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Astrocytic $P2Y_1$ receptor is involved in the regulation of cytokine/chemokine transcription and cerebral damage in a rat model of cerebral ischemia

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After brain ischemia, significant amounts of adenosine 5′-triphosphate are released or leaked from damaged cells, thus activating purinergic receptors in the central nervous system. A number of P2X/P2Y receptors have been implicated in ischemic conditions, but to date the $P2Y_1$ receptor ($P2Y_1R$) has not been implicated in cerebral ischemia. In this study, we found that the astrocytic $P2Y_1R$, via phosphorylated-RelA (p-RelA), has a negative effect during cerebral ischemia/reperfusion. Intracerebroventricular administration of the $P2Y_1R$ agonist, MRS 2365, led to an increase in cerebral infarct volume 72 hours after transient middle cerebral artery occlusion (tMCAO). Administration of the $P2Y_1R$ antagonist, MRS 2179, significantly decreased infarct volume and led to recovered motor coordination. The effects of MRS 2179 occurred within 24 hours of tMCAO, and also markedly reduced the expression of p-RelA and interleukin-6, tumor necrosis factor-α, monocyte chemotactic protein-1/chemokine (C-C motif) ligand 2 (CCL2), and interferon-inducible protein-10/chemokine (C-X-C motif) ligand 10 (CXCL10) mRNA. $P2Y_1R$ and p-RelA were colocalized in glial fibrillary acidic protein-positive astrocytes, and an increase in infarct volume after MRS 2365 treatment was inhibited by the nuclear factor (NF)-κB inhibitor ammonium pyrrolidine dithiocarbamate. These results provide evidence that the $P2Y_1R$ expressed in cortical astrocytes may help regulate the cytokine/chemokine response after tMCAO/reperfusion through a p-RelA-mediated NF-κB pathway.
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Introduction

Reactive astrocytes and microglia contribute to various disorders of the central nervous system (CNS), such as Alzheimer disease, multiple sclerosis, Parkinson disease, neuropathic pain, and stroke. Following cerebral ischemia, astrocytes and microglia become activated. On activation, these reactive astrocytes increase in number and form a wall-like boundary zone (the glial limitans) that surrounds the ischemic region called the penumbra (Stoll *et al*, 1998), which is where much of the reperfusion injury and delayed cell death occur.

Both *in vivo* and *in vitro* studies have shown that significant amounts of adenosine 5′-triphosphate (ATP) are released or leaked from damaged neurons or glial cells under conditions of cerebral ischemia. Extracellular ATP functions through the purinergic receptor family, which consists of ionotrophic P2Xs and G protein-coupled P2Ys. Several P2 receptors have been implicated in cerebral ischemic brain damage. For instance, the P2X1, P2X2, P2X4, P2X7, and $P2Y_1$ receptors ($P2Y_1Rs$) are upregulated in neurons or glial cells in various brain regions after ischemia (Tu and Wang, 2009), and a significant reduction in infarct volume has been observed following treatment with pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid, a P2 receptor antagonist (Lammer *et al*, 2006). Thus, P2 receptors are thought to have a key role in the pathophysiology of cerebral ischemia.

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Nuclear factor-κ-light-chain enhancer of activated B cells (NF-κB) is expressed in many cell types in the CNS and is activated by various stimuli in focal ischemia, for example, glutamate, interleukin (IL)-1, tumor necrosis factor-α (TNF-α), and reactive oxygen species (O'Neill and Kaltschmidt, 1997). The NF-κB consists of five subunits: NF-κB1/p50, NF-κB2/p52, RelA/p65, RelB, and c-Rel (Hayden and Ghosh, 2004; O'Neill and Kaltschmidt, 1997). These subunits can form homodimers and heterodimers and induce the transcription of many genes involved in the pathogenesis of cerebral ischemia, such as IL-6, TNF-α, intercellular adhesion molecules, inducible nitric oxide synthase, and cyclooxygenase-2 (O'Neill and Kaltschmidt, 1997). In focal cerebral ischemia, an increase in the transcriptional activity of NF-κB is critical for ischemic damage and cell death (Schneider *et al*, 1999; Zhang *et al*, 2005).

In this study, we focused on the role of the $P2Y_1R$ and phosphorylated-RelA (p-RelA) in the pathophysiology of cerebral ischemia. The $P2Y_1R$, a Gq/G11 protein-coupled receptor, is widely distributed throughout the CNS, and astrocytic $P2Y_1R$ is involved in the secretion of glutamate and several proinflammatory cytokines (Fujita *et al*, 2009; Sun *et al*, 2008). The $P2Y_1R$ has been shown to be involved in cardiac ischemia and Alzheimer disease (Moore *et al*, 2000; Olivecrona *et al*, 2007); however, the role of the $P2Y_1R$ in ischemic brain injury remains unclear. Here, we report that the $P2Y_1R$ and p-RelA are localized in astrocytes and are key mediators of brain damage during ischemia and reperfusion injury.

Materials and methods

Animals

All experimental procedures were performed in accordance with institutional guidelines at Kyushu University regarding the care and use of animals. Male Sprague–Dawley rats (8 to 12 weeks old, 270 to 320 g) were obtained from Kyudo Co., Ltd. (Tosu, Japan). The rats were housed in groups of four per cage at a temperature of $22^{\circ}C \pm 1^{\circ}C$ with a 12-hour light–dark cycle (lights on between 08:30 and 20:30 hours), and had *ad libitum* access to food and water.

Transient Middle Cerebral Artery Occlusion

Transient middle cerebral artery occlusion (tMCAO) was performed using a standard intraluminal procedure as described previously (Harada *et al*, 2005). The rats were anesthetized by inhalation of 2% (v/v) isoflurane in 60% (v/v) oxygen air. Body temperature was maintained at $37.0^{\circ}C \pm 0.2^{\circ}C$ using a heating pad (ATC-101B, Unique Medical, Tokyo, Japan). A rectangular thin probe (LP-CM, Unique Medical) for the laser Doppler flowmetry was inserted under the temporal muscle over the temporal cortex as previously reported (Harada *et al*, 2005). A silicon-coated 30 mm 4-0 nylon monofilament was inserted into the internal carotid artery through a small incision of the right common carotid artery. The rats were then allowed to recover from general anesthesia. Following tMCAO for 30 or 60 minutes, the cervical incision was reopened under brief general anesthesia to allow reperfusion of the MCA. The tMCAO was confirmed by measuring regional cerebral blood flow over the MCA territory using laser Doppler flowmetry (ALF21, Advance Co., Inc., Tokyo, Japan). In the sham group, rats were treated by using the same approach as that of tMCAO, with the exception of insertion of the silicon-coated 30 mm 4-0 nylon monofilament and reperfusion steps.

