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Dipyrone & 2,5-dimethylcelecoxib suppress Th2-related chemokine production in monocyte

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***Background & objectives*: Selective cyclooxygenase-2 (COX-2) inhibitor is a form of non steroidal anti-inflammatory drug (NSAID) and is commonly used in autoimmune and rheumatic diseases to control inflammation and alleviate pain. Tumour necrosis factor-alpha (TNF-α) production and an imbalance of T helper 1 (Th1)/Th2 contribute to the pathogenesis of autoimmune and also anti-tumour activity. Dipyrone is a NSAID used to treat pain worldwide. The celecoxib analogue, 2,5-dimethylcelecoxib (DMC), lacks COX-2 inhibitory activity but exhibits anti-tumour properties. However, the effects and the mechanisms of dipyrone and 2,5-dimethylcelecoxib on tumour necrosis factor (TNF)-α and Th1- and Th2-related chemokines in monocytes remain poorly defined. This study was carried out to investigate the effects of dipyrone and 2,5-dimethylcelecoxib on the expression of Th1 (IP-10) and Th2 (I-309 and MDC) and TNF-α in human monocytes and the associated intracellular mechanism.**

***Methods*: THP-1 cells and peripheral blood mononuclear cells (PBMCs) were pre-treated with dipyrone (10^{-9} – 10^{-4} M) and 2,5-dimethylcelecoxib (10^{-9} – 10^{-5} M) 2 h before lipopolysaccharide (LPS) stimulation. Cell supernatant was collected 24 h after LPS stimulation. TNF-α, I-309, MDC and IP-10 concentrations of cell supernatants were determined using ELISA. Intracellular signaling was evaluated by Western blot.**

***Results*: Dipyrone and 2,5-dimethylcelecoxib downregulated LPS-induced Th2-related chemokine I-309 and macrophage derived chemokine (MDC) production. Only high dose of 2,5-dimethylcelecoxib (10^{-5} M), but not dipyrone downregulated LPS-induced IP-10. Only very high dose of 2,5-dimethylcelecoxib had effect on LPS-induced TNF-α expression in PBMCs. Dipyrone and 2,5-dimethylcelecoxib suppressed LPS-induced p65 and JNK MAPK (C-Jun N-terminal kinase mitogen activated protein kinase). expression.**

***Interpretation & conclusions*: Dipyrone and 2,5-dimethylcelecoxib downregulated LPS-induced Th2-related chemokine I-309 and MDC in THP-1 cells. The suppressive effect on Th2-related chemokine I-309 and MDC may involve the downregulation of LPS–induced JNK and p65 expression.**

Key words COX-2 inhibitor - dipyrone - 2,5-dimethylcelecoxib - IP-10 - I-309, - MDC - monocyte - PBMC - Th 2 related chemokine

