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Original Research

P_2X and P_2Y receptor antagonists reduce inflammation in ATP-induced microglia

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Abstract

Background: P2 receptors have been implicated in the release of neurotransmitter and pro-inflammatory cytokines due to their response to neuro-excitatory substances in the microglia. The P2X4, P2X7 and P2Y12 receptors are involved in the development of pain behavior induced by peripheral nerve injury. However, it is not known if blocking P2X4, P2X7 and P2Y12 receptors is associated with the expression and the release of interleukin-1B (IL-1β), interleukin-6 (IL-6), or tumor necrosis factor-α (TNF-α) in cultured neonatal spinal cord microglia. **Objective:** For this reason, we examined the effects of P2X4, P2X7 and P2Y12 antagonists on the expression and the release of IL-1β, IL-6, and TNF-α in ATP-stimulated microglia. **Methods:** In this study, we observed the effect of A-740003, PSB-12062 and MRS 2395 (P2X4, P2X7 and P2Y12 receptors antagonist, respectively), on the expression and release of IL-1β, IL-6 and TNF-α by using real-time fluorescence quantitative polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA). **Results:** ATP induced the increased expression of IL-1β, IL-6 and TNF-α at the level of messenger RNA (mRNA). ATP-evoked increase in IL-1β, IL-6 and TNF-α mRNA expression was inhibited by the P2X4 receptor antagonist A-740003 or P2X7 receptor antagonist PSB-12062, respectively. Similarly, ATP-evoked release of IL-1β, IL-6 and TNF-α was inhibited by A-740003 and PSB-12062. Furthermore, ATP-evoked increased expression of Iba-1, IL-1β, IL-6 and TNF-α mRNA, and release of IL-1β, IL-6 and TNF-α were nearly all blocked after co-administration of A-740003 plus PSB-12062. Finally, ATP-evoked increased gene expression and release of IL-1β, IL-6 and TNF-α were also inhibited by MRS 2395 (P2Y12 antagonist). **Conclusion:** These observations suggest a new clue for therapeutic strategies to treat the neuro-inflammation.

Keywords: microglia; P2X receptor; P2Y receptor; ATP; IL-1β; IL-6; TNF-α

INTRODUCTION

Microglia, the primary resident immune cell population in the brain and spinal cord, are suggested to be involved in cellular-immunology and neuro-inflammation, as well as acting as the first line of defense against infection or injury.[1] Microglial responses are mediated via several effector mechanisms, including clearance of the debris or extracellular aggregates, initiation of the repair process, and regulation of neuronal excitability and synaptic strength.[2,3] Among the signaling molecules released within the neuro-inflammatory microenvironment, adenosine triphosphate (ATP) is a key molecule in stimulating and maintaining reactive microglia in case of pathological conditions. It can cause microglial chemotaxis resulting in microglial accumulation in damaged areas.[4] ATP activates microglia through both ATP-gated ionotropic P2X family and G protein-coupled metabotropic P2Y receptors.[5] Functional and pharmacological data provide evidence that indicates purinergic signaling has been associated in both the beneficial and toxic effects in microglia.[4] Recent evidences show that ATP-gated P2X7 receptors (P2X7Rs) and P2X4 receptors (P2X4Rs) critically control the immunological response in microglia. Extracellular ATP triggers tumor necrosis factor-α (TNF-α) release in rat brain microglia via P2X7 receptor, by a mechanism that is dependent on both the Ca^{2+} influx and ERK/ P38 signaling pathway.[6] The P2X4 receptor is highly expressed in microglial cells and peripheral macrophages.[7] The location and specific up-regulation of the P2X4 receptors in injured-spinal or supraspinal are critical, as the activated microglia link the receptor to pathophysiologic processes underlying neuropathic pain,[8] traumatic brain injury[9] and cerebral ischemia.[10] The association between upregulation of P2X4 and P2X7 receptors and microglial activation points to a neuro-inflammatory mechanism, the pharmacological blockage of which may provide a therapeutic potential in the treatment of neuropathic pain. P2X4 receptor knockout mice exhibited a significant decrease in mechanical allodynia and hyperalgesia in the spinal nerve ligation model of neuropathic pain.[11] In addition, P2X7 knockout mice displayed a decreased inflammatory response in an experimental arthritis model, indicating an essential role of the P2X7 receptor in inflammation.[12] Several studies reported that microglia cells also express P2Y receptors coupled to different G-proteins that mediate different biological effects through a cascade of downstream intracellular processes.[13-15] P2Y receptors have a main role in ATP-evoked interleukin-6 (IL-6) release in MG-5 cells (mouse microglial cell line).[16] Furthermore, the increased - expressions of P2Y12 and P2Y13 receptors in spinal cord microglia results in the P38MAPK phosphorylation and pain behaviors, in the rat peripheral nerve injury model.[17] In this study, we observed the effect of P2X and P2Y receptors antagonists on the expression and release of IL-1β, IL-6 and