Intracerebroventricular Drug Administration

The intracerebroventricular drug administration experiments were performed according to the following protocol. The rats were anesthetized by inhalation of 2% (v/v) isoflurane and mounted onto a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). The skull was exposed and a hole was drilled to place a 22-gauge guide cannula (VIDTEC, Fukuoka, Japan) into the lateral ventricle (Bregma coordinates: 0.8 mm posterior, 1.4 mm right lateral, 3.5 mm ventral). The rats were randomized and administrated with either 3 μL of [[(1R,2R,3S,4R,5S)-4-[6-Amino-2-(methylthio)-9H-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid mono ester trisodium salt (MRS 2365; Tocris Bioscience, Bristol, UK), 2′-Deoxy-N^6-methyladenosine 3′,5′-bisphosphate tetrasodium salt (MRS 2179; Tocris Bioscience), adenosine 3′-phosphate 5′-phosphosulfate lithium salt hydrate (A3P5PS; Sigma, St Louis, MO, USA), (1*R**,2*S**)-4-[2-Chloro-6-(methylamineo)-9*H*-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester diammonium salt (MRS 2279; Tocris Bioscience), and ammonium pyrrolidine dithiocarbamate (APDC; Wako, Osaka, Japan), MRS 2179 + APDC, MRS 2365 + APDC. A cohort of animals (referred to as the vehicle) received an intracerebroventricular injection of artificial cerebrospinal fluid. Within the protocol there were six time points at which the animals received the intracerebroventricular drug administration: 15 minutes before MCA occlusion, and 12, 24, 36, 48, and 60 hours after cerebral blood flow reperfusion. The rats were treated with the compounds twice daily. Within these six time points, three were considered 'early': 15 minutes before MCA occlusion, 12 and 24 hours after reperfusion; and three were considered 'late': 36, 48, and 60 hours after reperfusion. For the samples to be analyzed 24 hours after tMCAO by immunohistochemistry, Western blot, or real-time quantitative reverse transcriptase polymerase chain reaction, the drugs were administrated at two time points: 15 minutes before MCA occlusion and 12 hours after reperfusion. Verification of cannula placement was made by visual observation following dissection of the rat brain. Only the samples with correct cannula placement were included for analysis.

Analysis of Cerebral Infarct Volume

The rats were anesthetized with pentobarbital (100 mg/kg, intraperitoneally) 72 hours after reperfusion of the MCA.

The brains were removed and the forebrain was sliced into 2-mm-thick coronal sections. The sections were stained with 2% (w/v) 2,3,5-triphenyltetrazolium at 37°C for 30 minutes and fixed in 20% (w/v) paraformaldehyde. The stained slices were photographed using a complementary metal oxide semiconductor camera with a squared scale to calculate the area of the infarct. Infarct size was analyzed using Image J 1.42q software (National Institutes of Health, Bethesda, MD, USA) and compared with the ipsilateral hemisphere. Total infract volume (mm^3) was determined by multiplying the infarct area by slice thickness (2 mm). Animals with large hematomas in the brain or those that exhibited no infarct were omitted from further analyses.

Rotarod Test

Motor coordination was performed using the KN-75 rotarod apparatus (Natsume, Tokyo, Japan). The rotating speed proportionally increased from 3 to 20 r.p.m. in 0 to 300 seconds. Rotarod test was performed just before drug treatments at 24, 48, and 72 hours after tMCAO to rule out acute drug effects.

Immunohistochemistry

The rats were anesthetized with pentobarbital (100 mg/kg, intraperitoneally) and perfused transcardially with 4% (w/v) paraformaldehyde. Brain sections were obtained, postfixed, and placed in 30% (w/v) sucrose solution for 24 hours at 4°C. The sections (30 μm) were incubated in blocking solution (3% (v/v) normal goat serum/0.3% (v/v) Triton X-100/phosphate-buffered saline) for 2 hours at room temperature, followed by incubation with primary antibodies: mouse antiglial fibrillary acidic protein (anti-GFAP) antibody (1:500; Chemicon, Temecula, CA, USA), mouse antiionized calcium-binding adaptor molecule 1 (anti-Iba1) antibody (1:2,000; Wako), mouse anti-CD11b (anti-OX42) antibody (1:1,000; Serotec, Kidlington, UK), mouse antineuronal nuclei antibody (1:200; Chemicon), rabbit anti-$P2Y_1R$ antibody (1:1,000; Alomone, Jerusalem, Israel), or rabbit antiphospho-NF-κB p65 (Ser536; anti-p-RelA) antibody (1:100; Cell Signaling, Beverly, MA, USA) for 48 hours at 4°C. The sections were washed and incubated with anti-rabbit immunoglobulin G-conjugated Alexa Fluor 488, anti-mouse immunoglobulin G-conjugated Alexa Fluor 488, or anti-mouse immunoglobulin G-conjugated Alexa Fluor 546 (1:1,000; Molecular Probes, Invitrogen, Carlsbad, CA, USA) for 3 hours at room temperature. The brain sections were analyzed by confocal microscopy (LSM510; Zeiss, Oberkochen, Germany). The intensity of Iba1 and OX42 immunoreactivity was analyzed using the Photoshop CS3 software (Adobe, San Jose, CA, USA) and the mean of total fluorescent intensity in the ischemic hemisphere was digitalized.

Western Blotting

The right hemisphere of the brain ($\sim \pm 1.00$ mm from the Bregma) was removed, and total protein extraction was performed according to a previously described method (Hasegawa *et al*, 2009). Samples were separated on a 10% (v/v) polyacrylamide gel, and the protein was transferred onto a polyvinylidene difluoride membrane. The membranes were blocked in Blocking One-P (Nacalai tesque, Kyoto, Japan), incubated with anti-p-RelA antibody (1:1,000) overnight at 4°C and then incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G antibody (1:1,000; Amersham Biosciences, Little Chalfont, UK). Protein was detected using the chemiluminescence method (ECL system; Amersham Biosciences).

Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction

Coronal brain sections ($\sim \pm 1.00$ mm from the Bregma) were removed, and total RNA was prepared with the TRIsure (Bioline, London, UK) and RNeasy Mini total RNA Preparation Kit (Qiagen, Dusseldorf, Germany), in accordance with the methods described previously (Fujita *et al*, 2009). Reverse transcriptase polymerase chain reaction amplification and real-time detection were performed using an ABI PRISM 7500 (Applied Biosystems, Carlsbad, CA, USA). All expression values were normalized by the expression values of 18S ribosomal RNA. The TaqMan probe and the forward and reverse primers used in this study were designed according to Table 1. Ribosomal RNA was measured using TaqMan Ribosomal RNA Control Reagents (P/N 4308329; Applied Biosystems).

Statistics

Data are expressed as the mean with s.e.m. Statistical analyses of the results were conducted with one-way analysis of variance, one-way repeated analysis of variance, or two-way analysis of variance with a *post hoc* test (Tukey multiple comparison test). Statistical significance was set at a P value < 0.05.

Results

Regional Cerebral Blood Flow

Table 2 shows the values for regional cerebral blood flow over the MCA territory in rats that underwent intracerebroventricular administration with the vehicle or the different compounds before or after common carotid artery occlusion, after MCA occlusion, or after reperfusion. These findings did not show any significant differences in the rate of change of regional cerebral blood flow among any of the groups.