[*]The first two authors contributed equally to this work

The non steroidal anti-inflammatory drugs (NSAIDs) one of the most often prescribed drugs, suppress synthesis of prostaglandins through inhibition of cyclooxygenase COX-1 which is involved in maintaining cell integrity and COX-2, which presents particularly in the kidneys, and is overexpressed in response to inflammation. Many of the adverse effects of NSAIDs (*e.g*. gastrointestinal ulceration and bleeding, platelet dysfunction) are associated with the suppression of COX-1-derived prostanoids, whereas inhibition of COX-2-derived prostanoids mediates the anti-inflammatory, analgesic, and antipyretic effects of these compounds[1,2]. An imbalance of T helper 1 (Th1)/ Th2 is thought to contribute to the pathogenesis of autoimmune and rheumatoid diseases[3]. For example, the immune balance in patients with rheumatoid arthritis (RA) is characterized by Th1-dominance. Tumour necrosis factor (TNF)-α is a proinflammatory cytokine and plays an important role in rheumatoid diseases[4]. Anti-TNF-α and methotrexate (MTX) are the preferred therapy in RA[5]. MTX and TNF-blockers downregulate the expression of chemokine receptor CXCR3 and interleukin (IL)-12 receptors (both Th1 markers) and upregulate the expression of CCR4 and, to a lesser extent, IL-4 receptor (IL-4R) (both Th2 markers) in RA. This phenomenon further suggests the involvement of Th1/Th2 imbalance in RA[5]. Chemokines are small, chemotactic cytokines and play a key role in leukocyte migration under normal and pathological conditions[6]. The action spectrum of chemokines is generally dictated by differential expression of cognate receptors on distinct leukocyte subsets or stages of differentiation and activation. C-X-C molif chemokine 10, also known as interferon γ-induced protein 10 (CXCL10/ IP-10) is a 10 kDa protein and categorized as a Th1-chemokine. IP-10 binds to the receptor CXCR3 and regulates immune responses through the activation and recruitment of leukocytes including T cells, eosinophils, and monocytes. Serum and tissue expressions of CXCL10 are increased in various autoimmune diseases like RA, systemic lupus erythematosus (SLE), Sjogren syndrome (SS), systemic sclerosis (SSc), and idiopathic inflammatory myopathy (IIM). Moreover, CXCL10 and CXCR3 may have important roles in leukocyte homing to inflamed tissues as well as in the perpetuation of inflammation, and subsequently result in tissue damage[7]. The significant differences in the plasma concentrations of Th2-related chemokine (thymus and activation regulated chemokine) TARC/ CCL17 between the patients with untreated SLE and treated SLE, RA, and healthy controls and the close correlation between plasma levels of macrophage derived chemokine (MDC)/CCL22 and TARC/CCL17 also suggest that Th2-related chemokines are also involved in rheumatoid diseases[8]. CC chemokine ligand 1 (CCL1; I-309) is a CC chemokine that interacts with CC chemokine receptor 8, which is preferentially expressed in polarized Th2 and cytotoxic T cells, in eosinophils, and in T regulatory cells[9].

Dipyrone is a selective COX-2 inhibitor and common analgesic used worldwide[10]. Dimethylcelecoxib (DMC) is a close derivative of celecoxib, a selective COX-2 inhibitor, and has been reported without cyclooxygenase inhibiting properties up to very high concentrations. DMC has been developed to treat inflammatory and carcinogenic processes, although it does not inhibit cyclooxygenases. In cell line study, after stimulation of IL-1β/TNFα, DMC could also inhibit prostaglandin E2 (PGE2) synthesis[11]. It is reasonable to evaluate the effect of dipyrone and 2,5-dimethylcelecoxib on the pro-inflammatory cytokine, Th1- and Th2-related chemokines in monocytes. mitrogen-activated protein kinases (MAPKs) and nuclear factor-kappa B (NFκB) may be the major pathways involved in lipopolysaccharide (LPS)-induced chemokines and cytokines expression of monocytes[12-14]. We investigated whether COX-2 inhibitor and its analogue could modulate the Toll-like receptor (TLR) mediated TNF-α, I-309 (Th2-related chemokine), MDC (Th2-related chemokine) and IP-10 (Th1-related chemokine) expression in monocytes. We also explored the mechanisms of intracellular pathway, including MAPK and NFκB.

Material & Methods

This study was conducted in Division of Nephrology, Department of Internal Medicine, Kaohsiung Armed Forces General Hospital, Kaohsiung and Department of Pediatrics, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan during July 2009 to January 2011.

Cell preparation: The human monocytic cell line THP-1 (American Type Culture Collection, Rockville, MD, USA) was cultured in RPMI 1640 medium (Sigma Chemical Co., USA) supplemented with 10 per cent foetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37°C and 5 per cent CO_2 in a humidified incubator. Cells were centrifuged and resuspended in fresh medium in 24-well plates at a concentration of 10^6/ml for 24 h before experimental use. The study protocol was approved by the Institutional Review Board of Kaohsiung Medical University, Taiwan. After obtaining informed consent,

peripheral blood samples (20 ml) were collected from healthy individuals who had no personal or family history of allergies (n = 3). Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation (Lymphoprep, Oslo, Norway). The THP-1 cells and PBMCs were pre-treated with dipyrone and 2,5-dimethylcelecoxib (10^{-9} M- 10^{-4} M, Sigma Chemical Co., USA) 2 h before LPS (0.2 µg/ml) (*Escherichia coIi*, E5 strain; Sigma Chemical Co.) stimulation. Cell supernatant was collected at 24 h after LPS stimulation. Cells were centrifuged and resuspended in fresh medium in 24-well plates at a concentration of 10^6/ml for 24 h before experimental use.