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TNF-α using real-time quantitative polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Isolation of primary microglial from neonatal rat spinal cord

Neonate primary microglial cells were isolated from Postnatal day P1 and P2 Sprague Dawley rats, following the protocol provided by (Tamashiro et al., 2012) with a few modifications. Rats were rinsed with 70% ethanol then decapitated using a sterile scissor. Spinal cords were flushed with a sterile chilled Phosphate buffered saline (Euroclone) and collected in a chilled 7 ml of Leibovitz's L-15 (Euroclone) media supplemented with 0.1% BSA and 1.0% Penicillin/streptomycin. Spinal cords were carefully released from meninges and placed in a 50 ml tube containing 10 ml fresh chilled L-15 conditioned medium and centrifuged at 4 C for 5 minutes at a speed of 2500 xg. Supernatant was discarded and the pellet was resuspended with 5 ml of fresh chilled conditioned L-15 medium, and the spinal cords were subjected to gentle pipetting using a 10 ml serological pipette, then passed through a 100µm cell strainer to get a single cell suspension. The suspension was again centrifuged as mentioned above and the pellet was resuspended with 6 ml of DMEM conditioned medium, supplemented with 10% fetal bovine serum, 1% of Penicillin/ streptomycin and 1% of L-Glutamine. Cell suspension was distributed into a poly-L Lysin coated T75 tissue culture flasks with a ratio of 3 spinal cords/one T75 flask and cultured at 37 °C with 5% CO_2 in a humidified incubator. At day 5, Medium was changed, and then changed every 3 days until reaching confluency (10-14 days after isolation).

Harvesting and seeding of microglia cells

Microglia were detached from the mixed glial cultures using an orbital shaker with a speed of 100rpm for 1-2 h in CO2 incubator. Detached Microglia were collected and centrifuged at 2500 xg for 5 mints at 4 °C. The supernatant was discarded, and the pellet was resuspended with 1-2 ml of fresh conditioned culture media and counted. The pure isolated microglial cells were seeded into a poly-L lysin coated 12 well plate with a seeding density of 12×10^4 cell/well, and allowed to attach overnight in a 5% CO2 humidified incubator at 37 °C.

Treatment with purinergic receptor antagonists

Anti-inflammatory protective effect of the following purinergic receptor antagonists was tested on seeded cells: PSB-12062 (Sigma Aldrich, USA) a blocker for P2X4 receptor, A-740003 (Tocris, UK) a blocker for P2X7, MRS 2395 (Sigma Aldrich, USA) a blocker for P2Y12, and a combination of PSB-12062 and A-740003 together (P2X4 and P2X7 blockers, respectively). A concentration of 10 µM was used for a single type of treatment, while a concentration of 5 µM was used for both types of blockers in the combination group. Treated cells were incubated for one hour at 37 °C in a humidified incubator, then stimulated with 100 µM ATP (Sigma Aldrich, USA) and incubated for 6 hours under the same conditions. Conditioned media were collected and stored at -80 °C until being used. Cells were harvested for RNA extraction.

RNA extraction and cDNA synthesis

RNA was extracted using RNeasy mini RNA extraction kit (Qiagen, Germany), following its manufacturer's protocol. The amount of 500 ng of pure total RNA was subsequently converted to complementary DNA using PrimeScriptTM RT Master Mix (Takara, Japan).

Real-time PCR gene expression assay

Changes in the gene expression upon the use of purinergic receptor antagonists were evaluated using qPCR technique. A group of pro-inflammatory cytokine genes; *TNF-α* (Tumor necrosis factor-α) and *IL-1β* (Interleukin-1 β), were tested for changes in their expression levels, in addition to the microglial activation marker genes like *IRF5* and *IRF8* (Interferon regulatory factors 5 and 8, respectively). GAPDH was used as internal reference. Sequences of the primers used in this experiment are listed in Table 1. qPCR was performed using PowerTrack™ SYBR™ Green Master Mix (Applied Biosystems, USA). Following the manufacturer's protocol, a mixture of 11 ng of cDNA, 500 ng of each primer, in addition to the SYBR green mix was prepared. RNase free H_2O was used to adjust the final volume up to 10 µl. Microsoft Excel was used to measure the ΔΔCT values, and subsequently further analyzed using the statistical analysis software Prism version 8 (GraphPad Software, Inc).

