Neuropharmacology 75 (2013) 116–125

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

The activation of $P2Y_6$ receptor in cultured spinal microglia induces the production of CCL2 through the MAP kinases-NF-κB pathway

Norimitsu Morioka*, Masato Tokuhara, Sakura Harano, Yoki Nakamura, Kazue Hisaoka-Nakashima, Yoshihiro Nakata

Department of Pharmacology, Hiroshima University Graduate School of Biomedical & Health Sciences, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan

ARTICLE INFO

Article history:
Received 2 March 2013
Received in revised form
2 July 2013
Accepted 18 July 2013

Keywords:
Spinal microglia
CCL2
$P2Y_6$
UTP
MAP kinases
NF-κB

ABSTRACT

Rat primary cultures of spinal microglia were stimulated by UTP, a known $P2Y_{2/4}$ receptor agonist, which resulted in the production and release of the C–C chemokine CCL2 (monocyte chemoattractant protein-1; MCP-1) measured by real-time PCR and ELISA, respectively. In an in vitro preparation of rat spinal microglia, with regard to the P2Y subtypes, the expression of $P2Y_{1, 2, 6, 12, 13}$ and $P2Y_{14}$, but not $P2Y_4$, were detected by RT-PCR. The subtype of microglial P2Y receptor which could be involved in the production of CCL2 was also determined. The UTP-induced production of CCL2 was significantly blocked by pretreatment with reactive blue 2 and suramin, nonselective P2Y receptor antagonists, and MRS2578, a selective $P2Y_6$ receptor antagonist. By contrast, knockdown of the $P2Y_2$ receptor by RNA interference had no effect. The stimulatory effect of UTP was inhibited by phospholipase C (PLC) inhibitor U73122 and Src tyrosine kinase inhibitor PP2. A potential role of mitogen activated protein kinases was suggested since UTP-induced CCL2 production was significantly blocked by both U0126 and SB 202190, which are potent inhibitors of extracellular signal-regulated kinase (ERK) and p38, respectively. Moreover, UTP-stimulated phosphorylation of these kinases involved the activation of the $P2Y_6$ receptor. Lastly, activation of nuclear factor-κB (NF-κB) by UTP is likely to be essential in the expression of CCL2. Together, these findings suggest that stimulation of spinal microglia $P2Y_6$ receptors induce the production of CCL2 through either PLC-mediated ERK or p38 phosphorylation and the subsequent activation of NF-κB.

1. Introduction

Accumulating evidence suggests that spinal microglia act as a key player in the initiation of various neuropathological disorders (David and Kroner, 2011; Schomberg and Olson, 2012). Molecules released from neurons and leaked from dying cells, such as neuroinflammatory cytokines, chemokines, and prostanoids, are involved in the activation of microglia (Nakagawa and Kaneko, 2010; Svensson et al., 2005; Tokuhara et al., 2010). Thus, understanding the mechanism for the regulation of microglial function in the context of neuropathological disorders is crucial in order to develop effective therapeutics.

A number of studies have demonstrated that the ATP-P2 receptor system has an important role in modulating spinal microglia function (Inoue and Tsuda, 2012; Morioka et al., 2008, 2009). Several subtypes of the P2 receptor and their functions have been identified in microglia. For example, the $P2X_4$ receptor is upregulated in microglia after a spinal nerve ligation, and this response contributes to the initiation of neuropathic pain (Tsuda et al., 2003). Kobayashi et al. (2008, 2012) have indicated that the upregulation of $P2Y_6$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors in spinal microglia is also associated with the development of neuropathic pain. It has been reported that the $P2X_7$ receptor has a crucial role in the regulation of glutamate transporters in spinal microglia (Morioka et al., 2008). Moreover, upregulation of the $P2Y_2$ receptor is detected in macrophage/microglia after spinal cord injury (Rodríguez-Zayas et al., 2010). A previous study found that treatment of spinal microglia with ATP-induced phosphorylation of p38 mitogen-activated protein (MAP) kinase through either Gq or Gi-coupled P2Y receptors (Morioka et al., 2009). In this study, uridine-5′-triphosphate (UTP), which is an agonist of the $P2Y_{2/4}$ receptor and, following degradation to uridine diphosphate (UDP), also activates the $P2Y_6$ receptor, demonstrated pharmacological potency similar to that of ATP. Together, it is possible that UTP-sensitive P2Y receptors may have some role in spinal microglial functioning.