Cell viability: Various concentrations of dipyrone and 2,5-dimethylcelecoxib (10^{-9}, 10^{-4} M) were incubated in a 96-well plate for 24 h. XTT activation solution (Sigma Chemical Co., USA) was mixed in a reagent and diluted with a ratio of 1: 50. Then 50 µl reaction solution was added to each well and the plate was incubated for 5 h. The cell viability was calculated by measuring the absorbance of the sample with an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Rad Benchmark Plus microplate spectrophotometer, USA), at a wavelength of 470 nm and using 650 nm as reference. The mean value of the content of 4 wells was used to assess the cell viability expressed as per cent of control.

ELISA assay: The TNF-α, I-309, MDC and IP-10 concentrations of cell supernatants were determined using commercially available ELISA-based assay systems (R&D System, Minneapolis, MN, USA). Assays were performed using the protocols recommended by the manufacturer.

Western blotting and c-Jun MAPK activity assay: After treatment for 2 h with or without dipyrone and 2,5-dimethylcelecoxib, the cells were stimulated with LPS (0.2 µg/ml) and lysed with equal volumes of ice-cold 150-µl lysis buffer 1 h later. After centrifugation at 13,000 × *g* for 15 min, equal amounts of cell lysates were analyzed by Western blot with anti-p65, anti-phospho-p65, anti-MAPK (p38, ERK and JNK), and anti-phospho-MAPK (pp38, p-ERK and p-JNK) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibody and the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Chicago, IL, USA). JNK and c-Jun MAPK kinase activities in cells were measured by nonradioactive JNK and c-Jun MAPK assay kits (Cell Signaling Technology, Danvers, MA, USA). Assays were performed using the protocols recommended by the manufacturer. The c-Jun was used as substrates for JNK MAPK assay *in vitro*, and c-Jun phosphorylation was detected by Western blot using phospho-c-Jun (p-c-Jun) antibody to determine JNK MAPK activity.

Statistical analysis: All data were presented as mean ± SD of 4 independent experiments using THP-1 cells and of 3 independent experiments using human primary monocytes. Differences between experimental and control groups were analyzed by using the Mann-Whitney test. $P<0.05$ was considered indicative of significant between-group differences.

Results

Effect of dipyrone and 2,5-dimethylcelecoxib on LPS-induced Th2-related chemokine expression in THP-1 cells and PBMCs: The cell viability of THP-1 cell was not significantly influenced by dipyrone (Fig. 1A) and 2,5-dimethylcelecoxib (Fig. 1B). Dipyrone (10^{-9}- 10^{-4} M) significantly ($P<0.05$) downregulated LPS-induced CCL-1/I-309 production in THP-1 cells (Fig. 2A); 2,5-dimethylcelecoxib (10^{-9} – 10^{-4} M) also significantly ($P<0.05$) reduced LPS-induced CCL1 expression in THP-1 cells (Fig. 2B). Moreover, at a very low concentrations also dipyrone (10^{-9} – 10^{-4} M) significantly downregulated LPS -induced CCL1/I-309 production in PBMCs (Fig. 2C). 2,5-Dimethylcelecoxib suppressed LPS-induced CCL1/I-309 production in PBMCs (Fig. 2D). However, only at higher concentrations dipyrone and 2,5-dimethylcelecoxib (10^{-5}, 10^{-4} M) suppressed LPS-induced CCL22/MDC production in THP-1 cells (Fig. 3A and 3B) after 24 h of LPS stimulation. These data suggested COX-2 inhibitor and its analogue might have differently suppressive effect on different chemokine production in THP-1 cells. Dipyrone and 2,5-dimethylcelecoxib significantly suppressed LPS-induced CCL22/MDC production in PBMCs (Fig. 3C and 3D).

Effect of 2,5-dimethylcelecoxib and dipyrone on LPS-induced IP-10 production in THP-1 cells, and LPS-induced TNF-α expression in PBMCs: 2,5-Dimethylcelecoxib and dipyrone significantly ($P<0.05$) downregulated LPS-induced IP-10 production in THP-1 cells (Fig. 4A and 4B). However, LPS-induced IP-10 production by PBMCs was not detected. Dipyrone and 2,5-dimethylcelecoxib had no effect on LPS-induced TNF-α expression in THP-1 cells (Fig. 5A and 5B). Only 2,5-dimethylcelecoxib reduced LPS-induced TNF-α production at high concentration (10^{-4} M) in PBMCs (Fig. 5C and 5D).