Table 1. List of primers used in qPCR experiment

Gene	Primers
TNF-α	Forward: ATGGGCTCCCTCTCATCAGT Reverse: GCTTGGTGGTTTGCTACGAC
IL-1β	Forward: CACCTCTCAAGCAGAGCACAG Reverse: GGGTTCCATGGTGAAGTCAAC
IRF5	Forward: ACTGGGGAGCTCGGTCACAC Reverse: GGGCTCTGTGGGAGAAAGCA
IRF8	Forward: AACCGCAAGGGTGTGTTCGT Reverse: CGCTCCAGCTTGTTGGGTCT
GAPDH	Forward: TCTGCTCCTCCCTGTTCTAGAGA Reverse: CGACCTTCACCATCTTGTCTATGA

Changes in the level of secretory pro-inflammatory cytokines

As a confirmative study, Enzyme linked immunosorbent assay (ELISA) was used to measure the concentration of some secreted pro-inflammatory cytokines. Rat TNF alpha (RayBiotech, USA) and Rat IL-1 beta/IL-F2 DueSet (R&D Systems, USA) ELISA kits were used to measure the concentration of TNF-α and IL-1β cytokines secreted in the previously collected conditioned media, following the manufacturer's protocol provided by the kits. Concentrations were then calculated using Microsoft Excel software using the standard curve equation, and further analyzed using Prism 8 statistical analysis software (GraphPad Software, Inc).

RESULTS AND DISCUSSION

A growing body of literature strongly supports the importance of ATP as an extracellular signaling molecule in neurons and glial cells.[18] The role of purinergic signaling in microglia has been highlighted in the context of inflammation and neuropathic

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pain.[19] Certainly, large amounts of ATP are released by neuronal injured cells and act as a danger-associated molecular pattern (DAMP) into the extracellular environment, and recognized by microglia through their purinergic receptors(ref). Microglia express many purinergic receptors, including P2X4, P2X7 and P2Y12. It is well known that microglia are involved in almost all features of neural development, plasticity, and neuropathic pain. In the recent years, it has been reported that the P2Y12, P2X4 and P2X7 receptors, expressed in rat spinal dorsal horn microglia, are likely to have a central role in inflammation after spared nerve injury.[15] It has been demonstrated that IL-1β, IL-6 and TNF-α are significantly involved in the peripheral nerve injury-induced neuropathic pain and inflammation mechanisms in rodents.[20,21] In the present study, we found that the expression of IL-1β, IL-6 and TNF-α mRNA and their release were regulated by P2Y12, P2X4 and P2X7 receptors in A-740003 -, PSB-12062 -, and MRS-2395-treated microglia cells.

Gene expression changes of pro-inflammatory cytokines

Expression of the pro-inflammatory cytokines in ATP-activated microglia was assessed upon the treatment with a group of blockers that target the inflammatory pathway through the purinergic receptors, P2X4, P2X7 and P2Y12. Our qPCR results demonstrate that the expression of inflammatory cytokines, IL-1β and TNF-α, in addition to the activation markers of microglia; Interferon regulatory factors 5 and 8 (IFR 5 and IRF8), was decreased upon exposure of microglial cells to the antagonists (Figure 1 and Figure 2). Accordingly, the use of P2X4 antagonist (PSB-12062) significantly reduced the expression of IL-1β, with an inhibition percentage of about 53.9% compared to the ATP-activated group, while A-740003 (P2X7 antagonists showed a stronger anti-inflammatory effect and significantly inhibited (63.6%) the expression of IL-1β compared to the ATP-activated group. This indicates that blocking P2X7 receptors effectively prevents the inflammatory response induced by ATP. P2X7 receptor is an ATP ligand-gated nonselective cation channel that is a member of the purine receptor family. ATP could be synthesized and released by cells under stressful conditions in CNS and activates the purinergic receptors.[22] P2X7 receptor activation leads to Ca^{+2} influx, induced the upregulation of pro-inflammatory mediators such as IL-1β and IL-6, and enhanced the production of reactive oxygen species (ROS).[23] Interestingly, combining both antagonists, PSB-12062 and A-740003 exhibited a powerful synergism and markedly reduced IL-1β expression to about 76.42% in comparison to the ATP-treated group (Figure 1A). Several structural and functional studies revealed evidences of potential interactions between P2X4 and P2X7 receptors in some macrophages, which was followed by studies showing that the expression of P2X4 receptor is essential for P2X7 receptor-dependent cytokines and chemokines release in mouse bone marrow-derived dendrites cells.[24] P2Y12 antagonist (MRS-2395) also blocked half the expression of IL-1β compared to the ATP-activated group (Figure 2A). Consistent with previous studies, MRS2395 significantly suppressed mechanical hypersensitization in neuropathic and inflammatory pain models.[25] Besides, P2Y12 knockout mice have revealed a valuable insight into the role of P2Y12 receptors. Genetic knockout of P2Y12 receptors alleviates inflammatory and neuropathic pain.[26,27] Microglial

