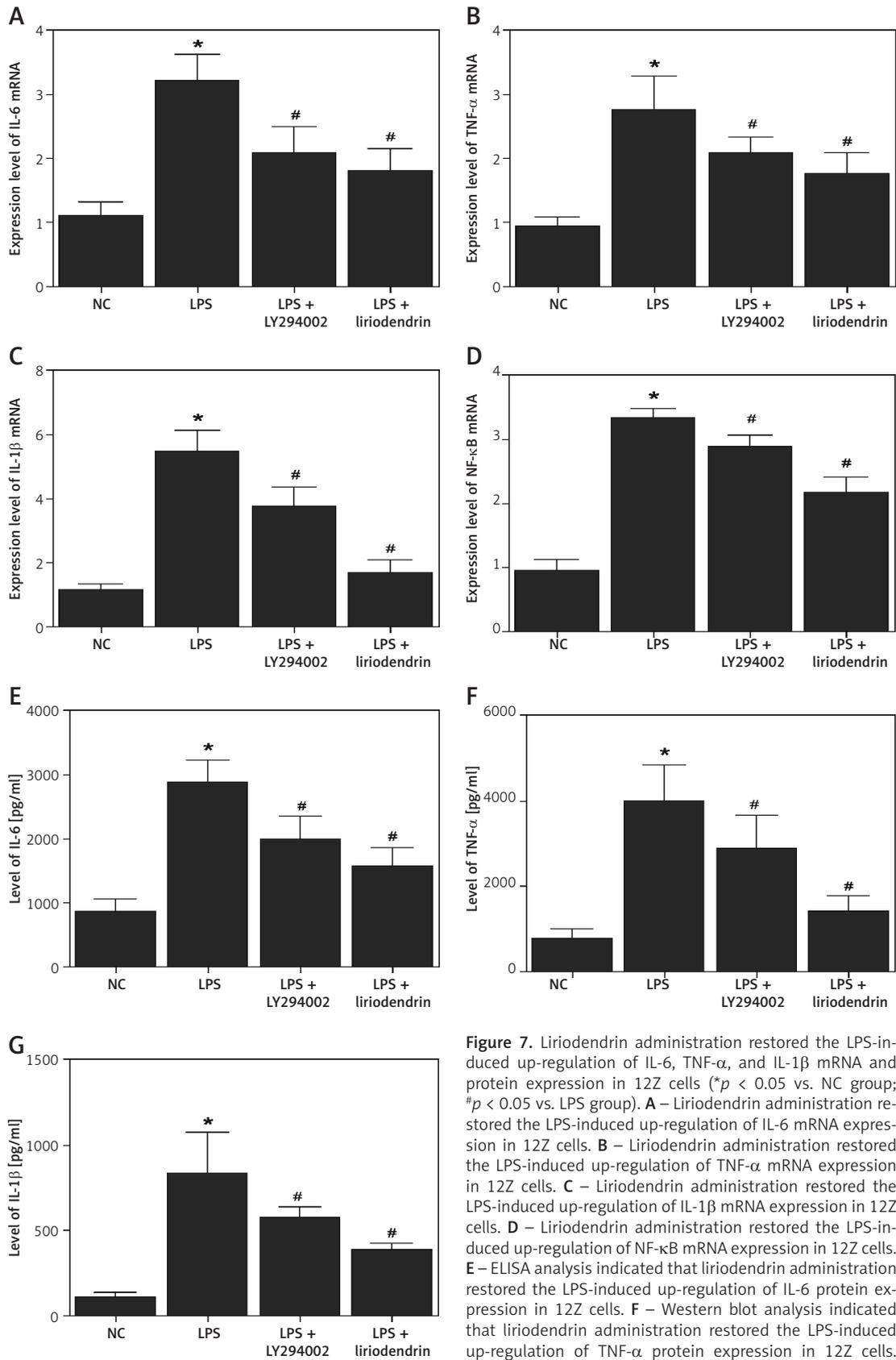
Liriodendrin administration at the dose investigated showed greater efficiency than LY294002 administration at the dose investigated in restoring the LPS-induced up-regulation of IL-6 (Figure 7 A), TNF- $\alpha$  (Figure 7 B), and IL-1 $\beta$  (Figure 7 C) mRNA



