



Antinociceptive Effect of Tetrandrine on LPS-Induced Hyperalgesia via the Inhibition of IKK β Phosphorylation and the COX-2/PGE $_2$ Pathway in Mice

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Abstract

Tetrandrine (TET) is a bisbenzylisoquinoline alkaloid that is isolated from the *Stephania Tetrandra*. It is known to possess anti-inflammatory and immunomodulatory effects. We have shown that TET can effectively suppress the production of bacterial lipopolysaccharide (LPS)-induced inflammatory mediators, including cyclooxygenases (COXs), in macrophages. However, whether TET has an antinociceptive effect on LPS-induced hyperalgesia is unknown. In the present study, we investigated the potential antinociceptive effects of TET and the mechanisms by which it elicits its effects on LPS-induced hyperalgesia. LPS effectively evoked hyperalgesia and induced the production of PGE $_2$ in the sera, brain tissues, and cultured astroglia. TET pretreatment attenuated all of these effects. LPS also activated inhibitor of κ B (I κ B) kinase β (IKK β) and its downstream components in the I κ B/nuclear factor (NF)- κ B signaling pathway, including COX-2; the increase in expression levels of these components was significantly abolished by TET. Furthermore, in primary astroglia, knockdown of IKK β , but not IKK α , reversed the effects of TET on the LPS-induced increase in I κ B phosphorylation, P65 phosphorylation, and COX-2. Our results suggest that TET can effectively exert antinociceptive effects on LPS-induced hyperalgesia in mice by inhibiting IKK β phosphorylation, which leads to the reduction in the production of important pain mediators, such as PGE $_2$ and COX-2, via the IKK β /I κ B/NF- κ B pathway.

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Introduction

Inflammatory mediators, such as prostaglandins (PGs), PG synthases, and cyclooxygenases (COXs), can cause abnormal neuronal activity, which leads to pain hypersensitivity[1]. Over the past decade, many studies have focused on the roles of these mediators in the regulation of hypersensitivity that is induced by environmental stimuli and pro-inflammatory factors, such as bacterial lipopolysaccharide (LPS)[2].

In the central nervous system (CNS), treatment of astrocytes and microglia with low concentrations of LPS can produce PGE $_2$ via Toll-like receptor 4-dependent manners[3,4]. PGE $_2$ can directly trigger pain-sensitive neurons to induce nociception[5,6]. At the same time, PGE $_2$ receptors that are located in peripheral tissues can scatter to the end of nociceptive nerve endings, thus sensitizing the CNS to the existence of nociceptive stimulation[7]. The COXs are rate-limiting enzymes that catalyze the synthesis of PGs. There are two distinct isoforms: COX-1 and COX-2. Although the existence of COX-3 has been reported, its roles and effects in humans are still unclear[8–9]. COX-1 is constitutively expressed to regulate normal physiological conditions, whereas COX-2 is initiated in response to inflammatory signals, such as cytokines and

LPS. Moreover, in inflammatory pain conditions, COX-2 itself can act as a nociceptive stimulator to directly cause pain. COX-2 is regulated by nuclear factor (NF)- κ B, which is a well-known transcription factor that is involved in inflammation or injury. Recent reports revealed that NF- κ B is also implicated in hyperalgesia[10–11], which is regulated by a series of adaptors. Under normal conditions, NF- κ B is inactive, and it is bound to inhibitor κ B (I κ B) via its subunits, P65 and P50, in the cytoplasm. Upon I κ B kinase (IKK) activation, I κ B is phosphorylated, thus resulting in its ubiquitination and subsequent degradation by the 26S proteasome. NF- κ B then translocates into the nucleus to regulate the transcription of genes that code for inflammatory cytokines and nociceptive substances [12,13].

Tetrandrine (TET) is an important bisbenzylisoquinoline alkaloid that is isolated from *Stephania Tetrandra* (Fig. 1A). It is traditionally used in China and Korea to treat patients with arthritis. Previous studies have shown that it possesses anti-arrhythmic[14], anti-hypertensive[15], cardio-protective[16], anti-tumorigenic[17], and anti-inflammatory effects[18]. We have demonstrated that TET exhibits anti-inflammatory and hepatoprotective effects in mice[19,20], and it inhibits IL-6 and TNF- α