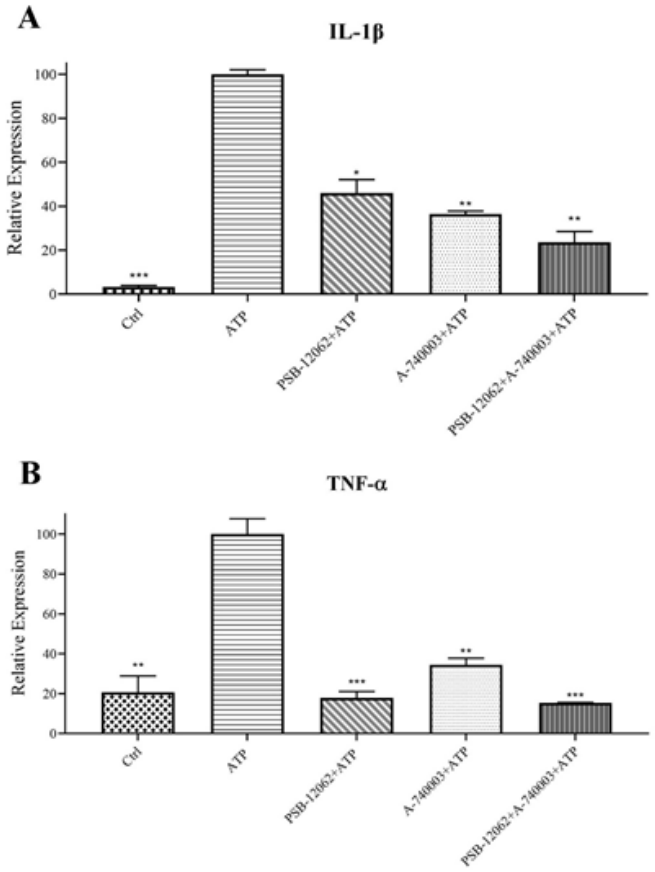

Figure 1. Real-time quantitative polymerase chain reaction results show the expression of IL-1β and TNF-α mRNA in cultured microglia cells. (A) ATP-induced increased expression of IL-1β mRNA was significantly inhibited after pretreatment with A740003, PSB-12062, or co-administration A740003 and PSB-12062. (B) ATP-induced increased expression of TNF-α mRNA was significantly inhibited after pretreatment with A740003, PSB-12062 or co-administration A740003 and PSB-12062. All data were expressed as mean ± SEM of at least 3 independent experiments. Data were analysed by student's t-test. *p < 0.05 and **p < 0.01 compared with ATP-induced cells.

P2Y12 expression is decreased in neuro-inflammatory disorders, such as multiple sclerosis and Alzheimer's.[28,29] Microglia-specific analysis of P2Y12 receptor signaling is essential for the additional development of potential analgesic agents.

Expression of TNF-α cytokine was depressed upon the treatment with purinergic receptor antagonists (Figure 1B). Interestingly, the strongest significant anti-inflammatory effect was observed when combining PSB-12062 and A-740003 (P2X4 and P2X7 antagonists, respectively) (Figure 1B) together and when applying MRS-2395 P2Y12 antagonist (Figure 2B), where both types of treatment potently inhibited about 85% of the expression of TNF-α compared to its expression in ATP treated group. Similarly, PSB-12062 P2X4 antagonist relatively inhibited 82% of the expression of the same cytokine compared to the ATP stimulated microglia group. The least effect, -however still significant and strong, was for A-740003 P2X7 antagonist which inhibited 66% of the expression of TNF- α.