**Figure 5.** Lirioidendrin administration restored the increased CGRP and substance P in the ventral horn of spinal cord samples collected from rats with endometriosis ( $*p < 0.05$  vs. SHAM group;  $\#p < 0.05$  vs. ENDO group). **A** – ELISA analysis indicated that lirioidendrin administration restored the elevated substance P in the ENDO rats. **B** – ELISA analysis indicated that lirioidendrin administration restored the elevated CGRP in the ENDO rats. **C** – QPCR analysis indicated that lirioidendrin administration restored the elevated substance P in the ENDO rats. **D** – QPCR analysis indicated that lirioidendrin administration restored the elevated CGRP in the ENDO rats. **E** – QPCR analysis indicated that lirioidendrin administration restored the elevated NF- $\kappa$ B in the ENDO rats. **F** – Western blot analysis of CGRP and substance P expression in the ventral horn of the spinal cord of rats under distinct conditions. **G** – ELISA analysis indicated that lirioidendrin administration restored the elevated substance P in the ENDO rats. **H** – ELISA analysis indicated that lirioidendrin administration restored the elevated CGRP in the ENDO rats.



**Figure 6.** LY294002 and liriodendrin administration restored the LPS-induced up-regulation of p-PI3K/p-AKT/p-mTOR in 12Z cells (\* $p < 0.05$  vs. NC group; # $p < 0.05$  vs. LPS group). **A** – Western blot analysis of PI3K/Akt/mTOR and p-PI3K/p-AKT/p-mTOR in 12Z cells under distinct conditions. **B** – Quantitative analysis indicated that no obvious difference was observed for the expression of PI3K in 12Z cells under distinct treatments. **C** – Quantitative analysis indicated that no obvious difference was observed for the expression of AKT in 12Z cells under distinct treatments. **D** – Quantitative analysis indicated that no obvious difference was observed for the expression of mTOR in 12Z cells under distinct treatments. **E** – Quantitative analysis indicated that LY294002 and liriodendrin administration restored the LPS-induced up-regulation of p-PI3K in 12Z cells. **F** – Quantitative analysis indicated that LY294002 and liriodendrin administration restored the LPS-induced up-regulation of p-AKT in 12Z cells. **G** – Quantitative analysis indicated that LY294002 and liriodendrin administration restored the LPS-induced up-regulation of p-mTOR in 12Z cells



**Figure 7.** Liriodendrin administration restored the LPS-induced up-regulation of IL-6, TNF- $\alpha$ , and IL-1 $\beta$  mRNA and protein expression in 12Z cells (\* $p < 0.05$  vs. NC group; # $p < 0.05$  vs. LPS group). **A** – Liriodendrin administration restored the LPS-induced up-regulation of IL-6 mRNA expression in 12Z cells. **B** – Liriodendrin administration restored the LPS-induced up-regulation of TNF- $\alpha$  mRNA expression in 12Z cells. **C** – Liriodendrin administration restored the LPS-induced up-regulation of IL-1 $\beta$  mRNA expression in 12Z cells. **D** – Liriodendrin administration restored the LPS-induced up-regulation of NF- $\kappa$ B mRNA expression in 12Z cells. **E** – ELISA analysis indicated that liriiodendrin administration restored the LPS-induced up-regulation of IL-6 protein expression in 12Z cells. **F** – Western blot analysis indicated that liriiodendrin administration restored the LPS-induced up-regulation of TNF- $\alpha$  protein expression in 12Z cells. **G** – Western blot analysis indicated that liriiodendrin administration restored the LPS-induced up-regulation of IL-1 $\beta$  protein expression in 12Z cells

in 12Z cells. Liriodendrin administration at the dose investigated showed more apparent efficiency than LY294002 administration at the dose investigated in restoring the LPS-induced up-regulation of IL-6 (Figure 7 E), TNF- $\alpha$  protein (Figure 7 F), and IL-1 $\beta$  protein (Figure 7 G) in 12Z cells. Also the NF- $\kappa$ B mRNA level (Figure 7 D) showed the same tendency as the gene expression level of CGRP and substance P.