$P2Y_1R$ Expression in the Central Nervous System

Localization of the $P2Y_1R$ in the rat brain was determined by fluorescent immunohistochemistry for the $P2Y_1R$ and several cell-type-specific markers. The $P2Y_1R$ only colocalized with GFAP (an astrocyte marker) but did not colocalize with the microglial

Table 1 Sequences of TaqMan probe, forward primer and reverse primer of GFAP, Iba1, $P2Y_1R$, IL-6, TNF-α, MCP-1/CCL2, IP-10/CXCL10, MIP-1α/CCL3, MIP-2/CXCL2, and MCP-5/CCL12 for real-time quantitative RT-PCR

	Probe and primers	*Sequence*
GFAP	Probe	CAGACGTTGCTTCCCGCAACGC
	Forward primer	TGGCCACCAGTAACATGCAA
	Reverse primer	CAGTTGGCGGCGATAGTCAT
Iba1	Probe	CAGGAAGAGAGGTTGGATGGGATCAA
	Forward primer	GATTTGCAGGGAGGAAAAGCT
	Reverse primer	AACCCCAAGTTTCTCCAGCAT
$P2Y_1R$	Probe	CCTGCCTGCGGTCTACATCTTAGTGTTCA
	Forward primer	CAAGACCGGCTTCCAGTTCTA
	Reverse primer	CTGTTGCCAAGGAAGCCTATG
IL-6	Probe	CACAGAGGATACCACCCACAACAGACCAG
	Forward primer	CACAGAGGATACCACCCACAACAGAC
	Reverse primer	CAG TTGCCATTGCACAACTCTTTTC
TNF-α	Probe	CGTAGCCCACGTCGTA
	Forward primer	GACCCTCACACTCAGATCATCTTCT
	Reverse primer	GGTACAGCCCATCTGCTGGTA
MCP-1/CCL2	Probe	AATCACCAGCAGCAGGTGTCCCAAA
	Forward primer	GAGTCGGCTGGAGAAC
	Reverse primer	GGTGACAAATACTACAGCTT
IP-10/CXCL10	Probe	TCCGCATGTTGAGATCATTGCCACA
	Forward primer	CAGGGCCATAGGAAAACTTGAA
	Reverse primer	ATGGCCTCAGATTCCGGATT
MIP-1α/CCL3	Probe	CGCCATATGGAGCTGACACCCCG
	Forward primer	CCACTGCCCTTGCTGTTCTT
	Reverse primer	GCAAAGGCTGCTGGTTTCAA
MIP-2/CXCL2	Probe	CGCCCAGACAGAAGTCATAGCCACTCTT
	Forward primer	CCTACCAAGGGTTGACTTCAAGAA
	Reverse primer	GGCTTCAGGGTTGAGACAAACT
MCP-5/CCL12	Probe	CCCCGGGAAGCTGTGATCTTCAGAAC
	Forward primer	AGATCCACATTCGGAGGCTAAAG
	Reverse primer	CAGCACAGAGCTCCTTATCCAGTA

GFAP, glial fibrillary acidic protein; Iba1, ionized calcium-binding adaptor molecule; IL-6, interleukin-6; IP-10, interferon inducible protein-10; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; $P2Y_1R$, $P2Y_1$ receptor; RT-PCR, reverse transcriptase polymerase chain reaction; TNF-α, tumor necrosis factor-α.

Table 2 Values of rCBF over the MCA territory in rats at first treated (before 15 minutes MCA occlusion) with vehicle or the different compounds before or after CCA or MCA occlusion and reperfusion

Compounds (dose)	N	*Before CCA occlusion*	*After CCA occlusion*	*After MCA occlusion*	*Reperfusion*
Vehicle	7	132 ± 7.0	100 ± 12.3	29.5 ± 7.6	92.3 ± 12.0
MRS 2365 (10 nmol)	6	135 ± 8.0	100 ± 8.8	27.4 ± 5.8	94.6 ± 13.2
MRS 2179 (1 nmol)	6	130 ± 9.5	100 ± 10.4	28.1 ± 9.2	94.0 ± 10.4
MRS 2179 (3 nmol)	6	134 ± 8.2	100 ± 9.8	30.0 ± 10.3	92.4 ± 10.9
A3P5PS (10 nmol)	5	130 ± 7.2	100 ± 7.8	27.1 ± 6.2	93.3 ± 14.4
MRS 2279 (3 nmol)	5	129 ± 8.8	100 ± 11.2	28.8 ± 5.7	93.7 ± 12.2
APDC (30 nmol)	6	132 ± 5.2	100 ± 9.5	29.0 ± 2.2	93.8 ± 18.5
MRS 2179 (3 nmol)+APDC (30 nmol)	6	131 ± 6.3	100 ± 8.6	29.5 ± 8.9	92.2 ± 17.9
MRS 2365 (10 nmol)+APDC (30 nmol)	6	134 ± 9.2	100 ± 7.2	29.6 ± 8.6	93.9 ± 13.3

APDC, ammonium pyrrolidine dithiocarbamate; CCA, common carotid artery; MCA, middle cerebral artery; rCBF, regional cerebral blood flow.
All data are shown as mean ± s.e.m.

marker, OX42, or the neuronal marker, neuronal nuclei. This cell-specific localization did not alter between the sham surgery and tMCAO (at 24, 48, or 72 hours after reperfusion) groups (Figure 1A). In the penumbra region, the $P2Y_1R$-positive area correlated with the GFAP-positive area; however, there was no $P2Y_1R$ signal in the Iba1-positive area, which was likely the ischemic core region (Figure 1B). These results indicate that astrocytes express $P2Y_1R$ in the intact and penumbra region. Moreover, we measured GFAP, Iba1, and $P2Y_1R$ mRNA expression following ischemia. Although GFAP and Iba1 mRNA

expression levels significantly increased after 24 to 72 hours of tMCAO (Figures 1C and 1D), there was no significant change in P2Y$_1$R mRNA expression (Figure 1E). After cerebral ischemia and reperfusion, cerebral infarct volume increased after reperfusion. In fact, infarct volume significantly increased during 24 to 72 hours after tMCAO (Figures 1F and 1G).

Effect of the P2Y$_1$R Agonist and Antagonists on Infarct Volume and Motor Coordination

We measured cerebral infarct volume after 72 hours of tMCAO in rats that were intracerebroventricularly administered different compounds (twice daily for 3 days). The administration of 10 nmol MRS 2365 significantly induced an increase in infarct volume in the MCA 30-minute occlusion model, but there was no change in infarct volume compared with the vehicle group in the MCA 60-minute occlusion model (Figures 2A–2C). Moreover, the administration of MRS 2179, A3P5PS, and MRS 2279 significantly reduced infarct volume in a dose-dependent manner (Figures 2D, 2E, 2G, and 2H). The decrease observed with 3 nmol MRS 2179 was ~63.8% ($1,350 \pm 166$ to $488 \pm 48\,mm^3$, $n = 6$). In addition, these antagonists significantly inhibited motor coordination deficits after tMCAO (Figures 2F and 2I).