The microglial P2X4 and P2X7 upregulation is a form of microglial activation, and many factors can regulate pain-related P2X4- and P2X7-positive microglial activation. IRF5 is a

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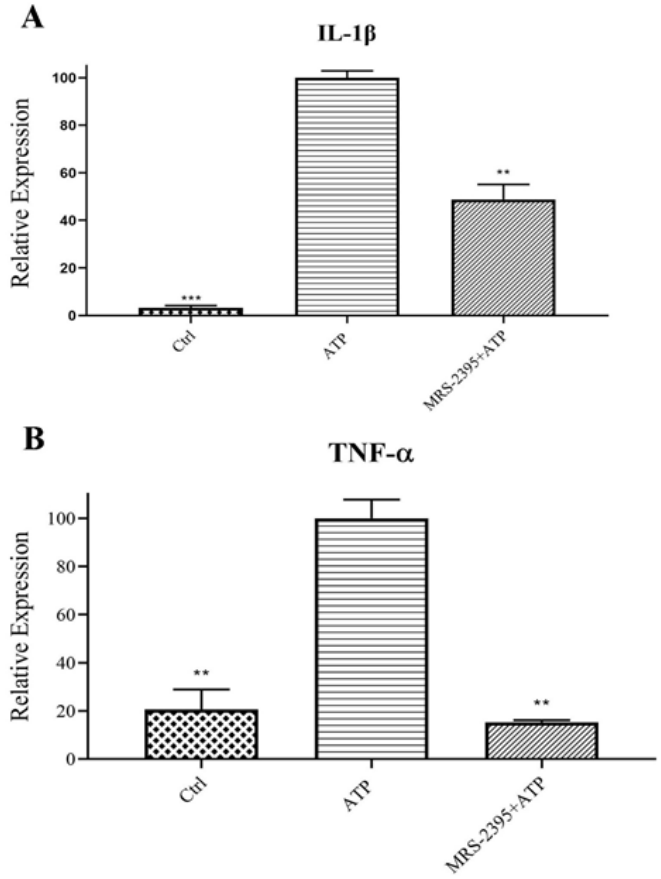

Figure 2. Real-time quantitative polymerase chain reaction results show the expression of IL-1β and TNF-α mRNA in cultured microglia cells. (A) ATP-induced increased expression of IL-1β mRNA was significantly inhibited after pretreatment with MRS-2395. (B) ATP-induced increased expression of TNF-α mRNA was significantly inhibited after pretreatment with MRS-2395. All data were expressed as mean ± SEM of at least 3 independent experiments. Data were analysed by student's t-test. *p < 0.05 and **p < 0.01 compared with ATP-induced cells.

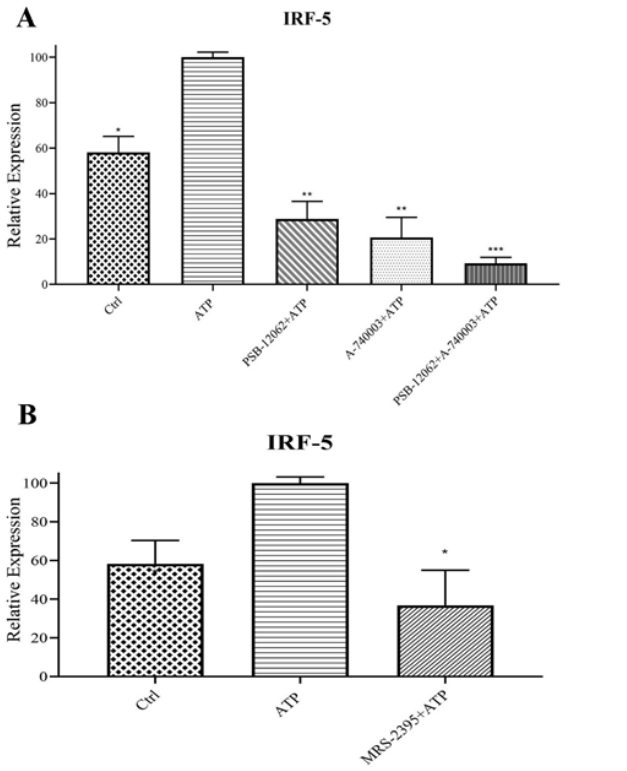

Figure 3. Real-time quantitative polymerase chain reaction results show the expression of IRF5 mRNA in cultured microglia cells. (A) ATP-induced increased expression of IRF5 mRNA was significantly inhibited after pretreatment with A740003, PSB-12062, or co-administration A740003 and PSB-12062. (B) ATP-induced increased expression of IRF5 mRNA was significantly inhibited after pretreatment with MRS-2395. All data were expressed as mean ± SEM of at least 3 independent experiments. Data were analysed by student's t-test. *p < 0.05 and **p < 0.01 compared with ATP-induced cells.