* Corresponding author. Tel.: +81 82 257 5312; fax: +81 82 257 5314.
E-mail address: mnori@hiroshima-u.ac.jp (N. Morioka).

0028-3908/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neuropharm.2013.07.017

Chemokines are associated with various neurodegenerative and neuroinflammatory diseases (Rostène et al., 2011). Among these, CCL2 acts to recruit myeloid cells to the site of neural injury. In the spinal cord, CCL2 released from primary afferent neurons and reactive astrocytes could contribute to either the induction or maintenance of chronic pain (Gao et al., 2009; Van Steenwinckel et al., 2011). In mutant Cu^{2+}/Zn^{2+} superoxide dismutase 1 transgenic mice, the expression of CCL2 was shown to be elevated in spinal microglia (Sargsyan et al., 2009). Furthermore, CCL2 immunoreactivity was located in aggregated spinal microglia from a transgenic rat model of amyotrophic lateral sclerosis (ALS) (Sanagi et al., 2010). Finally, it has been demonstrated that the expression of CCL2 was predominantly expressed in spinal microglia from rats with an experimental autoimmune encephalomyelitis, a model of multiple sclerosis (Tokuhara et al., 2010). Thus, even though the production of CCL2 in microglia appears to be a crucial factor in the pathogenesis of spinal neurodegenerative and neuroinflammatory disorders, it is not yet clear what molecules contribute to the regulation of CCL2 production itself. To understand the mechanism related to the regulation of CCL2 production, the current study utilized cultured spinal microglia stimulated with UTP. It was found that the production of CCL2 by UTP is significantly mediated by $P2Y_6$ receptor activation. Moreover, CCL2 production was associated with the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), p38, but not c-Jun N-terminal kinase (JNK), and the activity of nuclear factor-κB (NF-κB).

2. Materials and methods

2.1. Materials

UTP trisodium, reactive blue 2 (RB2), suramin, and U73122 were obtained from Sigma Chemical Co. (St. Louis, MO). MRS2578, SB 202190, SP600125, and U0126 were purchased from Tocris Cookson (Bristol, UK). PP2 was obtained from Calbiochem (La Jolla, CA). BAY-11-7082 was purchased from Cayman Chemical (Ann Arbor, MI). 2-thioUTP tetrasodium was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell culture

The preparation of cultured rat microglia has been described previously (Morioka et al., 2008). In brief, the isolated spinal cord was minced, and then incubated with trypsin and DNase I. Dissociated cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and penicillin/streptomycin (100 units/ml and 100 μg/ml, respectively). Thereafter, cell suspensions were plated in 75 cm^2 tissue culture flasks ($7.5–10 \times 10^6$ cells/flask) precoated with poly-L-lysine (10 μg/ml). Cells were maintained in an incubator in 10% CO_2 at 37 °C. After 8–13 days, microglia were prepared as floating cell suspensions by gentle shaking of the culture flask. Aliquots were transferred to 35-mm diameter dishes (4×10^5 cells). After 1 h, unattached cells were removed by washing with serum-free DMEM. Prepared microglia showed a purity >98% as determined by CD11b (OX-42) immunoreactivity.

2.3. RT-PCR analysis

Total RNA in cultured microglia derived from either rat spinal cord or cortex was prepared by a previously described method (Chomczynski and Sacchi, 1987) and used to synthesize cDNA with MuLV reverse transcriptase (Applied Biosystems, Foster City, CA) and a random hexamer primer. PCRs were performed with the specific primers indicated in Table 1 and AmpliTag Gold™ (Applied Biosystems) at 95 °C for 10 min followed by 35 cycles of 95 °C for 30 s, the annealing temperature indicated in Table 1 for 30 s, and 72 °C for 2 min with a final extension at 72 °C for 5 min. The resulting PCR products were analyzed on a 1.5% agarose gel and had the size expected from the known cDNA sequence.