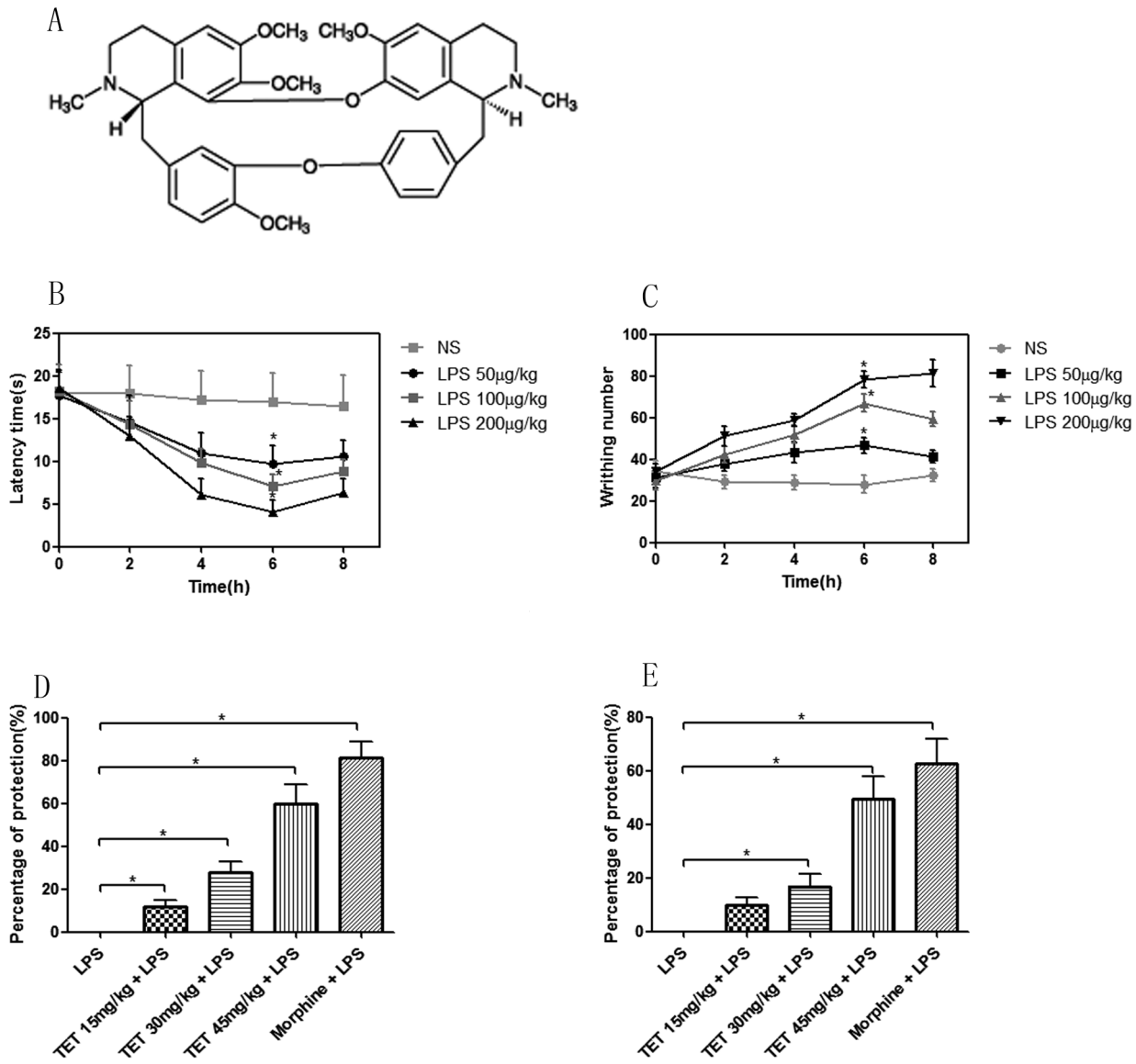


Figure 1. Induction of hyperalgesia by LPS in BALB/C mice. (A) Molecular structure of TET. (B) Latency time of hind-paw licking in the hot-plate test in mice that were treated with LPS at different concentrations and time points. (C) Writhing counts obtained from the acetic acid-induced abdominal constriction test in mice that were treated with LPS at different concentrations and time points. (D and E) Percentages of protection by TET (15, 30, 45 mg/kg) at 6 h after LPS (100 µg/kg) stimulus, as indicated by the hot-plate test (D) or acetic acid-induced abdominal constriction test (E). Indomethacin (5 mg/kg) and morphine (10 mg/kg) were applied as the positive controls. Values are shown as M±SD. *, $P < 0.05$. doi:10.1371/journal.pone.0094586.g001

production in macrophages. However, whether it is involved in the inflammatory processes of nociception is unknown. In this study, we tested the role of TET on LPS-induced hyperalgesia in mice and investigated the potential mechanisms by which TET elicits its effects.

Materials and Methods

2.1 Animals

BALB/C mice (6–8 weeks old, 20–22 g) were obtained from the Laboratory Animal Center of Chongqing Medical University (Chongqing, China). All mice received humane care, and all studies were performed with approval from the Animal Care and Use Committee of Chongqing Medical University (approval #SCXK20070001). The mice were maintained in a SPF-grade

facility under controlled conditions (22°C, 55% humidity, and 12 h day/night rhythm) and fed standard laboratory chow. After each experiment, mice were sacrificed under anesthesia with isoflurane and decapitated to ameliorate any suffering.

2.2 Materials and Drug Preparations

TET ($C_{38}H_{42}O_6N_2$, molecular weight: 622.8 g/mol) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and its purity was determined by HPLC, as previously described [19,20]. LPS (*Escherichia coli*, 0111:B4), morphine hydrochloride, and indomethacin were purchased from Sigma (MO, USA). All drugs were freshly prepared on the day of experiments. TET powder was dissolved in 0.01 M hydrochloric acid, and the pH was adjusted to 5.5 with 0.01 M NaOH. The LPS stock solution was

reconstituted to a final concentration of 10 µg/ml. Indomethacin solutions for intraperitoneal injections were prepared fresh in 0.01 M sodium carbonate, pH 7.2, at a final concentration of 0.2 mg/ml.

2.3 Induction of hyperalgesia in mice

To induce hyperalgesia, mice were randomly divided into the control group (0.1 ml/10 g pyrogen-free sterile saline, intraperitoneal injections [i.p.]), LPS-stimulated group (100 µg/kg, i.p.), low TET+LPS-treated group (15 mg/kg TET, 100 µg/kg LPS, i.p.), moderate TET+LPS-treated group (30 mg/kg TET, 100 µg/kg LPS, i.p.), high TET+LPS-treated group (45 mg/kg TET, 100 µg/kg LPS, i.p.), indomethacin-treated group (5 mg/kg, 100 µg/kg LPS, i.p.), and morphine-treated group (10 mg/kg, 100 µg/kg LPS, i.p.). TET, indomethacin, or morphine was administered 30 min prior to LPS injections. Each group was repeated at least by five individuals.

The hot-plate and acetic acid-induced abdominal constriction (writhing) tests were performed to measure the hyperalgesic responses to LPS in the presence or absence of TET, indomethacin, or morphine. The hot plates (RB200, Chengdu TME, China) were maintained at $55 \pm 1^\circ\text{C}$, and induction time was determined by measuring the latency of paw licking every 2 h until 8 h after treatment. Due to its long-term effects, acetic acid was administered only once for each individual mouse at different time points (0, 2, 4, 6, and 8 h) and concentrations. The acetic acid-saline solution (0.1 ml/10 g of 0.7% acetic acid-saline) was intraperitoneally injected, and the frequency of abdominal constrictions was counted for 20 min. Writhing was defined as the contraction of abdominal muscles, which were accompanied by the extension of forelimbs and elongation of the body.

2.4 Treatment and culture of astroglia

To prepare mouse cerebral astrocytes, cerebral cortices from P1 neonatal BALB/C mice were mechanically dissociated in astrocyte culture medium (Dulbecco's Modified Eagle Medium [DMEM] with 10% fetal bovine serum [FBS] and antibiotics). After filtering through a 70 µm cell strainer, the cells were seeded in cell culture flasks. To obtain astroglia, confluent cultures were shaken at 250 rpm overnight at 37°C . The purity of astrocytes was checked by immunostaining for GFAP (Abcam, MA, USA), and the threshold was set at $>95\%$ [21,22]. When the primary cells reached 80–90% confluency, they were digested by 0.25% trypsin and plated in 12-well tissue-culture plates at a density of $1.0\text{--}1.5 \times 10^5$ cells/well. When the cells in the 2nd passage were close to confluence, the culture medium was replaced with FBS-free DMEM. Cell treatments were performed according to following groups: control group (only the FBS-free DMEM); LPS-stimulated group (1 µg/ml); low, moderate, and high TET-treated groups (1 µg/ml LPS with TET [1×10^{-8} mol/l, 1×10^{-7} mol/l, and 1×10^{-6} mol/l, respectively]). Treatments lasted for 6 hours, after which the cells and supernatants were harvested for various experiments.

2.5 Measurement of cell viability

The TET toxicity of cultured cells was determined using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, according to the manufacturer's instructions. In brief, cells were seeded in 96-well plates and treated with increasing concentrations of TET (1×10^{-8} mol/l– 1×10^{-4} mol/l) for 15 min. MTT (terminal concentration: 0.5 g/L) was added into each well and incubated for 4 h. The optical density (OD) was measured at 570 nm by microplate UVspectrometer (SpectraMax 384 Plus).

2.6 Enzyme Immunoassay (EIA)

PGE₂ levels in purified plasma, brain homogenates, and cell culture supernatants were evaluated using a commercial EIA kit (Cayman, Michigan, USA), according to the manufacturer's protocol. Samples were added to a plate that was pre-coated with goat anti-mouse IgG antibodies. PGE₂ monoclonal antibodies were then added to each well, and the plates were incubated for 18 h at 4°C . Afterwards, Ellman's Reagent substrate was added to each well. The optical density of each sample was read at 412 nm. The standard curve was plotted, and the final concentrations of PGE₂ in the samples were calculated using the equations that were obtained from the curve.