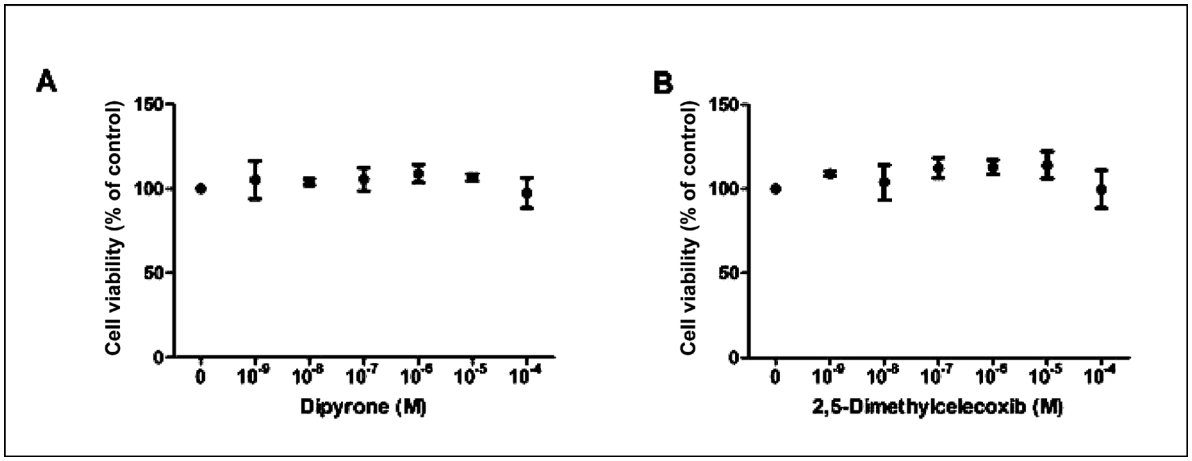

Fig. 1. Effect of **(A)** Dipyrone and **(B)** 2,5-dimethylcelecoxib on the cell viability of THP-1 cell. Values are mean ± SD (n=4).

Effect of 2,5-dimethylcelecoxib on LPS-induced IP-10 and I-309 expression via JNK-c-Jun pathway in THP-1 cells: Dipyrone and 2,5-dimethylcelecoxib suppressed LPS-induced phosphorylation of JNK and p65, but not p38 or ERK (extracellular signal regulated kinase) in THP-1 cells. Therefore, the suppressive effect of dipyrone and 2,5-dimethylcelecoxib on LPS-induced MDC and I-309 expression in THP-1 cells may be through MAPK-JNK and p65 pathway. 2,5-Dimethylcelecoxib downregulated p-c-Jun expression in MAPK kinase assay. These data suggested 2,5-dimethylcelecoxib suppressed LPS-induced Th2-related chemokine MDC and I-309 expression, at least partly, through JNK-c-Jun and p65 pathways.

Discussion

The effect of traditional NSAIDs relies on inhibition of the COX enzyme, resulting in reduced amount of pro-analgesic prostaglandins. COXs exist as two distinct isoforms: COX1, which is constitutively expressed in nearly all tissues, and mediates physiological responses (*e.g*. cytoprotection of the stomach, platelet aggregation); and COX2, which is used for the synthesis of prostanoids involved in pathological processe and chronic inflammatory states. COX2 inhibitors are used in rheumatic diseases for the treatment of arthritic pain through suppressing predominantly COX2-dependent prostaglandins and for the treatment of hyperanalgesia through acting in the spinal cord[15].

CC chemokine ligand 1 (CCL1; also called I-309) is a CC chemokine that interacts with CC chemokine receptor 8, which is preferentially expressed in polarized T helper cell type 2 and cytotoxic T type 2 cells, in eosinophils and also in T regulatory cells. CCL1/I-309 has a unique pattern of regulation associated with a distinct form of M2 (Type 2, M2b) monocyte activation, which participates in macrophage-dependent regulatory circuits of innate and adaptive immunity[16]. In general, M2 mononuclear phagocytes tune inflammatory responses and adaptive Th2 immunity, scavenge debris, and promote angiogenesis, tissue repair, and remodelling[9]. Because CCL1 is an important indicator of M2b polarization induced by immune complexes and dipyrone as well as 2,5-dimethylcelecoxib showed suppressive effect on CCL1, it is suggested that dipyrone and 2,5-dimethylcelecoxib may also inhibit the immune complex-induced inflammation in autoimmune disease. This aspect deserves further investigation in detail.