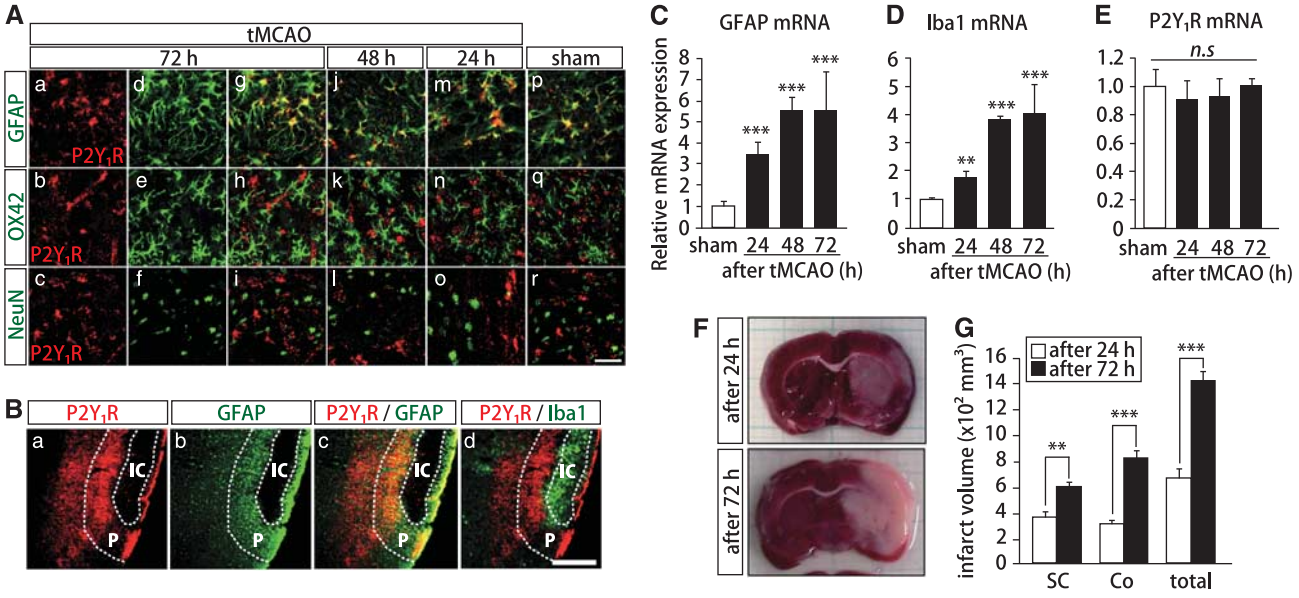
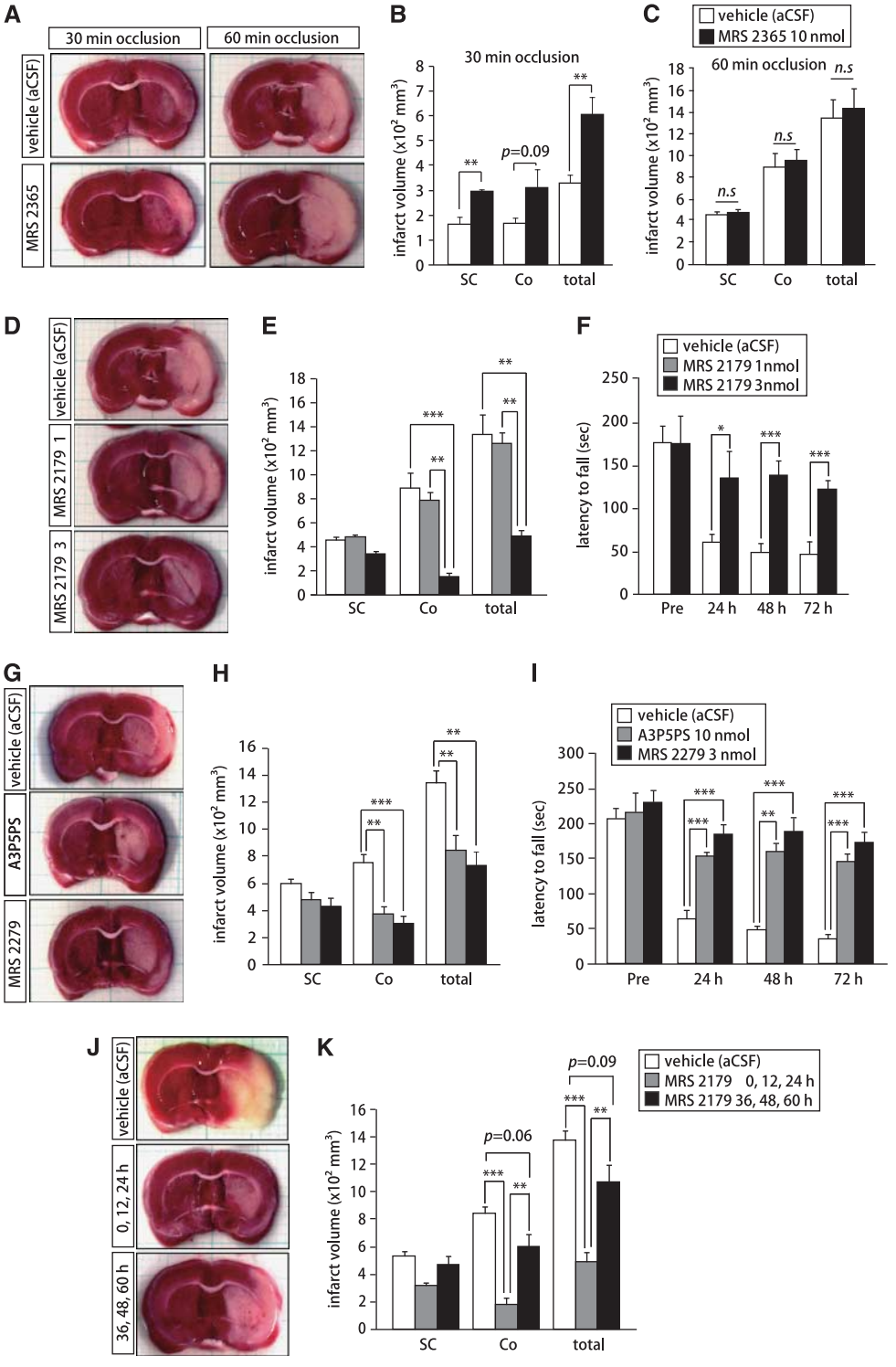


Figure 1 P2Y$_1$ receptor (P2Y$_1$R) expression in the central nervous system (CNS) and cerebral infarct volume after cerebral blood reperfusion. (**A**) Immunohistochemistry of the P2Y$_1$R (a–c), glial fibrillary acidic protein (GFAP) (d), OX42 (e), and neuronal nuclei (NeuN) (f) in the cortex penumbra or naive regions. After sham surgery (p–r), and 24, 48, or 72 hours after transient middle cerebral artery occlusion (tMCAO) (g–o), P2Y$_1$R colocalized with GFAP (g, j, m, p), but not with OX42 (h, k, n, q) or NeuN (i, l, o, r). (**B**) Fluorescent immunostaining in the peripenumbra region 72 hours after tMCAO. The P2Y$_1$R immunoreactive area (a) completely colocalized with the GFAP immunoreactive area (b, c) but not with the ionized calcium-binding adaptor molecule 1 (Iba1) immunoreactive area (d). Dotted lines: borderline of between ischemic core (IC) and P or P and naive region. (**C**–**E**) The relative expression of GFAP (**C**), Iba1 (**D**), and P2Y$_1$R, (**E**) mRNA. Data were normalized against the sham group ($n = 5$ animals/group). (**F**) Images of the 2,3,5-triphenyltetrazolium (TTC)-stained sections of rat brain after 24 and 72 hours of tMCAO. The damaged regions remained unstained. (**G**) Analysis of the infarct volume on the subcortex (SC), cortex (Co), or total area in each group ($n = 6$ animals/group). All data in this figure showed mean with s.e.m. Statistical analysis was determined by one-way analysis of variance (ANOVA) with Tukey test, $^{**}P < 0.01$, $^{***}P < 0.001$. P, pericore region (penumbra). Scale bars = 50 μm (**A**) or 500 μm (**B**).