2.7 Western blotting

Brain tissues and cultured cells were homogenized in protein extract solution. Protein concentrations were determined using the BCA protein assay kit (Thermo, USA). Samples (40 µg) were loaded onto a 12% polyacrylamide-sodium dodecyl sulfate (SDS) gel and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% (w/v) fat-free milk in Tris-buffered saline (TBS) containing 0.05% tween-20, followed by overnight incubations at 4°C with the primary antibody (1:1000). Afterwards, the membranes were treated with horseradish peroxidase-conjugated secondary antibody (1:5000, Abcam, USA) and visualized using an ECL chemiluminescence system with short exposure to X-ray films (Kodak, USA). The following primary antibodies were used: COX-1, COX-2, P65, phosphorylated P65 (pP65), IκBα, phosphorylated IκBα (pIκBα), IKKα, phosphorylated IKKα (pIKKα), IKKβ, phosphorylated IKKβ (pIKKβ), GAPDH, and tubulin (Abcam, USA).

2.8 Small-interfering RNA (siRNA) transfection

The SignalSilence siRNAs of IKKα, IKKβ, and control siRNA (unconjugated) were obtained from Santa Cruz Biotechnology (CA, USA). Cells were plated onto a 6-well plate at a density of 1.6×10^5 cells/well. Once they were 60–80% confluent, cells were washed with PBS, and the pre-mixed siRNA transfection solution (including siRNA duplex solution and the dilution reagent) was added directly to the culture medium. Cells were then incubated for 24 h, and the culture medium was changed for another 24 h. The reactions were stopped, and lysis buffer was added to extract proteins from the cells for further experiments.

2.9 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from brain tissues and cultured cells using Trizol reagent, (Invitrogen, USA) according to the manufacturer's guidelines, followed by further purification using the RNeasy Mini Kits (Qiagen, USA). Purified RNA was reverse-transcribed into cDNA with random hexamers by the SuperScript™ First-Strand Synthesis System kit (Qiagen, USA) and analyzed by real-time RT-PCR with the QuantiTect SYBR Green PCR Kits (Qiagen, USA), using a MJ DNA Engine Opticon2 qPCR System (MJ, USA). The following oligonucleotide primers were used: COX-1 forward: 5'-TGC CCT CTG TAC CCA AAG AC-3', reverse: 5'-GGA CCC ATC TTT CCA GAG GT-3'; COX-2 forward: 5'-CGG AGA GAG TTC ATC CCT GA-3'; reverse: 5'-ATC CTT GAA AAG GCG CAG T-3'; GAPDH (control) forward: 5'-CTC ATG ACC ACA GTC CAT GC-3', reverse: 5'-CAC ATT GGG GGT AGG AAC AC-3'.

2.10 Statistical Analysis

All data were expressed as mean \pm standard deviation ($M \pm SD$) from at least four independent experiments. Results were analyzed by Student's *t* test or analysis of variance (ANOVA). $P \leq 0.05$ was considered to be statistically significant.

Results

3.1 LPS induced hyperalgesia in mice via time- and dose-dependent manners

The administration of LPS (50, 100, or 200 $\mu\text{g}/\text{kg}$, i.p.) evoked dose-dependent hyperalgesia, as evaluated by the hot-plate and acetic acid-induced abdominal constriction tests (Fig 1). In the hot-plate test, hyperalgesia was assessed by the action of hind-paw licking. A decrease in the latency time of paw licking was observed at 2 h after LPS treatment, and maximal reduction was observed at 6 h (from 17.0 to 9.7, 7.1 and 4.1 seconds at LPS of 50, 100, 200 $\mu\text{g}/\text{kg}$, respectively). This was maintained until 8 h after LPS treatment (Fig 1B). Acetic acid-induced abdominal constriction numbers increased with LPS treatment, and the analogous initial and peak time points were determined (Fig 1C). Based on these results, we chose the 6-h time point for further experiments. In addition, mice that were exposed to the highest concentration of LPS (200 $\mu\text{g}/\text{kg}$) exhibited obvious hyperalgesia. However, this was accompanied by adverse reactions, including cachexia, diarrhea, and sustained tumbling, all of which may potentially influence the evaluation of the analgesic effect. Alternatively, a lower dose of LPS (100 $\mu\text{g}/\text{kg}$) induced obvious hyperalgesia without the adverse effects. Therefore, this concentration of LPS was used for further experiments.

3.2 Analgesic effects of TET on LPS-induced hyperalgesia in mice

Different doses of TET were administered to mice 30 min prior to LPS treatment. LPS-induced hyperalgesia was significantly repressed, as indicated by the elongated threshold time in hot-plate tests and the decrease in the number of writhing in acetic acid-induced abdominal constriction tests. The percentages of protection at 6 h by TET concentrations of 15, 30, and 45 mg/kg were 11.7%, 27.8%, and 59.6%, respectively, in the hot-plate test (Fig. 1D) and 9.7%, 16.8%, and 49.6%, respectively, in the acid-induced abdominal constriction test (Fig. 1E). Indomethacin (5 mg/kg) and morphine (10 mg/kg) were used as the positive controls, due to their known antinociceptive effects[23,24]. The percentages of protection by morphine and indomethacin were 81.2% (hot-plate test) and 62.7% (acid-induced abdominal constriction test), respectively. These results indicate that TET may possess both peripheral and central antinociceptive properties on LPS-induced hyperalgesia in mice.

3.3 TET repressed PGE₂ production in LPS-induced hyperalgesia in mice and cultured astroglia

To explore the antinociceptive mechanism of TET, PGE₂ production was measured by EIA in the sera and brain tissues of LPS-induced hyperalgesic mice and in the supernatants of cultured astroglia. *In vivo*, LPS significantly increased PGE₂ levels, which were markedly suppressed by TET pretreatment in dose-dependent manners (Fig. 2A, 2B). To exclude the pathophysiological conditions *in vivo* that may potentially affect the intrinsic reactions, we cultured astroglia cells to verify the mechanism *in vitro*. The toxicity of TET on astroglia was evaluated by the MTT assay, and we confirmed that TET concentrations from 1×10^{-8} mol/l to 1×10^{-6} mol/l did not significantly repress cell

viability (Fig. 2C). Therefore, these concentrations of TET were used in further experiments. Treatment of astroglia with 1 $\mu\text{g}/\text{ml}$ LPS significantly increased PGE₂ levels. Similarly, TET co-treatment attenuated PGE₂ levels in a concentration-dependent manner. The repressive peak was at the TET concentration of 1×10^{-6} mol/l (Fig. 2D). These results suggest that the antinociceptive effect of TET on LPS-induced hyperalgesia in mice may be partially mediated through downregulation of the PGE₂ signaling pathway.

