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Inhibition of prostaglandin E_2 EP3 receptors improves stroke injury *via* anti-inflammatory and anti-apoptotic mechanisms

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ABSTRACT

Although deletion of EP3 receptors is known to ameliorate stroke injury in experimental stroke models, the underlying mechanisms and the effects of EP3-specific antagonists remain poorly understood. Here we demonstrate the protective effect of postischemic treatment with an EP3 antagonist, ONO-AE3-240, through anti-inflammatory and anti-apoptotic effects. In transient focal ischemia models, peritoneal injection of an EP3 antagonist after occlusion–reperfusion reduced infarction, edema and neurological dysfunctions to almost the same levels of those in EP3 knockout (KO) mice. Furthermore, neuronal apoptosis in the ischemic cortex investigated by terminal dUTP nick-end labeling (TUNEL) and caspase-3 immunostaining were ameliorated in EP3 antagonist-treated mice or EP3 KO mice as compared with vehicle-treated mice or wild-type (WT) mice, respectively. There were no significant differences between ONO-AE3-240-injected or EP3 KO mice and vehicle-injected or WT mice, respectively, in mean arterial blood pressure, cerebral blood flow or body temperature. The double-immunostaining showed that EP3 receptor-positive cells were also positive for CD-11b and partially for Neu-N, the marker for microglia and neurons. Deletion of EP3 receptors also reduced damage of the blood–brain barrier, activation of microglia and infiltration of neutrophils into the ischemic cortex. These results suggest that EP3 receptors are involved in stroke injury through the enhancement of inflammatory and apoptotic reactions in the ischemic cortex. Thus, EP3 antagonists may be valuable for the treatment of human stroke.

1. Introduction

Stroke remains a major cause of death and neuronal disability worldwide. In the early stages of cerebral ischemia, activation of glutamate receptors initiates the ischemic cascade that causes the damage (Butcher et al., 1990; Lee et al., 1999). At later times after ischemia, the inflammation and programmed cell death is a major factor in the progression of the injury (Du et al., 1996; Barone and Feuerstein, 1999; Dirnagl et al., 1999).

Prostaglandin E_2 (PGE_2), one of the most likely candidates for propagation of inflammation, is known to be produced and accumulated at the lesion sites of the ischemic brain (Kempski et al., 1987; Iadecola et al., 2001; Ikeda-Matsuo et al., 2006). Recently, using a microsomal PGE_2 synthase-1 (mPGES-1) knockout (KO) focal cerebral ischemia model in mice, we demonstrated that induction of mPGES-1 contributes to the production of ischemic PGE_2, and thereby exacerbates the stroke injury (Ikeda-Matsuo et al., 2006). PGE_2 acts on four G-protein-coupled receptors (EP1–EP4) that have very distinct and potentially antagonistic signaling cascades. EP1 couples to G_q, and activation of this receptor results in increased intracellular Ca^{2+} concentrations (Kawano et al., 2006); EP2 and EP4 couple to G_s to increase cyclic AMP (cAMP) formation, whereas EP3 couples to G_i to decrease cAMP. Thus, there is great potential for variability in the response of target cells to PGE_2 based on which receptors it activates (Narumiya et al., 1999).

The various roles of the PGE_2 receptors in neuronal death induced by excitotoxicity and ischemic stroke have been clarified by genetic deletion and selective inhibition of each EP receptor. Deletion and inhibition of the EP1 receptors partially reduces the neuronal damage caused by NMDA-induced excitotoxicity and ischemic stroke (Ahmad et al., 2006; Kawano et al., 2006). Deletion of the EP2 increases the infarct volume in mice after ischemic stroke (McCullough et al., 2004). Conversely, a selective agonist of the EP2 receptor has been shown to induce caspase-dependent apoptosis (Takadera et al., 2004). An EP4 receptor agonist has been shown to reduce excitotoxic brain injury (Ahmad et al., 2005). Interestingly, a recent study showed that genetic deletion of the EP3 reduces the neuronal damage caused by oxygen/glucose deprivation (OGD), an *in vitro* model for ischemia, or ischemic stroke (Saleem et al., 2009), while an EP3 receptor agonist exacerbates acute excitotoxic or ischemic-induced brain injury (Ahmad et al., 2007). Thus, EP3 could be one of the most important effectors for the neurotoxicity of PGE_2.

However, the effect of EP3-specific antagonists on stroke injury and the mechanisms of EP3 receptor-mediated neurotoxicity are currently

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unknown. The present study was designed to examine these issues, and demonstrated that peritoneal injection of an EP3 antagonist even starting at 2 h after the onset of ischemia protected against ischemic injury. Our results also demonstrate that genetic deletion of EP3 attenuated inflammatory responses, such as enhancement of vascular permeability, edema, blood–brain barrier damage, neutrophil infiltration and microglial activation, and apoptotic responses, such as caspase-3 activation and DNA fragmentation.