Figure 2 P2Y$_1$ receptor (P2Y$_1$R) activation significantly augmented infarct volume and coordination deficits in rats within 24 hours of transient middle cerebral artery occlusion (tMCAO). (**A**) Images of 2,3,5-triphenyltetrazolium (TTC)-stained sections of rat brain after 72 hours of 30- or 60-minute MCA occlusion model in the MRS 2365-treated animals. The damaged brain regions remained unstained. (**B**, **C**) Analysis of the infarct volume on the subcortex (SC), cortex (Co), or total area in each group ($n = 6$ animals/group). (**D**) Images of TTC-stained sections of rat brain after 72 hours of tMCAO in the MRS 2179-treated animals. (**E**) Analysis of the infarct volume in each group ($n = 6$ animals/group). (**F**) Motor coordination measured using the Rotarod test ($n = 6$ animals/group). (**G**) Images of TTC-stained sections of rat brain after 72 hours of tMCAO in the A3P5PS or MRS 2279 treatment groups. (**H**) Analysis of the infarct volume in each group ($n = 5$ animals/group). (**I**) Motor coordination measured using the Rotarod test ($n = 5$ animals/group). (**J**) Images of TTC-stained sections of rat brain after 72 hours of tMCAO in the MRS 2179 early group (15 minutes before MCA occlusion 12, 24 hours after reperfusion) or late group (36, 48, and 60 hours after reperfusion) treatment. (**K**) Analysis of the infarct volume in each group ($n = 5$ animals/group). All data in this figure showed mean with s.e.m. Statistical analysis was determined using one-way analysis of variance (ANOVA) (**B**, **H**), one-way repeated ANOVA (**C**, **F**, **I**), or two-way ANOVA (**E**, **K**) with Tukey test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

We also investigated the period of time during which the $P2Y_1R$ antagonist had a cerebroprotective effect. The infarct volume in the rats that were admininstered an intracerebroventricular injection of 3 nmol MRS 2179 were compared between the early group (15 minutes before MCA occlusion and 12 and 24 hours after reperfusion) and the late group (36, 48, and 60 hours after reperfusion). Although in the early group there was reduced infarct volume relative to the vehicle-treated rats, there was no cerebroprotection in the late group relative to the vehicle-treated rats (Figures 2J and 2K).



mRNA Expression of Cytokines/Chemokines Following Occlusion

In this study, the mRNA expression of several cytokines/chemokines was markedly upregulated 24 hours after tMCAO. In the cortex, an increase in IL-6, TNF-α, monocyte chemotactic protein-1 (MCP-1)/chemokine (C-C motif) ligand 2 (CCL2), and interferon-inducible protein-10 (IP-10)/chemokine (C-X-C motif) ligand 10 (CXCL10) mRNA was significantly suppressed by 3 nmol MRS 2179 (Figure 3A). In contrast, the $P2Y_1R$ did not appear to be involved in the transcription of macrophage inflammatory protein-1α/CCL3, macrophage inflammatory protein-2/CXCL2, and MCP-5/CCL12 (data not shown). These results suggest that the $P2Y_1R$ critically contributes to transcriptional upregulation of several cytokines/chemokines under ischemic conditions.

Microglial Activation and/or Macrophage Accumulation

As microglia and macrophages are considered to be major sources of proinflammatory cytokines and chemokines, we used immunohistochemistry to assess whether the administration of 3 nmol MRS 2179 suppressed the activation of microglia and macrophages. The activation of resident microglia and infiltrated macrophages was evaluated by measuring Iba1 and OX42 immunoreactivity. We found that the intensity of Iba1 and OX42 immunoreactivity staining in the tMCAO rats was significantly lower in the MRS 2179 administration group (Figures 3B and 3C).

Expression of Phosphorylated-RelA

In the tMCAO rat brain, the p-RelA protein gradually increased 6 to 24 hours after reperfusion (Figure 4A). Notably, p-RelA colocalized with GFAP, but not with OX42 or neuronal nuclei (Figure 4C). As this localization was similar to that of the $P2Y_1R$ (see Figure 1A), we assessed the effect of 3 nmol MRS 2179 on p-RelA expression after 24 hours of tMCAO. MRS 2179 administration significantly suppressed the increase in p-RelA levels (Figure 4B). Thus, these results suggest that RelA is phosphorylated and activated by $P2Y_1R$ signaling in astrocytes.

Inhibition of Nuclear Factor-κB Activation

We investigated the effect of APDC, a NF-κB inhibitor, on tMCAO. Cerebral infarct volume was significantly reduced following 30 nmol APDC administration (Figures 4D and 4E) after 72 hours of tMCAO. The amount of decrease with APDC was $\sim$43.3% ($1{,}343 \pm 116$ to $761 \pm 70\,mm^3$, $n=6$). Moreover, APDC reduced motor coordination deficits (Figure 4F) and suppressed the upregulation of cytokine/chemokine mRNA levels (Figure 4G) after tMCAO. These results correlate well with the results obtained after MRS 2179 administration. Finally, we investigated the effect of combining APDC with MRS 2365 and MRS 2179. In this case, cotreatment with 3 nmol MRS 2179 and 30 nmol APDC resulted in a marked reduction in infarct volume after 72 hours tMCAO (Figures 5A and 5B). The change of decrease with MRS 2179 and APDC was $\sim$58.0% ($1{,}210 \pm 65$ to $508 \pm 49\,mm^3$, $n=6$), and there was no additive effect with the two compounds relative to using each alone. Moreover, the increase in infarct volume observed after 10 nmol MRS 2365 treatment in the MCA 30-minute occlusion model (see Figures 2A and 2B) was significantly suppressed with APDC cotreatment (Figures 5C and 5D).