3.4 TET suppressed COX-2, but not COX-1, levels

Following its release from membrane phospholipids by cytosolic or secretory phospholipases, arachidonic acid is converted to PGE₂ by COX-1 and COX-2[25]. We then investigated the expression of COX-1 and COX-2 at the mRNA and protein levels. As shown in Figure 3A, brain tissues from LPS-stimulated mice exhibit four-fold increases in COX-2 protein levels, and TET pretreatment decreases these levels of COX-2. No changes in COX-1 were observed in the presence or absence of LPS or TET. This indicates that TET can selectively suppress COX-2 expression. Similar trends in the mRNA levels of COX-1 and COX-2 were observed (Fig. 3B and 3C). Thus, the mechanisms of action *in vivo* and *in vitro* appear to be the similar processes.

3.5 TET decreased COX-2 expression through IKK β , which further inhibited the NF- κ B pathway

The NF- κ B signaling pathway is consensually involved in LPS-induced cell activation and inflammation[26,27]. To investigate whether NF- κ B activity is also regulated by TET, the expression of various components of the NF- κ B pathway, including P65, pP65, I κ B α , and pI κ B α , were assessed by western blotting in cultured astroglia. As shown in Figure 4A, P65 expression is not significantly changed in the presence of LPS or TET. However, pP65 was notably up-regulated after LPS stimulus and gradually reversed by TET pretreatment. Meanwhile, pI κ B α levels increased dramatically after LPS treatment and decreased with TET pretreatment. No changes in I κ B α levels were observed. These results suggest that TET can inactivate the NF- κ B signaling pathway through the inhibition of LPS-induced increases in I κ B α phosphorylation, thus preventing the degradation of I κ B α and retaining NF- κ B in the cytoplasm (Fig. 4A).

The phosphorylation of I κ B α is catalyzed by the IKK complex, which is comprised of the IKK α , IKK β , and IKK γ subunits. Among these, IKK α and IKK β serve as the catalytic subunits to phosphorylate I κ B for degradation via ubiquitination[28]. Hence, we investigated whether IKK α and IKK β are the upstream targets for TET in the NF- κ B pathway. Western blotting showed that LPS increased the phosphorylation of IKK β without affecting IKK α , which was consistent with previous reports[29,30,31]. Similarly, TET pretreatment effectively inhibited IKK β phosphorylation without affecting IKK α (Fig. 4B), which suggests that TET represses I κ B α activity by inhibiting IKK β .

To verify whether TET specifically targets IKK β , cells were transfected with IKK α or IKK β siRNA (si) to knock down their respective gene expression levels. As shown in Figure 5A, cells transfected with siIKK β exhibit decreases in the protein expression of phosphorylated IKK β , and pP65. LPS stimulus partially rescued these trends, although not to levels of those seen in LPS-treated control siRNA-transfected cells. siIKK β transfection also did not affect IKK α phosphorylation, and phosphorylated IKK β did not functionally affect pIKK α in the case of LPS challenge, as shown by other groups[32,33,34]. On the other hand, transfection of cells with siIKK α only decreased the expression of pIKK α without affecting the levels of pIKK β or pP65. Collectively, these

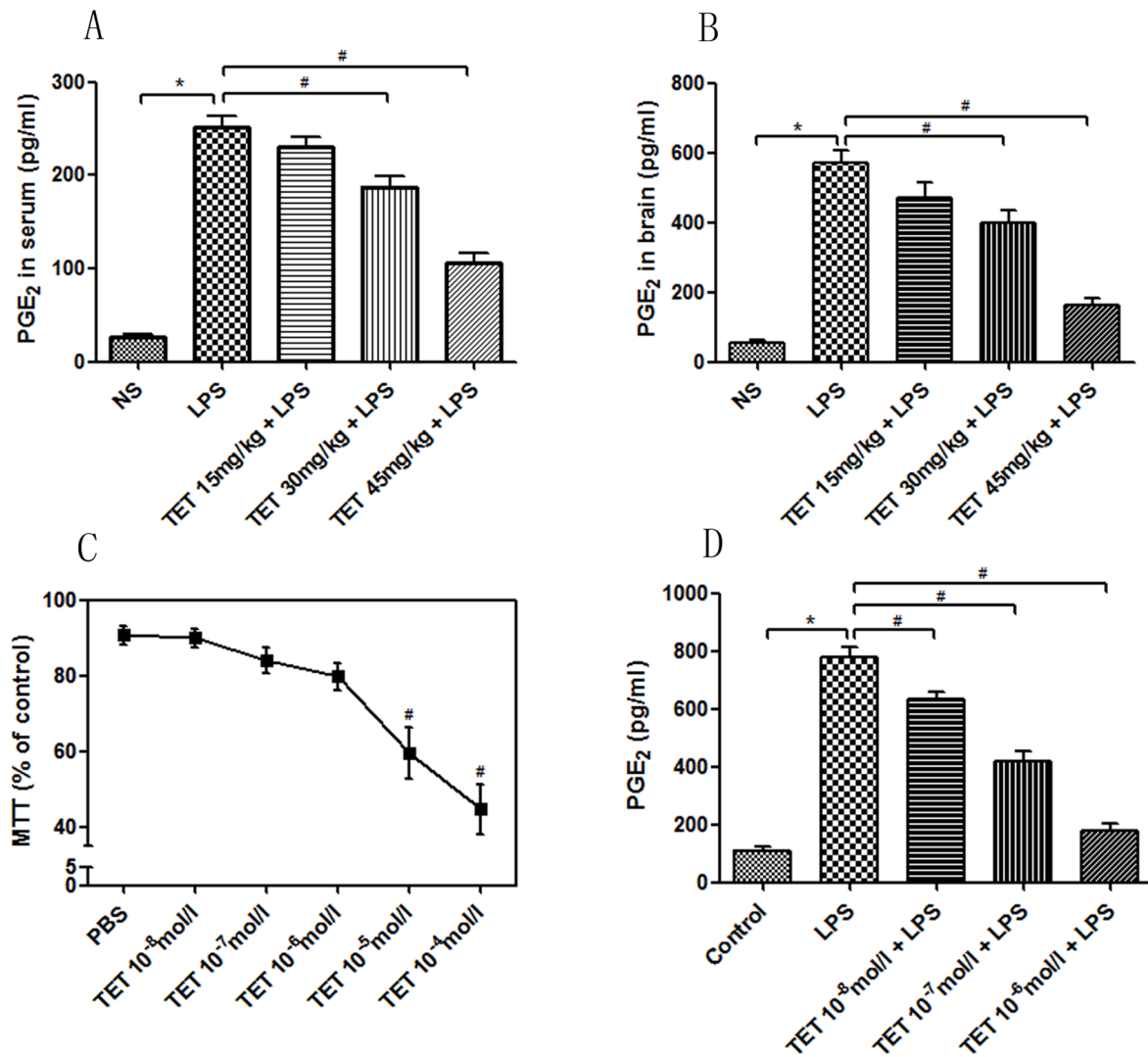


Figure 2. Repressive effects of TET on PGE₂ levels in sera, brain tissues, and cultured astroglia. (A, B) PGE₂ levels in the sera and brain tissues, respectively, were significantly increased after LPS stimulation and markedly suppressed by TET. (C) MTT analysis of astroglia viability *in vitro* in the presence of different concentrations of TET. (D) PGE₂ levels in LPS-treated astroglia were suppressed by TET in a dose-dependent manner. Values are shown as M±SD. *, *P*<0.05. doi:10.1371/journal.pone.0094586.g002

results show that IKK α and IKK β played distinct roles in the pathway of LPS-induced hyperalgesia. Moreover, IKK α may not directly participate in the LPS- and TET-signaling cascade. TET treatment in cells with IKK β knockdown had no effect on the levels of pIKK β and pP65. This was partially due to the low levels of IKK β that already existed in these cells with IKK β knockdown.