## Discussion

A disaccharide lignan named liriodendrin, (+)-syringaresinol di-O-b-D-glucopyranoside, is the bioactive component of *Sargentodoxa cuneata* (Oliv.) Rehd. Et Wils. A plethora of biological effects against inflammation, pain, and arrhythmia have been observed for the medicine [14, 15, 27]. In this study, we evaluated the efficiency of acute drug administration and daily drug administration of liriodendrin in attenuating pain in endometriosis rats. Liriodendrin has been reported to suppress inflammatory damage in the treatment of ulcerative colitis and sepsis-associated tissue injuries [28, 29]. However, its effect in the treatment of endometriosis-associated pain was not investigated. Therefore, we aimed to explore and compare its effect in the treatment of endometriosis-associated pain with LY294002 in our study. As a result, we found that, although both LY294002 and liriodendrin functioned as PI3K inhibitors, only liriodendrin alleviated inflammation, thus making liriodendrin a better therapeutic method compared with LY294002. In addition, the authors compared the distinct expression of neuropeptides mRNA and protein in the ventral horn of the spinal cord of ENDO rats. Liriodendrin administration restored the elevation of neuropeptides in the ventral horn of the spinal cord of ENDO rats. Upon oral administration, liriodendrin is transformed to syringaresinol, which is attributed to the anti-inflammatory effect [15]. In various hormone-dependent conditions, the phytoestrogen syringaresinol binds selectively to estrogen receptors [21].

Previous studies have shed light on the function of the mTOR/AKT pathway in endometriosis. One of the studies revealed that the P3KCA mutation, which is important for Akt activation, is frequently observed in patients with ovarian clear cell carcinoma [30, 31]. Higher AKT activity has been observed in ovarian endometriosis compared to the normal endometrium, which could be possibly due to the estrogen in endometriotic cells [32]. The authors found that DIE patients showed higher activity of AKT in endometriotic lesions similar to ovarian endometriosis. A PI3K inhibitor, LY294002, was found to inhibit the PI3K/Akt/mTOR signaling pathway in animals. The aim of the study was to investigate the acute and chronic effects of intra-

thecal injection of LY294002 on various signaling pathways in sciatic nerve endometriosis models in rats including PI3K/Akt/mTOR. The effects of LY294002 in the animal models began at 2-3 h. The peak effect was seen at four to five hours and was four hours long. Daily injections of LY294002 for 3 weeks greatly reduced mechanical and thermal hyperalgesia caused by sciatic nerve endometriosis (Figures 1 and 2). The therapeutic effects of LY294002 in sciatic nerve endometriosis models might be related to reduced expression levels of PI3K/Akt/mTOR signaling pathway-related factors (Figure 3). The authors performed western blot analysis to determine the expression of the signaling proteins and their phosphorylated counterparts in ENDO-treated rats and LPS-treated 12Z cells. Treatment with the drugs LY294002 and liriodendrin reestablished the lower levels of phosphorylated signaling proteins in the animal and cellular models. In addition, an ELISA assay was performed to measure the IL-1, IL-2, IL-6, TNF- $\alpha$  and PGE2 levels in stomach fluid of ENDO-treated rats. Previously, it was reported that injection of LY294002 significantly reduced lung inflammation and airway hyperresponsiveness in an asthma animal model [33, 34].

Even though the mechanism is still unclear, recent studies have shown that swelling and modified nerves in the endometriotic lesions are connected to the pathology of endometriosis. The aberrant stimulation causes modification in neurotransmitter levels and also abnormal secretion of inflammatory factors. The PI3K inhibitors reduced the levels of IL-6, MCP-1 (also known as CCL2), TNF- $\alpha$  and nitric oxide, which are the key factors regulating the inflammatory process. The PI3K/Akt signaling pathway has been shown to regulate NF- $\kappa$ B activation and phosphorylation of p65 units of NF- $\kappa$ B [35]. In this study, we analyzed the differential expression of IL-6, TNF- $\alpha$ , and IL-1 $\beta$  in LPS-treated 12Z cells. Liriodendrin administration restored the LPS-induced elevation of IL-6, TNF- $\alpha$ , and IL-1 $\beta$  in LPS-treated 12Z cells. The (+)-syringaresinol, produced in situ from liriodendrin by bacteria in intestines, enhanced FOXO3 and SIRT1 activity, which further modulated the activity of HIF-1 [36–38]. Hence, the authors hypothesized that liriodendrin degrades HIF-1 $\alpha$  by FOXO3 and SIRT1 activation, in turn reducing VEGF expression.

In conclusion, we found that compared with LY294200, the administration liriodendrin exerted a more evident therapeutic effect in the treatment of endometriosis-associated pain by suppressing the secretion of pro-inflammatory cytokines.

## Conflict of interest

The authors declare no conflict of interest.

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