direct transcriptional regulator of P2X4 and P2X7 receptors and is in turn under IRF8 transcriptional control.[30] The expression of IRF5 and IRF8 was significantly depressed upon the treatment of these antagonists (Figure 3A and Figure 4A). The combination of both P2X4 and P2X7 receptors antagonists again exhibited the most potent blockage of the downstream signaling pathways of both IRF5 and IRF8, with an inhibition equals to 91% and 78% relative to their expression in ATP treated group. Both P2X4 and P2X7 receptors antagonists significantly reduced the expression of both IRF5 and IRF8, but their anti-inflammatory effect was higher on IRF5 than in IRF8. Furthermore, the effect of P2X7 receptor antagonist was stronger than P2X4 receptor antagonist on both IRF5 and IRF8, as shown in (Figure 3B). Moreover, P2Y12 blocker (MRS-2395) also significantly reduced the expression of inflammatory activators IRF5 and IRF8 compared to their expression in ATP treated group, and its effect was nearly similar on both types of inflammatory activators (Figure 4B). following peripheral nerve injury (PNI), IRF5 expression increased in spinal microglia, and induced the expression of P2X4 receptor by directly binding to the promoter region of the P2rx4 gene.[30] Mice lacking Irf5 did not upregulate spinal P2X4R after PNI, and also showed substantial resistance to pain hypersensitivity.[30] Previous reports have revealed that toll like receptor (TLR) signals activate gene expression via the IRF5 pathway in macrophages.[31] Previous studies have shown that fibronectin-mediated signals play an important role in the increased the expression of P2X4R in microglia. Additionally, fibronectin stimulation causes IRF5 to cluster on the putative promoter loci of P2X4R.[32] Therefore, activation of IRF5 following ATP treatment in this study provided an additional insight into the function of microglial IRF5. IRF5 upregulation may allow microglial cells to elicit the inflammatory responses more quickly and efficiently. It is reported that IRF8 is a key transcription regulator of microglia for its involvement in the pathogenesis of neuropathic pain after PNI.[33] Genetic ablation of Irf8 in mice, causes the microglial cells to exhibit an abnormal morphology without fine processes,[34] by which microglia sense pathological changes or disorders to maintain homeostasis, suggesting that excessive suppression of microglial IRF8 may constrain the physiological roles of these cells. Therefore, describing transcriptional control mechanisms that participate downstream of IRF8 and regulate specialized microglial genes, including P2X4 and P2X7 receptors, involved in neuropathic pain may be a promising target for chronic pain treatment. Our results reveal that Irf5 expression decreased upon P2X4 and P2X7 receptors antagonist's treatment, suggesting that IRF5 may be a potential therapeutic target.

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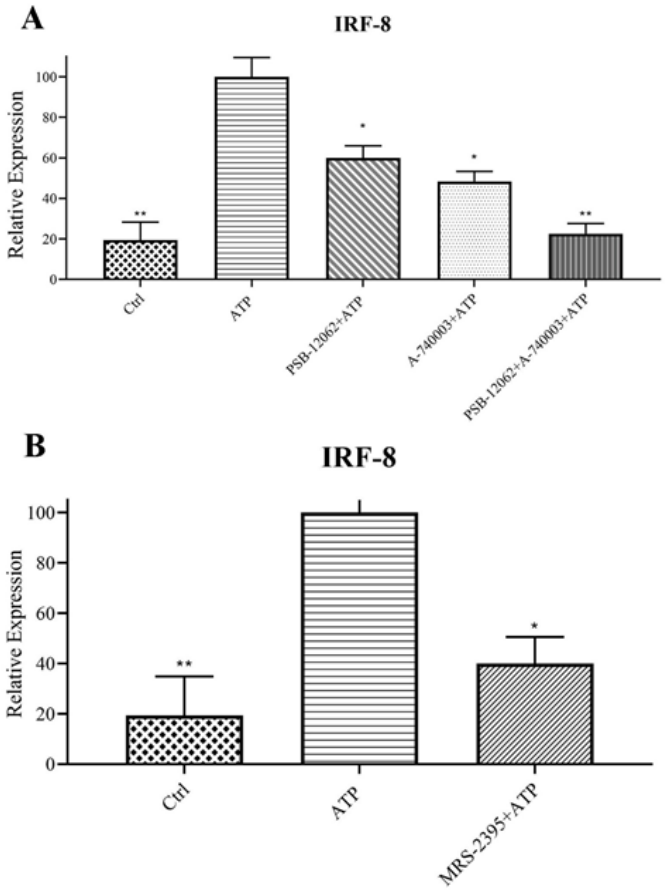