Discussion

Adenosine 5′-triphosphate contributes to various CNS diseases including stroke. Following cerebral ischemia, intracellular ATP vanishes and acute necrosis occurs because the supply of oxygen and glucose is interrupted. In this period, ATP is released or leaked by damaged or dying cells (Melani *et al*, 2005). After cerebral blood reperfusion, ATP is resynthesized as the supply of oxygen and glucose to the site is reestablished. It is considered that damaged cells, reactive astrocytes, and neurons release extracellular ATP after reperfusion. Although intracellular ATP behaves as a 'molecular unit of currency' for intracellular energy, in contrast, extracellular ATP has a role as a transmitter molecule. Extracellular ATP functions through the purinergic (P2) receptors presented on the cell membrane and is involved in purinergic signaling in the pathology of ischemic damage. The purinergic receptors, P2X2, P2X4, and P2X7, are upregulated in microglia and the $P2Y_1R$ is localized to astrocytes. Previous work has found that, although the broad-spectrum P2 receptor antagonist, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid, is cerebroprotective, the P2X7R antagonist, benzoyl-ATP, can significantly exacerbate ischemic damage (Suzuki *et al*, 2004). This type of research underscores the importance of understanding the P2 receptor subtypes that ATP functions on.

In previous studies, hippocampal and retinal pyramidal cells, cerebellar Purkinje cells, and glial cells, grouped together as astrocytes, have been found to express $P2Y_1R$ (Moran-Jimenez and Matute, 2000). In addition, *in vitro* studies have also confirmed $P2Y_1R$ expression in microglia (Ballerini *et al*, 2005; De Simone *et al*, 2010). However, in this report, we have shown that the $P2Y_1R$ is expressed on astrocytes before and after focal cerebral ischemia, using an *in vivo* model. Although it is possible that the other cell types may have limited $P2Y_1R$ expression, our data suggest that $P2Y_1R$ expression is mainly restricted to astrocytes. Interestingly, follow-

ing tMCAO, the P2Y$_1$R accumulated in the penumbra, as did GFAP. This observation suggests that the astrocytic P2Y$_1$R may contribute to the protective function of the penumbra region. Following ischemia and reperfusion, GFAP and Iba1 mRNA expression levels were significantly increased but there was no increase in the expression of P2Y$_1$R mRNA. Astrocytes perished in the ischemic core and increased in penumbra, thus making the determination of absolute number of astrocytes difficult.

After ischemia and reperfusion, despite cerebral blood reperfusion, the development of cerebral injury was observed at the 24- to 72-hour time point. We have previously shown that P2Y$_1$R expression in cultured astrocytes is actively involved in the protection against hydrogen peroxide, an reactive oxygen species generated in ischemic stroke. Furthermore, neuroprotection is induced by astrocytic P2Y$_1$R stimulation via IL-6 release in hippocampal astrocyte-neuronal cocultures (Fujita *et al*, 2009). These studies suggest that stimulation of P2Y$_1$R signaling enhances protection from ischemia/reperfusion injury. However, in the present study, MRS 2365 markedly promoted an increase in infarct volume in the MCA 30-minute occlusion model. In the MCA 60-minute occlusion model, MRS 2365 did not worsen cerebral damage. A possible explanation for this is that the cerebral infarct volume itself might be too large, or that the concentration of ATP might be too high, which dampens the effect of the P2Y$_1$R agonist. However, significant cerebroprotection was observed with A3P5PS, MRS 2279, and MRS 2179, which allowed for the maintenance of motor coordination following tMCAO. The P2Y$_1$R agonist, 2-methylthio-ADP, has been reported to induce apoptosis by activating the human recombinant P2Y$_1$R heterologously expressed in the 1321N1 astrocytoma cell line (Sellers *et al*, 2001). Thus, the P2Y$_1$R appears to possess a dual role in cell life and death, and its function may be dependent on the concentration and timing of ATP release, the extent of damage, and the influence of other cells. A recent study showed the involvement of the P2Y$_1$R in cerebral ischemia, whereby the P2Y$_1$R expressed on astrocytes enhance the expression of GFAP and the glial cell line-derived neurotrophic factor through the Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) and phosphoinositide 3 kinase (PI3K)/

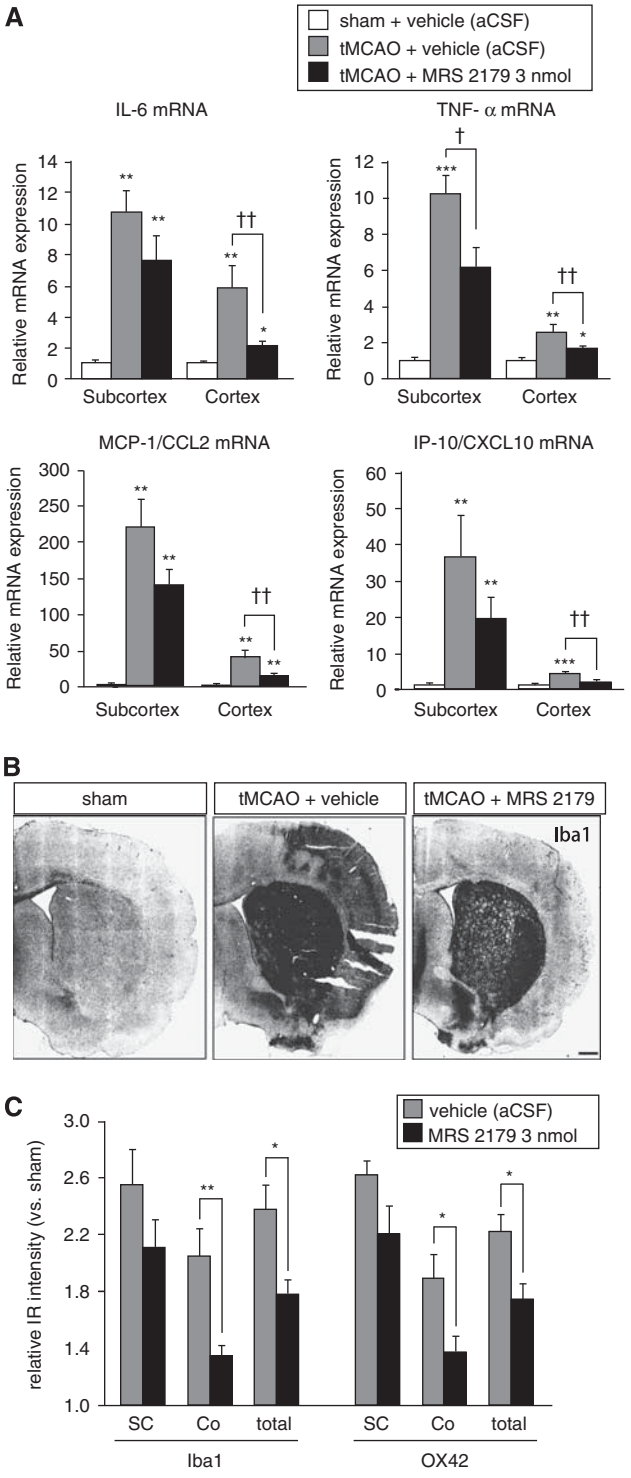


Figure 3 MRS 2179 inhibits mRNA expression of several cytokines/chemokines, microglial activation, and/or macrophage accumulation after transient middle cerebral artery occlusion (tMCAO). (**A**) The relative expression of interleukin (IL)-6, tumor necrosis factor (TNF)-α, monocyte chemotactic protein-1 (MCP-1)/chemokine (C-C motif) ligand 2 (CCL2), and interferon-inducible protein (IP)-10/ chemokine (C-X-C motif) ligand 10 (CXCL10) mRNA in the subcortex or cortex after 24 hours of tMCAO with or without MRS 2179. Data were normalized against the sham plus vehicle group ($n = 7$ animals/group). (**B**) Fluorescent immunostaining with ionized calcium-binding adaptor molecule 1 (Iba1) after 72 hours of tMCAO with or without MRS 2179. Iba1-positive cells were represented with black color. (**C**) Analysis of Iba1 and OX42 immunoreactivity (IR) in the subcortex (SC), cortex (Co), or total area ($n = 5$ animals/group). All data in this figure showed mean with s.e.m. Statistical analysis was determined using one-way analysis of variance (ANOVA) (**C**) or two-way ANOVA (**A**) with Tukey test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$. Scale bar = 1 mm.