Next, to further determine whether the pathway of “LPS/TET—IKK β —NF- κ B—COX-2/PGE₂” practically take effect in astroglia, we investigated the COX-2 generation under the control siRNA, siIKK α , siIKK β knockdown with or without TET treatment (Fig 5B). Results showed both siIKK β and TET could decrease COX-2 generation, but not siIKK α , which more solidly supported the conclusion of TET specifically inhibited IKK β phosphorylation and subsequently downregulate COX-2/PGE₂ levels.

Discussion

LPS, which is a component of the cell wall of gram-negative bacteria, is known to activate a number of cellular signals in various cell types and tissues during inflammation and infection. In addition to its ability to cause endotoxic shock, LPS induces hyperalgesia in mice at lower doses[35]. A single dose of LPS that is administered centrally or peripherally can evoke a hyperalgesic reaction by decreasing mechanical nociceptive thresholds. In this study, we generated a hyperalgesic mouse model, in which BALB/C mice were treated with LPS. The hyperalgesic effect of LPS was verified by the shortened latency time of paw licking using the canonical hot-plate test, as well as the increase in writhing numbers in the acetic acid-induced abdominal constriction test. Because the writhing model is sensitive to the antinociceptive action of non-steroidal anti-inflammatory drugs, including indomethacin[23], and the hot-plate test focuses on the pathophysiological process above the spinal cord level[24], we chose indomethacin and morphine to be the positive controls of the

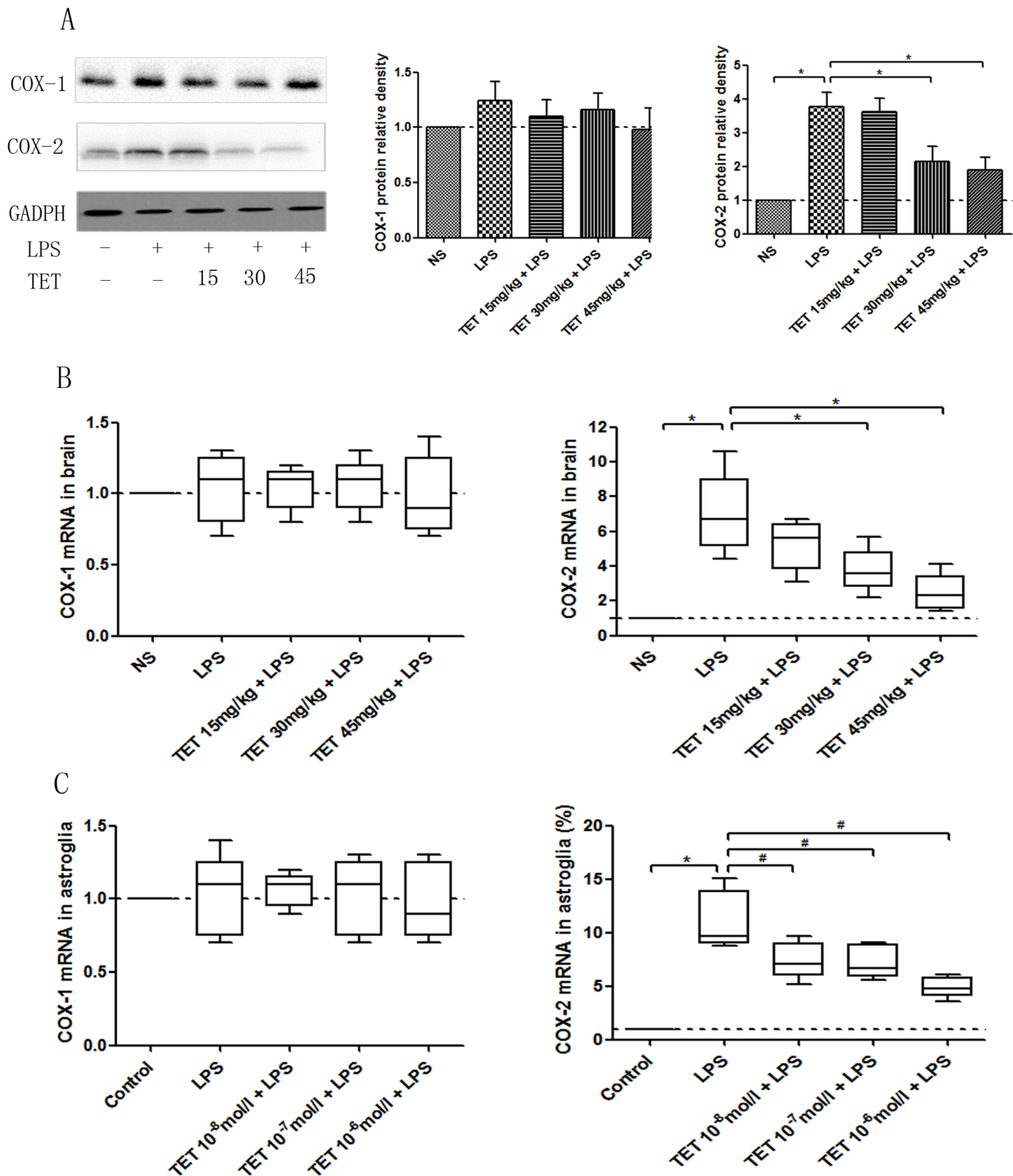


Figure 3. Repressive effects of TET on the expression of COX-1 and COX-2 in LPS-induced hyperalgesia. (A) Western blotting of COX-1 and COX-2 in brain tissues and the quantified comparison of relative densities are shown. (B) COX-1 and COX-2 mRNA expression were tested by qRT-PCR in brain tissues. (C) COX-1 and COX-2 mRNA expression were tested by qRT-PCR in cultured astroglia. Values are shown as $M \pm SD$, and normalized to the NS groups. $* P < 0.05$. doi:10.1371/journal.pone.0094586.g003