In the present study, dipyrone and 2,5-dimethylcelecoxib appeared to be potent in suppressing the TLR-related production of Th2-associated I-309/CCL1 in THP-1 cells and also in PBMCs. The celecoxib analogue, 2,5-dimethylcelecoxib (DMC) lacks COX-2 inhibitory activity but exhibits anti-tumour properties comparable to the COX-2 inhibitor celecoxib[17]. *In vitro* and *in vivo* analyses confirmed that 2,5-dimethylcelecoxib has anti-tumour and antivascular activity[18]. CCL1/I-309 plays an important role not only in inflammation but also in apoptosis, angiogenesis and tumour biology[19]. The immunosuppressive environment created by intratumoural accumulation of T regulatory cells (Tregs) reduces the efficacy of antitumour immunotherapy. The neutralization of CCL1 can be used as an adjuvant to antitumour immunotherapy by reversing the immunosuppressive function of Tregs[17]. Therefore, the suppressive effect of 2,5-dimethylcelecoxib may be further enhancing its anti-tumour property.

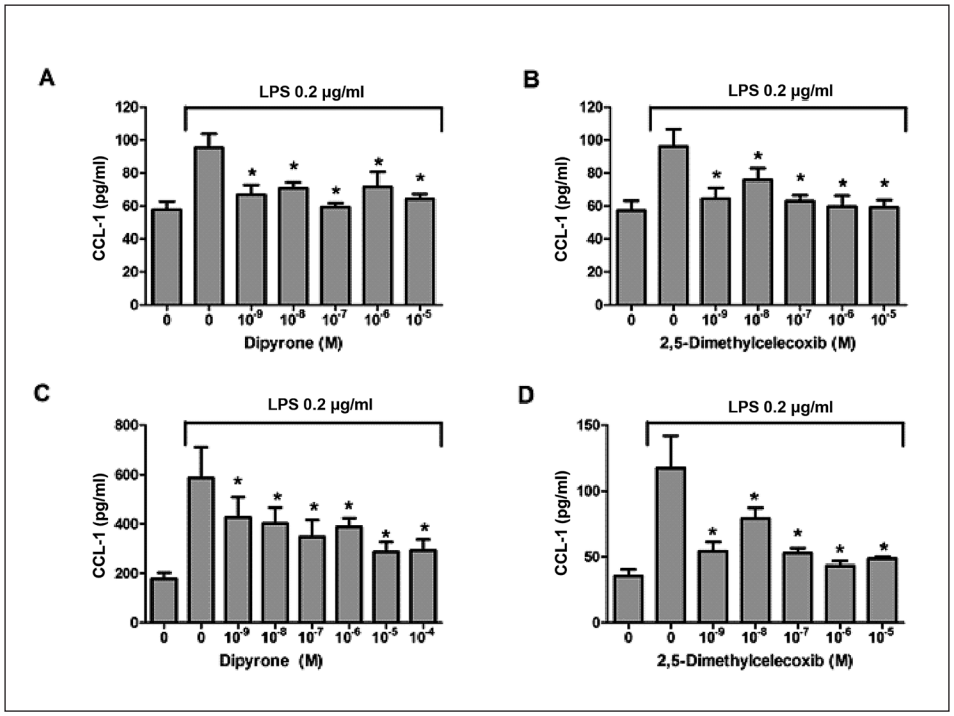

Fig. 2. **(A)** Dipyrone and **(B)** 2,5-dimethylcelecoxib (10^{-9} – 10^{-5} M) significantly suppressed LPS-induced Th2-related chemokine CCL-1 production in human monocyte cell line THP-1 after 24 h of LPS stimulation. The production of LPS-induced CCL1 was suppressed by **(C)** dipyrone and **(D)** 2,5-dimethylcelecoxib in PBMCs. (*: $P< 0.05$ between LPS and LPS plus dipyrone or 2,5-dimethylcelecoxib treatment). Values are mean ± SD (n=4).