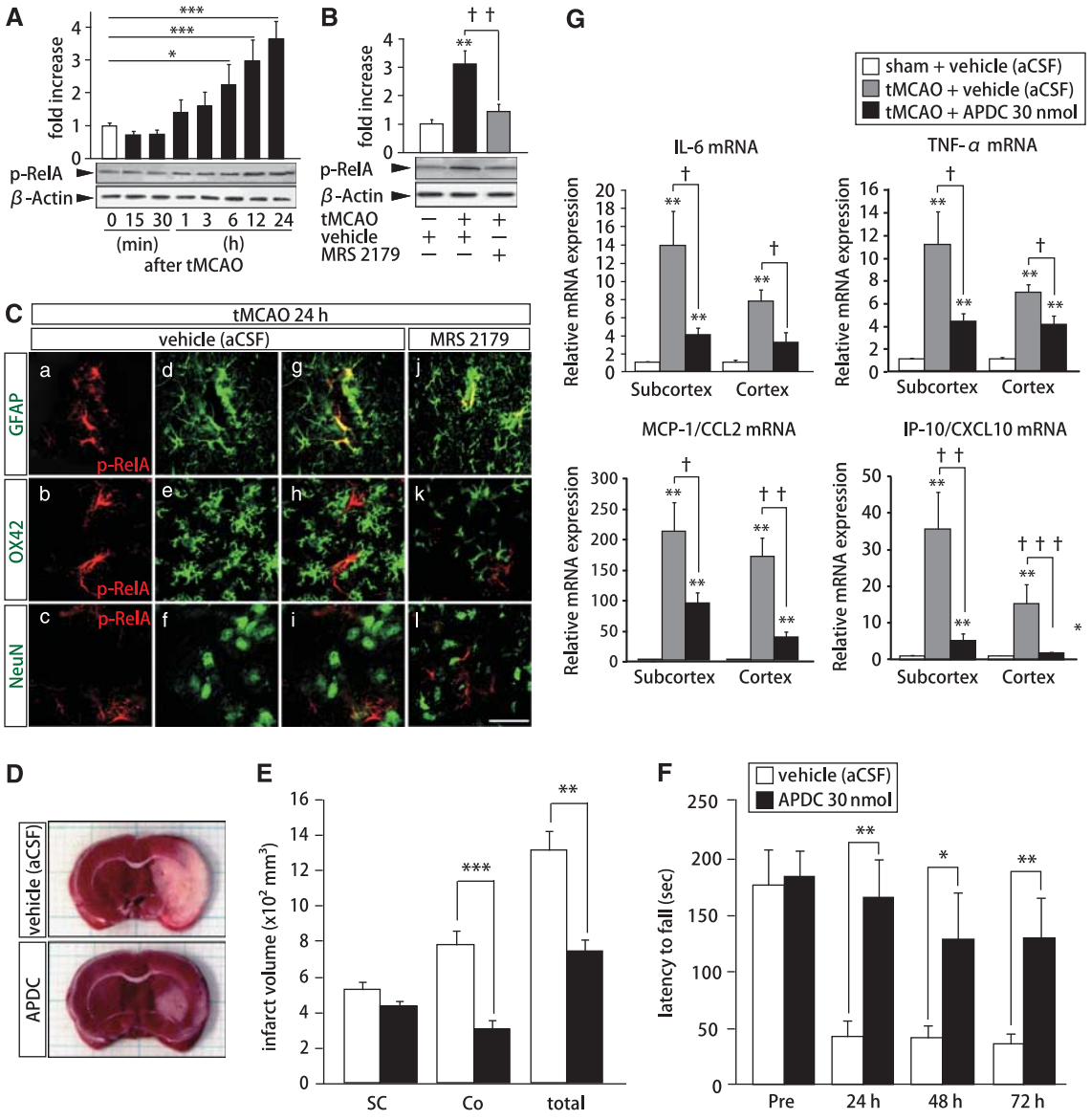


Figure 4 Astrocytic phosphorylated-RelA (p-RelA) is upregulated by activation of the P2Y$_1$ receptor (P2Y$_1$R) after transient middle cerebral artery occlusion (tMCAO), and ammonium pyrrolidine dithiocarbamate (APDC) mimics the effect of MRS 2179. (**A**) Immunoblot analysis of p-RelA protein expression levels after reperfusion of MCA ($n = 5$ animals/group). (**B**) Immunoblot analysis of p-RelA protein expression levels after administration of MRS 2179 or vehicle. p-RelA was significantly inhibited by MRS 2179 ($n = 5$ animals/group). (**C**) Immunostaining of the rat cortex with p-RelA (a–c), glial fibrillary acidic protein (GFAP) (d), OX42 (e), and neuronal nuclei (NeuN) (f) after 24 hours of tMCAO. p-RelA colocalized with GFAP (g, j) but not with OX42 (h, k) or NeuN (i, l). (**D**) Images of 2,3,5-triphenyltetrazolium (TTC)-stained brain sections after 72 hours of tMCAO in APDC-treated rats. The damaged brain regions remained unstained. (**E**) Analysis of infarct volume in the subcortex (SC), cortex (Co), or total area in each group ($n = 6$ animals/group). (**F**) Motor coordination measured using the Rotarod test ($n = 5$ animals/group). (**G**) The relative expression of interleukin (IL)-6, tumor necrosis factor (TNF)-α, monocyte chemotactic protein-1 (MCP-1)/chemokine (C-C motif) ligand 2 (CCL2), and interferon-inducible protein (IP)-10/chemokine (C-X-C motif) ligand 10 (CXCL10) mRNA in the subcortex or cortex after 24 hours of tMCAO with or without APDC treatment. Data were normalized against the sham plus vehicle group ($n = 5$ animals/group). All data in this figure showed mean with s.e.m. Statistical analysis was determined using one-way analysis of variance (ANOVA) (**A**, **E**), one-way repeated ANOVA (**F**), or two-way ANOVA (**B**, **G**) with Tukey test, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$. Scale bars = 500 μm.

