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The involvement of $P2X_1$ receptor in pyramidal cell degeneration in the rat hippocampus after trimethyltin administration

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ABSTRACT

The P2 family of receptors for adenosine 5′-triphosphate (ATP) is involved in several neuronal and glial cell functions in the central nervous system (CNS), and impaired function of these receptors is associated with both neuronal and glial dysfunction. Using quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and immunohistochemical analysis, we examined the expression profiles of P2 subtype receptors in the rat hippocampus following treatment with the neurotoxicant trimethyltin (TMT). Among the subtypes, $P2X_1$ exhibited a unique profile, with an increase in expression prior to the onset of cell death after TMT administration, and a gradual decrease thereafter in neuronal cells in the rat hippocampus. This expression pattern was similar to that of cyclooxygenase-2 (COX-2) following TMT administration. The $P2X_1$ antagonist NF499 strongly prevented neuronal cell death induced by TMT in the CA1 region, and successfully suppressed locomotor hyperreactivity. Furthermore, NF449 administration also inhibited COX-2 expression in the CA1 region on day 3 following TMT treatment, whereas no change was observed in the CA3. These findings suggest that $P2X_1$ plays a primary role in TMT-induced neuronal cell death in the CA1 region.

1. Introduction

Trimethyltin (TMT) is a triorganotin compound that induces selective loss of pyramidal cells of the rat hippocampus after a single dose administered orally (Dyer et al., 1982; Morita et al., 2008; Tsutsumi et al., 2002). TMT intoxication is behaviorally characterized by seizures, hyperactivity, and impairment of working memory (Chang and Dyer, 1983; Ishida et al., 1997; Morita et al., 2008; Ruppert et al., 1982). In addition, TMT lesions share the molecular hallmarks and pathophysiological mechanism of Alzheimer's disease (Liu et al., 2006; Nilsberth et al., 2002; Woofruff, 1994). However, the early response of brain damage induced by TMT still remains unclear.

We previously demonstrated that cyclooxygenase-2 (COX-2) expression preceded TMT-induced cell death, suggesting a pivotal role for COX-2 as an initiator of TMT neurotoxicity (Shirakawa et al., 2007). COX-2, a rate-limiting enzyme for the synthesis of prostaglandins, is known to be an important factor in acute and chronic neurodegenerative disorders such as stroke, Alzheimer's disease, and Parkinson's disease (Abdullah et al., 2006; Liang et al., 2007). Pharmacological inhibition of COX-2 reduced infarction size and the level of neuronal cell death (Govoni et al., 2001; Kunz et al., 2006; Takemiya et al., 2006). Moreover, COX-2 expression has been reported to involve P2 receptors (Brambilla et al., 2000; McLarnon, 2005; Xu et al., 2003), which act as purinergic receptors for adenosine 5′-triphosphate (ATP) (Ralevic and Burnstock, 1998). Despite the increasing evidence that different noxious stimuli applied to brain preparation cause an increase in the extracellular levels of ATP, in vivo situations have been studied only rarely. Two principal families of P2 receptors are ligand-gated ion channel receptors (P2X receptors) and G-protein-coupled receptors (P2Y receptors) (Abbracchio and Burnstock, 1994; Barnard et al., 1994; North and Barnard, 1997). Seven genes coding for P2X subtypes have been identified and named $P2X_1$ through $P2X_7$, while eight P2Y receptor subtypes were also cloned; these are referred to as $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ (Franke and Illes, 2006; von Kugelgen, 2006). These may contribute to neurodegeneration and inflammatory reactions. Receptors expressed around β-amyloidal plaques in a transgenic mouse model of Alzheimer's

Abbreviations: TMT, trimethyltin; COX-2, cyclooxygenase-2; NF449, 4,4′,4″,4‴-(carbonylbis(imino-5,1,3-benzenetriylbis(carbonylimino))) tetrakis-benzene-1,3-disulfonic acid octasodium salt.

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disease activated microglia/macrophages following experimentally induced brain injury (Parvathenani et al., 2003; Zhang et al., 2006), and activated the release of inflammatory cytokines such as IL-1β, IL-6, and tumor necrosis factor-α (Ferrero, 2009).

Here, we report a role for P2 receptors, particularly $P2X_1$, which is involved in the early stage of brain damage caused by TMT in vivo.

2. Experimental procedures

2.1. Experimental animals

All experiments were conducted using 5-week-old male Sprague–Dawley rats (JcL: SD, CLEA Japan, Inc., Tokyo, Japan) that had been acclimatized in the animal room for 1 week prior to experimentation. The animal room was maintained at a temperature of $23 \pm 2\,^{\circ}C$ and a relative humidity of $55 \pm 15\%$, with light provided from 8 a.m. to 8 p.m. Animals were allowed *ad libitum* access to a pellet diet (CE-2, CLEA Japan, Inc.) and microfiltered tap water. All animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Astellas Pharma Inc.

2.2. Drug administration

A single dose of TMT hydroxide (Strem Chemicals, Newburyport, MA, USA) dissolved in distilled water was administered orally at 9 mg/kg to 6-week-old male rats (Ikeda et al., 1996; Liu et al., 2005; Morita et al., 2008; Nishimura et al., 2001; Tsunashima et al., 1998; Tsutsumi et al., 2002). A control group was similarly administered distilled water alone.