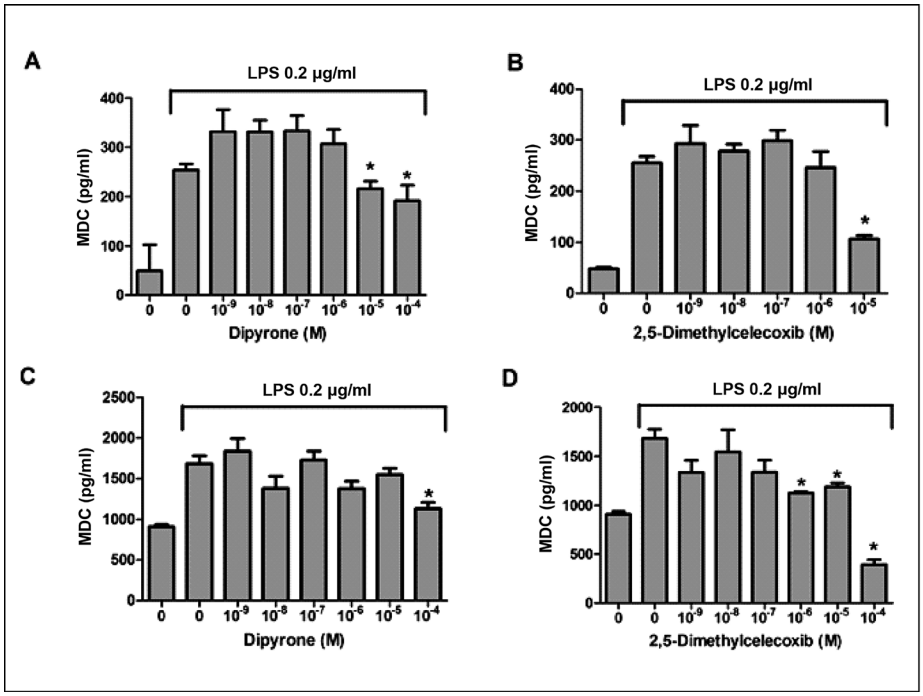

Fig. 3. Only high doses of **(A)** dipyrone (10^{-5} – 10^{-4} M) and **(B)** 2,5-dimethylcelecoxib (10^{-4} M) significantly reduced LPS-induced Th2-related chemokine CCL-22/MDC production in human monocyte cell line THP-1 after 24 h of LPS stimulation. The LPS-induced MDC production was suppressed by **(C)** dipyrone and **(D)** 2,5-dimethylcelecoxib in PBMCs. (*: $P<0.05$ between LPS and LPS plus dipyrone or 2,5-dimethylcelecoxib treatment). Values are mean ± SD (n=4).

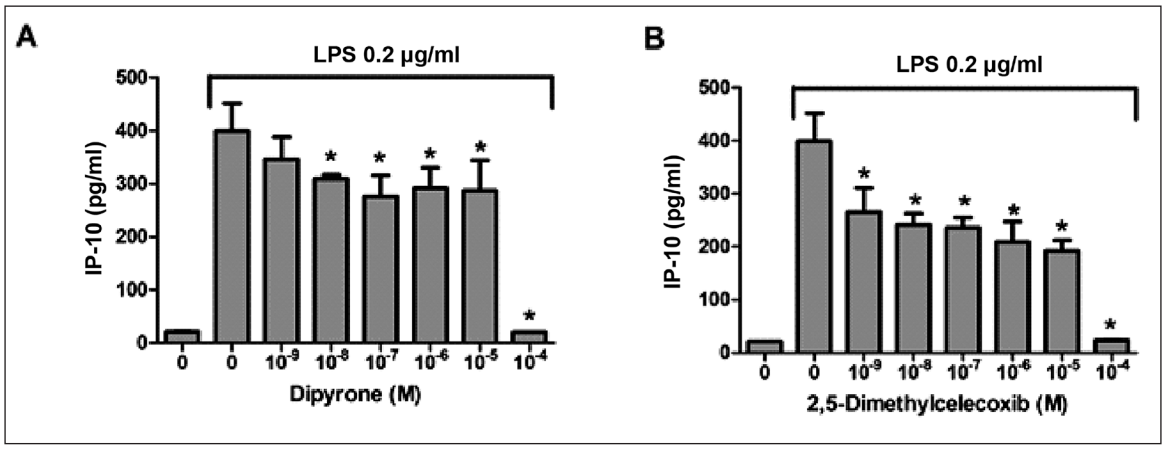

Fig. 4. **(A)** Dipyrone and **(B)** 2,5-dimethylcelecoxib downregulated LPS-induced IP-10 production in THP-1 cells 24 h after LPS stimulation. (*: $P<0.05$ between LPS and LPS plus dipyrone or 2,5-dimethylcelecoxib treatment). Values are mean ± SD (n=4).