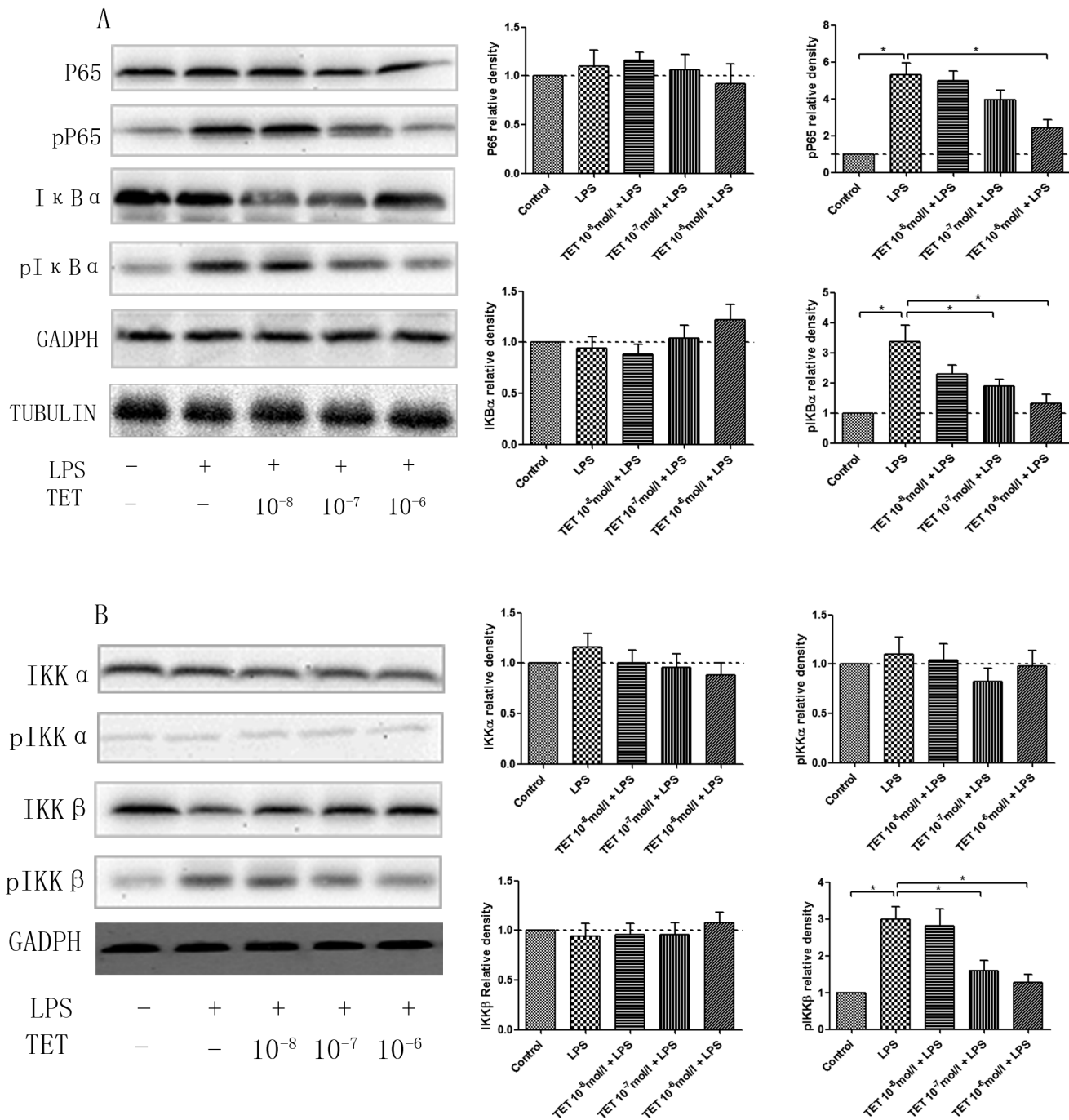


Figure 4. Regulatory effects of TET on the NF-κB pathway in LPS-induced cultured astroglia. (A) The expression of P65, pP65, IκBα, and pIκBα in the presence or absence of TET and LPS was assessed by western blotting, and the quantified comparisons of relative densities are shown. (B) The protein expression of IKKs in the presence or absence of TET and LPS was assessed by western blotting, and the quantified relative densities. Values are shown as M±SD, and normalized to the NS groups. *, P<0.05. doi:10.1371/journal.pone.0094586.g004

writhing and hot-plate tests, respectively. Using these models, tests, and drugs, we were able to investigate the peripheral and central anti-nociceptive effects of TET.

TET dose-dependently reduced the nociceptive responses in the writhing and hot-plate tests in LPS-treated mice, thus suggesting that TET has both central and peripheral anti-nociceptive effects. Because PGE₂ is a critical pro-inflammatory and algesic factor, we measured its levels *in vivo* and *in vitro*. PGE₂ levels were significantly

increased and repressed with LPS and TET treatments, respectively, in mouse sera, brain tissues, and cultured astroglia. This suggests that PGE₂ plays pivotal roles in LPS-induced hyperalgesia and TET-mediated analgesia. The COXs are key enzymes that regulate the formation of PGE₂ from arachidonic acid. LPS increased COX-2 expression in mouse brain tissues and cultured astroglia. No effects on COX-1 were seen. Consistent with the physiology of canonical pain, COX-2 acted as a key regulatory