Figure 4. Real-time quantitative polymerase chain reaction results show the expression of IRF8 mRNA in cultured microglia cells. (A) ATP-induced increased expression of IRF8 mRNA was significantly inhibited after pretreatment with A740003, PSB-12062, or co-administration A740003 and PSB-12062. (B) ATP-induced increased expression of IRF8 mRNA was significantly inhibited after pretreatment with MRS-2395. All data were expressed as mean ± SEM of at least 3 independent experiments. Data were analysed by student's t-test. *p < 0.05 and **p < 0.01 compared with ATP-induced cells.

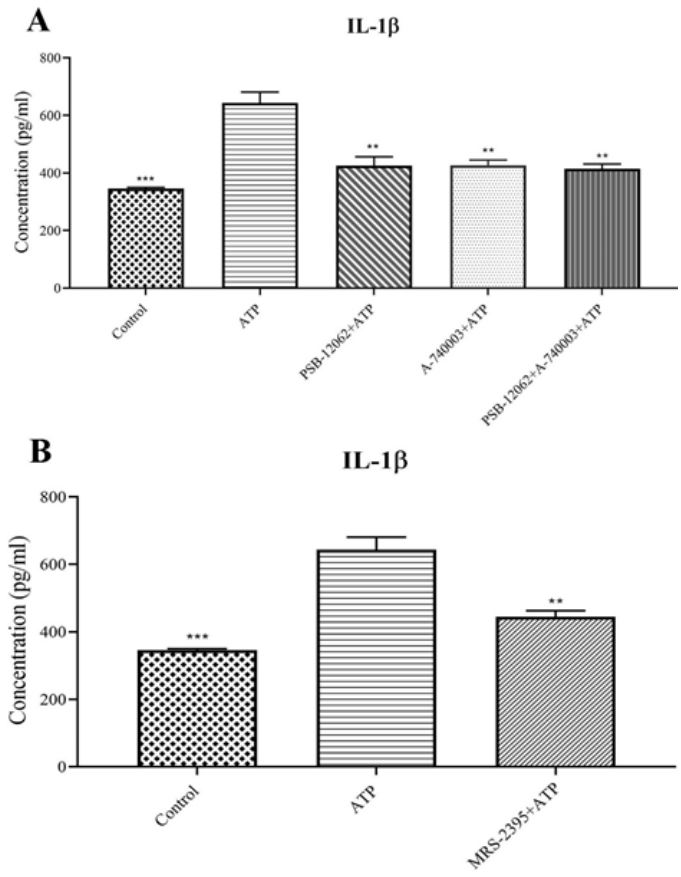

Figure 5. ELISA results show the release of TNF-α protein in cultured microglia cells. (A) ATP-induced increased release of TNF-α protein was significantly inhibited after pretreatment with A740003, PSB-12062, or co-administration A740003 and PSB-12062. (B) ATP-induced increased release of TNF-α protein was significantly inhibited after pretreatment with MRS-2395. All data were expressed as mean ± SEM of at least 3 independent experiments. Data were analysed by student's t-test. *p < 0.05 and **p < 0.01 compared with ATP-induced cells.