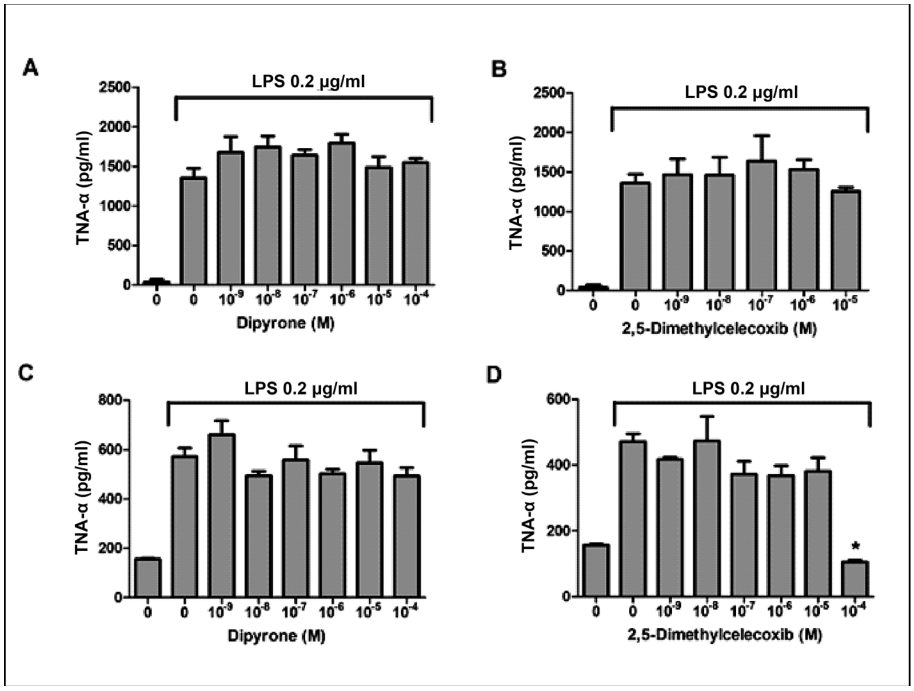

Fig. 5. **(A)** Dipyrone and **(B)** 2,5-dimethylcelecoxib could not suppress the LPS-induced TNF-α production in THP-1 cells after 24 h of LPS stimulation. Not **(C)** dipyrone but **(D)** 2,5-dimethylcelecoxib decreased LPS-induced TNF-α production in PBMCs. (*: $P< 0.05$ between LPS and LPS plus dipyrone or 2,5-dimethylcelecoxib treatment). Values are mean ± SD (n=4).

We further examined the intracellular signal pathway to further explore the mechanisms. MAPKs and NFκB are involved in LPS-induced chemokines and cytokines expression of monocytes[12-14]. According to our previous report, all MAPK pathways were involved in LPS-induced I-309 in human monocytes[20]. Our present data showed that the inhibition of JNK activation by dipyrone and 2,5-dimethylcelecoxib may at least in part be involved in the suppressive effects of dipyrone and 2,5-dimethylcelecoxib on the chemokines expression of LPS-stimulated monocytes. The data of Western blot and MAP kinase assay showed that dipyrone and 2,5-dimethylcelecoxib downregulated LPS-induced p65 and p-c-Jun expression. These data suggest that dipyrone and 2,5-dimethylcelecoxib may reduce LPS-induced I-309 expression via, at least partly, p65 and JNK-c-Jun pathway.

In the present study, dipyrone and 2,5-dimethylcelecoxib appeared to be potent in suppressing the production of Th2- and M2-associated chemokine in monocytes. Dipyrone and 2,5-dimethylcelecoxib suppressed LPS-induced Th2-related chemokine as well as TNF-α production in PBMCs, especially dipyrone.

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