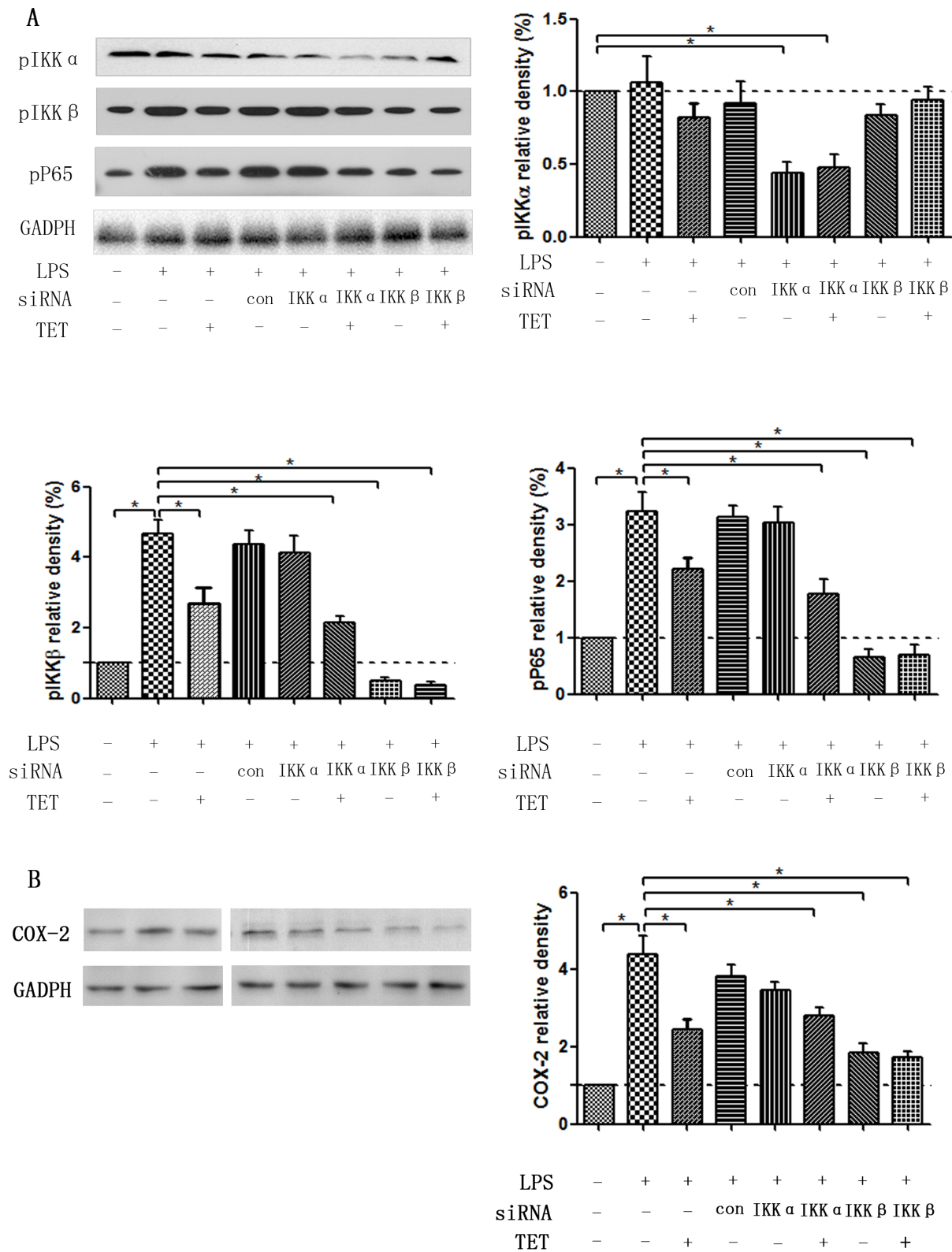


Figure 5. Signaling targets of LPS and TET. Small RNA-interfering (si) experiments with siIKK α , siIKK β , and control siRNA were performed to investigate the specific targets of LPS and TET in astroglia. (A) Both LPS and TET initially target the phosphorylation of IKK β , but not that of IKK α . (B) Both siIKK β and TET decrease COX-2 generation, but not siIKK α . Values are shown as $M \pm SD$, and normalized to the LPS(-)siRNA(-)TET(-) groups. *, $P < 0.05$.

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synthase in the production of PGE₂ in our hyperalgesic mice and astroglia models. These results show that PGE₂/COX-2 was the appropriate central pathway of hyperalgesia. Proportional decreases in central and peripheral PGE₂/COX-2 levels by TET were also observed.

A crucial role for astroglia in mediating pain has been implicated by studies involving animal models and patients with persistent pain conditions[36]. Pro-inflammatory cytokines are produced and released by activated microglia and astrocytes in the CNS. The IKK/I κ B/NF- κ B signaling pathway regulates the expression of these inflammatory cytokines, including COX-2 and IL-1[37]. Therefore, we isolated astrocytes from the brains of newborn mice and co-treated them with TET and LPS. The phosphorylation of IKK β , I κ B α , P65 and COX-2 increased proportionally upon LPS stimulus, and these increases were significantly reversed by TET co-treatment, thus implicating the IKK β /I κ B α /NF- κ B pathway in LPS-induced hyperalgesia and TET-induced antinociception. No effects on IKK α were observed. Knockdown experiments with IKK α or IKK β siRNAs further clarified the mechanism by which TET elicits its analgesic effects, and the results show that LPS induced NF- κ B pathway activation by, at least in part, triggering the phosphorylation of IKK β but not IKK α . Interestingly, TET specifically targeted IKK β phosphorylation in LPS-treated astroglia, and eventually depressed NF- κ B

activation and COX-2/PGE₂ expression. These results allow us to better understand the mechanisms by which LPS and TET induce hyperalgesia and antinociception, respectively, and show that both effects were elicited via the activation or inhibition of IKK β phosphorylation and the downregulation of the NF- κ B/COX-2/PGE₂ pathway.

Although TET appears to mediate analgesia via inhibiting IKK β phosphorylation, it may also target other components of the pathway that are upstream of IKK. Additionally, the modulation of pain by peripherally derived inflammatory mediators involves factors and effector cells other than PGE₂ and astroglia, respectively. The microglia and spinal glia also participate in pain modulation[38,39]. Whether the central modulation of pain involves the actions of the other eicosanoid metabolites, nitric oxide, or pro-inflammatory mediators requires further elucidation. Therefore, more work needs to be done to reveal the exact mechanisms of hyperalgesia, as well as the main mechanisms behind the analgesic effects of TET.

Author Contributions

Conceived and designed the experiments: YFY JYW. Performed the experiments: HGZ FLL HZL. Analyzed the data: HGZ LZ. Wrote the paper: HGZ.