The $P2X_1$ receptor antagonist NF449 (Merck Ltd., Tokyo, Japan) was administered by intracerebroventricular (icv) injection. Rats were anaesthetized using sodium pentobarbital (Nacalai Tesque, Kyoto, Japan) and stereotaxically implanted with guide cannulae (Brain Infusion Kit, Alzet, Palo Alto, CA, USA) 0.9 mm posterior, 1.6 mm lateral, and 4.5 mm ventral to the bregma, based on an atlas of the rat brain (Swanson, 1992). After surgery, the rats were housed individually and allowed to recover for at least 7 days. After recovery, NF449 at 15 or 75 ng/animal dissolved in saline was administered by icv injection under light ether anesthesia through the guide cannula for 3 days from day 2 after TMT administration (Braun et al., 2001; Cavaliere et al., 2007; Kassack et al., 2004; Rettinger et al., 2005). The control group was administered saline in the same manner.

2.3. Quantitative real-time polymerase chain reaction (qRT-PCR)

On days 1, 3, 5, 7, and 14 after TMT administration, each animal in a group (5 animals/group) was decapitated under light ether anesthesia. The hippocampus was removed and immediately frozen in liquid nitrogen. Hippocampus samples were homogenized with the RLT buffer supplied in the RNeasy Mini Kit (Qiagen, Inc., Hilden, Germany). Total RNA was isolated using TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA) and further purified using the RNeasy Mini Kit (Qiagen, Inc.). Complementary DNA (cDNA) was synthesized from 0.4 μg of total RNA using the SuperScript First-Strand Synthesis System (Invitrogen). qRT-PCR was performed according to the manufacturer's instructions using 4 μL of the cDNA solution, 1× SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), and 40 μM of each primer pair in a 7900 HT Fast Real-Time PCR System (Applied Biosystems). Sequences of primers used in this study were designed using Primer Express 2.0 Software (Applied Biosystems) and synthesized by Sigma (Tokyo, Japan). The results were normalized against β-actin.

2.4. Tissue preparation

For immunohistochemical examination on days 1, 3, 5, 7, and 14 after TMT administration and for cell counting on day 14 after TMT administration, each animal (5 animals/group) was perfused through the heart for a short period with 0.1 mol/L phosphate-buffered saline (PBS, pH 7.4), followed by a fixative containing 4% paraformaldehyde in 0.1 mol/L PBS. The perfused brain was quickly removed and immersed in the same fixative for an additional 3 h at 4 °C, embedded in paraffin, and then cut using a microtome into 14- and 4-μm sections at a position 3.9 mm posterior to the bregma based on an atlas of the rat brain (Swanson, 1992), and sections were used for cell count and immunohistochemistry analyses, respectively.

2.5. Histological and immunohistochemical examination

To confirm the effects of $P2X_1$ antagonist administration against TMT-induced neuronal loss, the brain sections corresponding to day 14 after TMT administration were deparaffinized and stained with 1% cresyl violet solution. After staining, the average number of pyramidal cells in the CA1 and CA3 regions per microscope field (0.0625 mm^2 using a microscope ocular lens with a square grid) was counted (5 animals/group), as previously described (Shirakawa et al., 2007).

For immunohistochemical analysis, brain sections from days 1, 3, 5, 7, and 14 after TMT administration were initially treated with 3% H_2O_2 in PBS for 15 min to inactivate the endogenous peroxidases. After a 10-min incubation with blocking buffer (1% bovine serum albumin [BSA] in PBS), the sections were treated with antibodies against either $P2X_1$ receptors (1:1000; Affinity BioReagents, Inc., Golden, CO, USA) or COX-2 (1:2000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at room temperature for 3 and 1 h, respectively. After the sections were washed, they were incubated with ENVISION+ (DAKO, Copenhagen, Denmark) at room temperature for 1 h for the detection of $P2X_1$ receptors, and with biotinylated anti-goat IgG antibody (1:500; Zymed, San Francisco, CA, USA) for 30 min at room temperature for the detection of COX-2. The sections treated with COX-2 were further incubated with streptavidin peroxidase (1:2500; Zymed) for 30 min at room temperature. Diaminobenzidine was used to visualize peroxidase activity. The antibodies used in the present study were specific for the $P2X_1$ epitope region between 364 and 372. To prevent nonspecific binding, the antibody was treated with the control peptide; a control using the secondary antibody alone was also included. To analyze the cell type-specific expression of $P2X_1$ receptors and COX-2 proteins, the brain sections corresponding to day 3 after TMT administration were treated with 3% H_2O_2 in PBS for 15 min at room temperature. After a 10-min incubation with blocking buffer (1% BSA in PBS), the sections were treated with antibodies against the $P2X_1$ (1:1000; Affinity BioReagents), COX-2 (1:2000; Santa Cruz, Biotechnology, Inc.), and NeuN (1:400; Abcam plc., Cambridgeshire, UK) at room temperature for 3 h. After washing, sections were incubated with Alexa 544-conjugated anti-rabbit IgG (1:200; Invitrogen), Alexa 488-conjugated anti-goat IgG (1:200; Invitrogen), and Qdot 655 streptavidin conjugate (1:200; Invitrogen) for 30 min at room temperature. Immunofluorescence was examined using a confocal laser scanning microscope (TCS SPII; Leica Microsystems GmbH, Wetzlar, Germany).

2.6. Western blotting

On day 3 after TMT administration, each animal (4 animals/group) was decapitated under light ether anesthesia. The hippocampus was removed and frozen immediately in liquid nitrogen. Hippocampus samples were homogenized using RIPA buffer