2.4. Real-time PCR analysis

cDNA synthesized using 1 μg of total RNA in each sample was subjected to real-time PCR assays with specific primers and EXPRESS SYBR® GreenER™ qPCR SuperMix (Invitrogen). The sequences of primers are as follows: CCL2, 5′-ACGCTTCTGGGCCTGTTGTT-3′ (forward) and 5′-CCTGCTGCTGGTGATTCTCT-3′ (reverse), CCL3, 5′-ATGGCGCTCTGGAACGAA-3′ (forward) and 5′-TTTGGGGTCAGCGCAGAT-3′ (reverse), CCL20, 5′-ATCTGCCCTTCCTGGCTT-3′ (forward) and 5′-ACAAGCTTCGTCGGCCAT-3′ (reverse), $P2Y_2$, 5′-CACCCGTGCTCTACTTTG-3′ (forward) and 5′-GTCCCATAAGCCGGTTTG-3′ (reverse), glyceraldehydes-3-phosphate dehydrogenase (GAPDH), 5′-AGCCCAGAACATCATCCCTG-3′ (forward) and 5′-CACCACCTTCTTGATGTCATC-3′ (reverse). Real-time PCR assays were conducted using a DNA engine Opticon 2 real-time PCR detection system (Bio-Rad). The three-step amplification protocol consisted of 3 min at 95 °C followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. RNA quantities of target genes were calculated using the C_t method. The C_t values of CCL2, CCL3, CCL20 and $P2Y_2$ receptor amplification were normalized to those of GAPDH amplification.

2.5. Western blot analysis

Cells were solubilized in radioimmunoprecipitation assay buffer with inhibitors (100 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and phosphatase inhibitor cocktail 2 (Sigma)). The lysates were centrifuged at 14,000 × *g* for 10 min at 4 °C and the supernatant was added to Laemli's buffer and boiled for 5 min. Equal amounts of protein were separated by 10% SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes. The membranes were soaked in blocking buffer (5% bovine serum albumin), and subsequently incubated with a purified polyclonal antibody against rat phospho-ERK1/2, total-ERK1/2, phospho-p38, total-p38 or phosphor-IκBα (1:1,000, Cell signaling Technology, Beverly, MA), or a monoclonal antibody against β-actin (1:10,000, Sigma) overnight at 4 °C. After washing, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature. The membranes were then rinsed and incubated with a luminescence reagent (Thermo Fisher Scientific, Rockford, IL, USA). Finally, the membranes were exposed to X-ray film. For quantification of signals, the densities of specific bands were measured with Science Lab Image Gauge (Fuji Film, Tokyo, Japan).

2.6. ELISA

CCL2 protein levels in cell-conditioned medium were measured using a CCL2 ELISA kit (eBioscience, San Diego, CA, USA). Following experimental treatment, culture medium was immediately collected and stored at −80 °C until assay. Each reaction was performed according to the protocol of the manufacturer.

2.7. Transfection with small interfering RNA (siRNA)

Spinal microglia were seeded into 24-well plates at 4.0×10^5 cells/wells. After 2 h, the culture medium was replaced with Opti-MEM (Gibco BRL, Rockville, MD), and then cells were transfected with 25 nM of siRNA targeting the rat $P2Y_2$ receptor (ON-TARGETplus SMARTpool, Thermo Fisher Scientific, Rockford, IL, USA) or non-targeting siRNA (siCONTROL Non-Targeting siRNA, Thermo Fisher Scientific) by using Lipofectamine RNAi MAX (Invitrogen) according to manufacturer's instructions. Cells were used in experiments 24 or 48 h after transfection.

2.8. Statistical analysis

Data are expressed as the mean ± SEM of at least three independent determinations. Differences between means were determined using a one-way

Table 1
Primer sequences for RT-PCR of P2Y receptor subtypes and GAPDH.

	Forward primers (5′→3′)	Reverse primers (5′→3′)	Product size	Annealing temp.
P2Y1	CGGCATCTCGGTGTACATGT	AAGATCAGCACCAGGGGGAT	450	61
P2Y2	AACGCCTCCACCACCTACAT	GTGGTCCCATAAGCCGGTTT	503	57
P2Y4	GGATGCAACAGCCACCTACA	CTTGTCCCCCGTGAAGAGAT	736	57
P2Y6	ATGCCTGTTCACTGCCCCTA	CACAGCCAAGTAGGCTGTCT	572	55
P2Y12	AACCCTGGTGTGCCAAGTCA	TGGCTCAGGGTGTAGGGAAT	510	57
P2Y13	GGCTCCTGATGTTCCTCATC	GGTACCTTCCGGGTGAACTT	478	57
P2Y14	AGGGTGTCTGCCGTGATCTT	GGCCATGTAGAAGACGGTCA	342	57
GAPDH	GAGCGAGATCCCGTCAAGATCAAA	CACAGTCTTCTGAGTGGCAGTGAT	330	50