References

- Ndengle MM, Cuzzocrea S, Esposito E, Mazzone E, Di Paola R, et al. (2008) Cyclooxygenases 1 and 2 contribute to peroxynitrite-mediated inflammatory hypersensitivity. *FASEB J* 22(9):3154–3164.
- Benson S, Kattoor J, Wegner A, Hammes F, Reidick D, et al. (2012) Acute experimental endotoxemia induces visceral hypersensitivity and altered pain evaluation in healthy humans. *Pain*. 153(4):794–799.
- Xiang Y, Wei X, Chen L, Liu H, Liu X, et al. (2013) Anti-inflammatory Effect of Acetylpuerarin on Eicosanoid Signaling Pathway in Primary Rat Astrocytes. *J Mol Neurosci*. Sep 13. PMID: 24026619
- Johann SL, Kampmann E, Denecke B, Arnold S, Kipp M, et al. (2008) Expression of enzymes involved in the prostanoid metabolism by cortical astrocytes after LPS-induced inflammation. *J Mol Neurosci*.34(2):177–185.
- Cao L, Tanga FY, Deleo JA (2009) The contributing role of CD14 in toll-like receptor 4 dependent neuropathic pain. *Neuroscience*. 158(2):896–903.
- Ma W1, Quirion R (2008) Does COX2-dependent PGE2 play a role in neuropathic pain? *Neurosci Lett*. 437(3):165–169.
- Southall MD, Vasko MR (2001) Prostaglandin receptor subtypes, EP3C and EP4, mediate the prostaglandin E2-induced cAMP production and sensitization of sensory neurons. *J Biol Chem*. 276(19):16083–16091.
- Berenbaum F (2004) COX-3: fact or fancy? *Joint Bone Spine*. 71:451–453.
- Nasrallah R, Clark J, Hébert RL (2007) Prostaglandins in the kidney: developments since Y2K. *Clin Sci (Lond)*. 113(7):297–311.
- Kanngiesser M, Häussler A, Myrczek T, Küssner N, Lim HY, et al. (2012) Inhibitor kappa B kinase beta dependent cytokine upregulation in nociceptive neurons contributes to nociceptive hypersensitivity after sciatic nerve injury. *J Pain*. 13(5):485–497
- Hsu CC, Lien JC, Chang CW, Chang CH, Kuo SC, et al. (2013) Yuwen02f1 suppresses LPS-induced endotoxemia and adjuvant-induced arthritis primarily through blockade of ROS formation, NF κ B and MAPK activation. *Biochem Pharmacol*. 85(3):385–395.
- Haddad JJ, Abdel-Karim NE (2011) NF- κ B cellular and molecular regulatory mechanisms and pathways: therapeutic pattern or pseudoregulation? *Cell Immunol*. 271(1):5–14.
- Niederberger E, Geisslinger G (2008) The IKK-NF-kappaB pathway: a source for novel molecular drug targets in pain therapy? *FASEB J*. 22(10):3432–3442.
- Yu XC, Wu S, Chen CF, Pang KT, Wong TM, et al. (2004) Antihypertensive and anti-arrhythmic effects of an extract of Radix Stephaniae Tetrandrae in the rat. *J Pharm Pharmacol*. 56(1):115–122.
- Isturriaga-Vásquez P, Miquel R, Ivorra MD, D'Ocon MP, Cassels BK, et al. (2003) Simplified tetrandrine congeners as possible antihypertensive agents with a dual mechanism of action. *J Nat Prod*. 66(7):954–957.
- Shen DF, Tang QZ, Yan L, Zhang Y, Zhu LH, et al. (2010) Tetrandrine blocks cardiac hypertrophy by disrupting reactive oxygen species-dependent ERK1/2 signalling. *Br J Pharmacol*. 159(4):970–981.
- Li X, Su B, Liu R, Zhang Y, Zhu LH, et al. (2011) Tetrandrine induces apoptosis and triggers caspase cascade in human bladder cancer cells. *J Surg Res*. 166(1):e45–51
- Lee YS, Han SH, Lee SH, Kim YG, Park CB, et al. (2012) The mechanism of antibacterial activity of tetrandrine against Staphylococcus aureus. *Foodborne Pathog Dis*. 9(8):686–691.
- Gong X, Luo FL, Zhang L, Li HZ, Wu MJ, et al. (2010) Tetrandrine attenuates lipopolysaccharide-induced fulminant hepatic failure in D-galactosamine-sensitized mice. *Int Immunopharmacol*. 10(3):357–363.
- Wang TH, Wan JY, Gong X, Li HZ, Cheng Y (2012) Tetrandrine enhances cytotoxicity of cisplatin in human drug-resistant esophageal squamous carcinoma cells by inhibition of multidrug resistance-associated protein 1. *Oncol Rep*. 28(5):1681–1686.
- Miyachi T, Asai K, Tsuiji H, Mizuno H, Yamamoto N, et al. (2001) Interleukin-1 β induces the expression of lipocortin 1 mRNA in cultured rat cortical astrocytes. *Neurosci. Res*. 40(1): 53–60.
- Thomsen R, Daugaard TF, Holm IE, Nielsen AL (2013) Alternative mRNA Splicing from the Glial Fibrillary Acidic Protein (GFAP) Gene Generates Isoforms with Distinct Subcellular mRNA Localization Patterns in Astrocytes. *PLoS One*. 8(8):e72110.
- Kaushik D, Kumar D, Kaushik P, Rana AC (2012) Analgesic and Anti-inflammatory Activity of Pinus roxburghii Sarg. *Adv Pharmacol Sci*. 2012: 245431.
- Ballou LR, Botting RM, Goorha S, Zhang J, Vane JR (2000) Nociception in cyclooxygenase isozyme-deficient mice. *Proc Natl Acad Sci USA*. 97(18):10272–10276.
- Morham SG, Langenbach R, Loflin CD, Tian HF, Vouloumanos N, et al. (1995) Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. *Cell*. 83(3):473–482.
- Koppula S, Kim WJ, Jiang J, Shim DW, Oh NH, et al. (2013) Carpesium macrocephalum attenuates lipopolysaccharide-induced inflammation in macrophages by regulating the NF- κ B/I κ B- α , Akt, and STAT signaling pathways. *Am J Chin Med*. 41(4):927–943.
- Gao F, Ding B, Zhou L, Gao X, Guo H, et al. (2013) Magnesium sulfate provides neuroprotection in lipopolysaccharide-activated primary microglia by inhibiting NF- κ B pathway. *J Surg Res*. 184(2):944–950.
- Hayden MS, Ghosh S (2008) Shared principles in NF-kappaB signaling. *Cell*. 132(3):344–362.
- Yang F, Tang E, Guan K, Wang CY (2003) IKK beta plays an essential role in the phosphorylation of RelA/P65 on serine 536 induced by lipopolysaccharide. *J Immunol*. 170(11):5630–5635.
- Dajani R, Sanlioglu S, Zhang Y, Li Q, Monick MM, et al. (2007) Pleiotropic functions of TNF- α determine distinct IKKbeta-dependent hepatocellular fates in response to LPS. *Am J Physiol Gastrointest Liver Physiol*. 292(1):G242–252.
- Shao DZ, Lin M (2008) Platonin inhibits LPS-induced NF-kappaB by preventing activation of Akt and IKKbeta in human PBMC. *Inflamm Res*. 57(12):601–606.
- Li ZW, Chu W, Hu Y, Delhase M, Deerinck T, et al. (1999) The IKKbeta subunit of I κ B kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. *J Exp Med*. 189(11):1839–1845.

33. Park EJ, Cheenpracha S, Chang LC, Pezzuto JM (2011) Suppression of cyclooxygenase-2 and inducible nitric oxide synthase expression by epimiquibin A via IKK/ $\text{I}\kappa\text{B}$ / $\text{NF-}\kappa\text{B}$ pathways in lipopolysaccharide-stimulated RAW 264. 7 cells. *Phytochem Lett.* 4(4):426–431.
34. Islam S, Hassan F, Mu MM, Ito H, Koide N, et al. (2004) Piceatannol prevents lipopolysaccharide (LPS)-induced nitric oxide (NO) production and nuclear factor (NF)- κB activation by inhibiting $\text{I}\kappa\text{B}$ kinase (IKK). *Microbiol Immunol.* 48(10):729–736.
35. Kovács KJ, Pápic JC, Larson AA (2008) Movement-evoked hyperalgesia induced by lipopolysaccharides is not suppressed by glucocorticoids. *Pain.* 136(1–2):75–84.
36. Svensson CI, Brodin E (2010) Spinal astrocytes in pain processing: non-neuronal cells as therapeutic targets. *Mol Interv.* 10(1):25–38.
37. Shishodia S, Koul D, Aggarwal BB (2004) Cyclooxygenase (COX)-2 inhibitor celecoxib abrogates TNF-induced $\text{NF-}\kappa\text{B}$ activation through inhibition of activation of $\text{I}\kappa\text{B}$ kinase and Akt in human non-small cell lung carcinoma: correlation with suppression of COX-2 synthesis. *J Immunol.* 173(3):2011–2022.
38. Saito O, Svensson CI, Buczynski MW, Wegner K, Hua XY, et al. (2010) Spinal glial TLR4-mediated nociception and production of prostaglandin E(2) and TNF. *Br J Pharmacol.* 160(7):1754–1764.
39. Ikeda-Matsuo Y, Ikegaya Y, Matsuki N, Uematsu S, Akira S, et al. (2005) Microglia-specific expression of microsomal prostaglandin E2 synthase-1 contributes to lipopolysaccharide-induced prostaglandin E2 production. *J Neurochem.* 94(6):1546–1558.