Akt/cAMP response element binding protein (CREB) signaling pathway (Sun *et al*, 2008); however, the present study is the first to suggest that ATP/P2Y$_1$R signaling is the pathway responsible for the degeneration observed in focal cerebral ischemia. Moreover, administration of MRS 2179 at the three early times (15 minutes before MCA occlusion, and 12 and 24 hours after reperfusion), but not in the three late times (36, 48, and 60 hours after reperfusion), showed a reduction in the infarct volume. These data strongly

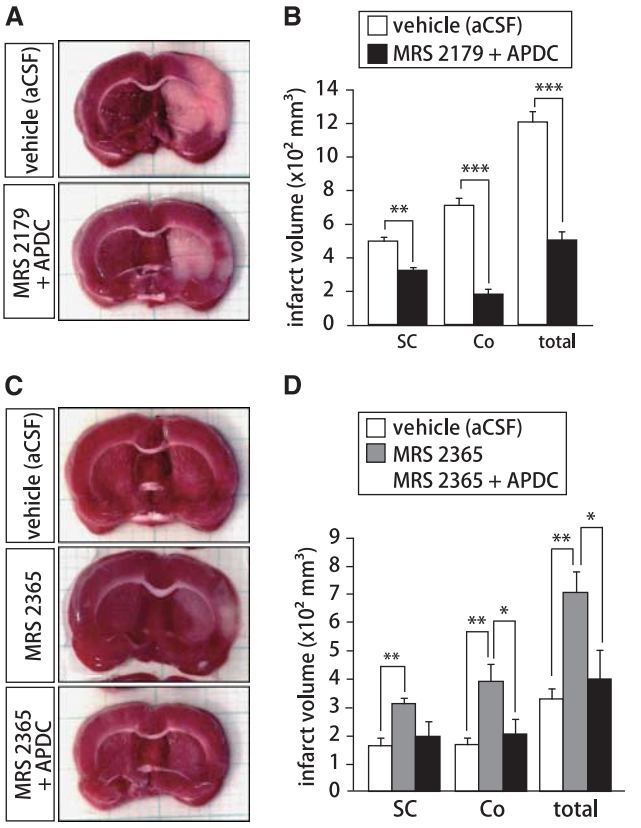


Figure 5 Ammonium pyrrolidine dithiocarbamate (APDC) showed no additive effect when used with MRS 2179, and significantly suppressed the increase in cerebral infarct volume caused by MRS 2365. (**A**) Images of 2,3,5-triphenyltetrazolium (TTC)-stained brain sections after 72 hours of transient middle cerebral artery occlusion (tMCAO) in MRS 2179 + APDC-treated rats. The damaged brain regions remained unstained. (**B**) Analysis of infarct volume on the subcortex (SC), cortex (Co), or total area in each group ($n = 6$ animals/group). (**C**) Images of TTC-stained brain sections after 72 hours of tMCAO in MRS 2365 + APDC-treated rats. (**D**) Analysis of the infarct volume in each group ($n = 6$ animals/group). All data in this figure show mean with s.e.m. Statistical analysis was determined using one-way analysis of variance (ANOVA) (**B**) or two-way ANOVA (**D**) with Tukey test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

suggest that $P2Y_1R$ has a critical role during the 24 hours following cerebral damage after ischemia and reperfusion.

The $P2Y_1R$ is involved in modulating transcriptional activity in astrocytes (Fujita *et al*, 2009; Sun *et al*, 2008). Our data, and that of previous studies (Minami *et al*, 2006), have showed that IL-6, TNF-α, MCP-1/CCL2, and IP-10/CXCL10 mRNA expression is markedly upregulated in the 24 hours after tMCAO. These cytokines/chemokines are known to contribute not only to cerebral ischemia but also other neurodegenerative disorders, such as Alzheimer disease, multiple sclerosis, and acquired immune deficiency syndrome encephalopathy (Wang *et al*, 2002). Previous investigations using a TNF-α neutralizing antibody or MCP-1/CCL2-deficient mice indicate that the depression of these cytokines/chemokines has a cerebroprotective effect (Barone *et al*, 1997; Hughes *et al*, 2002). However, it has been reported that recombinant IL-6 reduces ischemic brain damage (Minami *et al*, 2006), but this finding was not supported in IL-6-deficient mice (Clark *et al*, 2000). Moreover, the inhibition of the IL-6 receptor resulted in an increase in infarct volume (Yamashita *et al*, 2005). The conflicting roles of IL-6 indicate that more detailed studies are required. In this study, we showed that MRS 2179 significantly inhibited microglial and/or macrophage activation in the ischemic brain. The IP-10/CXCL10 is known as a migration factor for T cells and macrophages during cerebral ischemia (Wang *et al*, 1998). Moreover, reactive microglia are thought to induce astrocyte activation via IL-1β or IL-18 release (Miyoshi *et al*, 2008). In contrast, astrocytes contribute to the activation of microglia (Rohl and Sievers, 2005). Because minocycline, which inhibits microglial activation *in vivo* (Raghavendra *et al*, 2003), reduces infarct volume and brain atrophy (Yrjanheikki *et al*, 1999), it is possible that cerebroprotection by MRS 2179 can be attributed to microglial and/or macrophage inactivation.

The main NF-κB subunits in the ischemic brain are NF-κB1/p50 and RelA/p65 (Zhang *et al*, 2005). These subunits form heterodimeric complexes and classical pathways that require IκB kinases (Hayden and Ghosh, 2004). There are various sites for serine phosphorylation on the RelA protein. For example, serine 536 is phosphorylated by IκB kinases after TNF stimulation, which increases the transcriptional efficacy of NF-κB (Hayden and Ghosh, 2004). The NF-κB1:RelA complex has a pivotal role in the IL-6, TNF-α, and IP-10 response (Cui *et al*, 2006; O'Neill and Kaltschmidt, 1997). We have showed that the p-RelA protein gradually increases 24 hours after tMCAO in astrocytes. The neuronal death by β-amyloid, ischemia, or status epilepticus are suppressed by NF-κB (Blondeau *et al*, 2001; Kaltschmidt *et al*, 1999). In contrast, the NF-κB contributes to brain degeneration in ischemia, trauma, epilepsy, Alzheimer disease, Parkinson disease, and Huntington disease (Bethea *et al*, 1998; Kell, 2010; Nurmi *et al*, 2004). Recently, the major cause of this dual cerebroprotection/degeneration effect was reported to be a NF-κB subtype-dependent pathway (Pizzi *et al*, 2009). c-Rel contributes to cerebroprotection and RelA induces neurodegeneration. Thus, an increase in the p-RelA protein may be induced by brain damage. This increase in p-RelA is completely blocked by MRS 2179. In addition, a similar result was also observed with APDC administration, with cerebroprotection and transcriptional repression. In addition, NF-κB1-deficient mice have been reported to show cerebral ischemic tolerance (Schneider *et al*, 1999). In this study, there was no additive effect when using MRS 2179 and APDC in combination. Moreover, APDC significantly suppressed the increase of cerebral infarct volume induced by MRS 2365. These findings indicate that NF-κB has a role

in cytokine/chemokine transcriptional activity and has a pivotal function during ischemic damage involving the $P2Y_1R$.

In summary, we have showed that after cerebral ischemia, inhibition of the astrocytic $P2Y_1R$ results in cytokine/chemokine transcriptional suppression and cerebroprotection via NF-κB. Our findings open the door to new ideas for the development of strategies useful for the management of stroke and add to the current body of knowledge on the pathophysiology of focal cerebral ischemia.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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