Measurements of TNF-α and IL-1β released cytokines

The concentration of the pro-inflammatory cytokines; TNF-α and IL-1β secreted in the conditioned media collected after the treatment of activated microglia with different antagonists for the purinergic signaling pathways was assessed and precisely measured. Enzyme Linked Immunosorbent assay technique was used to reinforce and support our gene expression results regarding these secretory immunological factors. Results show that both TNF-α and IL-1β amounts in the collected conditioned media are significantly reduced in antagonists treated ATP-induced macrophages. This finding parallels our gene expression data, assuring the ability of these synthetic purinergic antagonists to successfully counteract the inflammation induced in ATP-activated macrophages. Potently, P2X4, P2X7 antagonists and their combination significantly blocked the expression of TNF-α cytokine (Figure 5A), returning its level back to normal levels as in the vehicle-treated group. Their effect was similar regarding the release of TNF-α and no significant differences were detected among different treatment groups. The effect of P2Y12 antagonist MRS-2395 on TNF-α release was moderate, lower than the effect of other antagonists, but still significant (Figure 5B).

The anti-inflammatory effect of these antagonists was more noticeable on the secretion of IL-1β in all treatment groups. Similarly, P2X4, P2X7 antagonists and their combination significantly inhibited the release of IL-β, but no individual significant differences were detected among different treatment groups (Figure 6A). Following the same pattern detected on TNF-α, P2Y12 antagonistic effect on the secretion of IL-1β was significant but lesser than others (Figure 6B). Our data suggest the direct involvement of both P2X4 and P2X7 purinergic receptors on the inflammatory responses of neonatal microglial cells, while P2Y12 receptor has a minor role, and its blockage reflects a diminished anti-inflammatory effect compared to other studied receptors.

CONCLUSION

In conclusion, we demonstrated that A740003 and PSB-12062 (P2X7 and P2X4 antagonists, respectively) significantly reduced

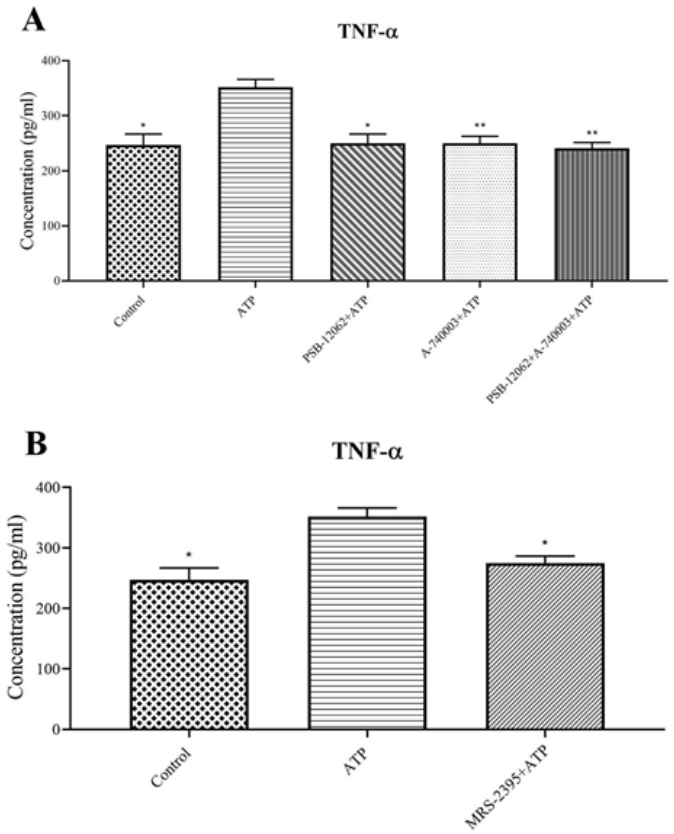

Figure 6. ELISA results show the release of IL-1β protein in cultured microglia cells. (A) ATP-induced increased release of IL-1β protein was significantly inhibited after pretreatment with A740003, PSB-12062, or co-administration A740003 and PSB-12062. (B) ATP-induced increased release of IL-1β protein was significantly inhibited after pretreatment with MRS-2395. All data were expressed as mean ± SEM of at least 3 independent experiments. Data were analysed by student's t-test. *p < 0.05 and **p < 0.01 compared with ATP-induced cells.

inflammatory responses in microglial cells induced by ATP. In addition, the treatment of MRS-2395 (P2Y12 antagonist) prevented the inflammation in microglia. The P2X4 and P2X7 receptors antagonists could reduce inflammation and microglial function by regulation of the IRF5 and IRF8 signaling pathway and suppression of pro-inflammatory cytokines release. The results provide a new clue for therapeutic strategies to treat the neuro-inflammation.

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CONFLICTS OF INTEREST

The authors declare that no competing financial interests or any other conflicts of interest exist.

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