2. Materials and methods

2.1. Materials

Selective antagonists for EP3 (ONO-AE3-240; Fig. 1A) receptors were the kind gift of Ono Pharmaceutical (Osaka, Japan). The Ki values of ONO-AE3-240 obtained by competition-binding isotherms to displace the radioligand binding to the respective prostanoid receptor are 590, 0.23, 58, and 1500 nM for EP1, EP3, EP4, and FP, respectively, and more than 10 mM for EP2, DP, TP, and IP (Amano et al., 2003). Other materials and their sources were as follows: triphenyltetrazolium chloride (TTC) (Sigma, St. Louis, MO), rabbit anti-EP3 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA; #sc-20676), anti-NeuN monoclonal antibody (Chemicon, Temecula, CA; #MAB377), anti-CD11b monoclonal antibody (Serotec Inc., Oxford, UK; #MCA275), rabbit anti-Iba1 polyclonal antibody (Wako, Tokyo, Japan; #019-19741), rabbit anti-cleaved caspase-3 (Cell Signaling, Beverly, MA; #9961); goat anti-occludin polyclonal antibody (Santa Cruz Biotechnology; #sc-8145), biotin-conjugated Donkey anti-mouse IgG polyclonal antibody (Jackson ImmunoResearch, West Grove, PA; #715-065-150), FITC-conjugated rabbit anti-polymorphonuclear (PMN) polyclonal antibody (Inter-Cell Technologies Inc., Hopewell, NJ; #FAD-31140), rabbit anti-human myeloperoxidase (MPO) polyclonal antibody (DAKO, Carpinteria, CA; #A0398), and multiple-labeling grade secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Other reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Animals

EP3 KO and WT mice (C57BL/6J × 129/SvJ background) back-crossed to C57BL/6J mice for >8 generations to avoid artifactual differences caused by genetic background were used (Ushikubi et al., 1998). Our preliminary data showed no significant gender differences in infarct volume, degree of edema, neurological score and motor activity 24 h after ischemia (Ikeda-Matsuo et al., 2006). Therefore, both male and female mice were studied at weight 25–30 g; and data from both sexes were pooled. In all studies, the animal care and experimental procedures complied with the guidelines given by the Japanese Pharmacological Society.

2.3. Induction of transient focal ischemia

Middle cerebral artery (MCA) occlusion was carried out as described previously (Ikeda-Matsuo et al., 2006). Mice were anesthetized by halothane during surgery (5.0% for induction, 1.0% for maintenance). The right common carotid artery was exposed through a midline incision, and occlusion of the MCA was achieved by inserting a 6–0 nylon monofilament (Johnson & Johnson, New Brunswick, NJ) with a heat-blunted tip coated with silicon (Xantopren, Heraeus

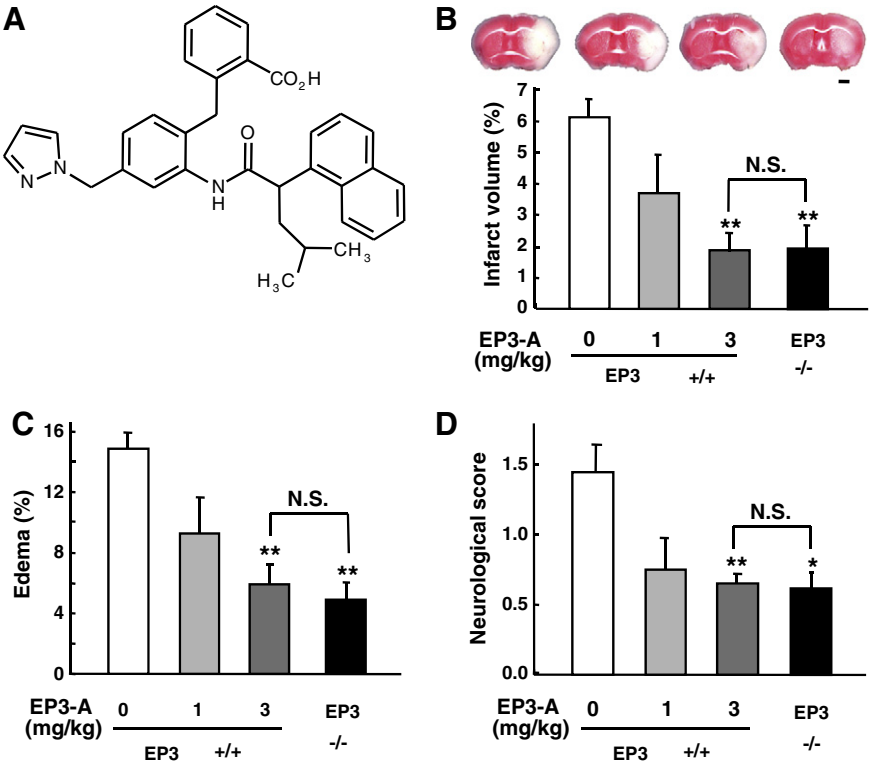

Fig. 1. Protective effect of an EP3 antagonist on postischemic symptoms. ONO-AE3-240 (EP3-A; 1 or 3 mg/kg) or vehicle was injected three times to WT mice at 2, 8 and 14 h after MCA-occlusion. (A) Structure of ONO-AE3-240. (B) Representative TTC-stained coronal sections of the EP3 KO (−/−) and WT (+/+) mice injected with vehicle or EP3 antagonist (scale bar: 1 mm). The volume of infarcted cortex 24 h after ischemia was estimated and expressed as a percentage of the corrected tissue volume. n = 10 mice per group. (C) The corrected edema percentage in the infarcted cortex. n = 10 mice per group. (D) Neurological dysfunction 24 h after ischemia. n = 10–20 mice per group, $^{**}p<0.01$, $^{*}p<0.05$ *vs.* 0 mg/kg, N.S. (not significant).