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CANNABINOID RECEPTOR SUBTYPES 1 AND 2 MEDIATE LONG-LASTING NEUROPROTECTION AND IMPROVE MOTOR BEHAVIOR DEFICITS AFTER TRANSIENT FOCAL CEREBRAL ISCHEMIA

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Abstract—The endocannabinoid system is crucially involved in the regulation of brain activity and inflammation. We have investigated the localization of cannabinoid CB1 and CB2 receptors in adult rat brains before and after focal cerebral ischemia due to endothelin-induced transient occlusion of the middle cerebral artery (eMCAO). Using immunohistochemistry, both receptor subtypes were identified in cortical neurons. After eMCAO, neuronal cell death was accompanied by reduced neuronal CB1 and CB2 receptor-linked immunofluorescence. In parallel, CB1 receptor was found in activated microglia/macrophages 3 days post eMCAO and in astroglia cells at days 3 and 7. CB2 receptor labeling was identified in activated microglia/macrophages or astroglia 3 and 7 days post ischemia, respectively. In addition, immune competent CD45-positive cells were characterized by pronounced CB2 receptor staining 3 and 7 days post eMCAO. KN38-72717, a potent and selective CB1 and CB2 receptor agonist, revealed a significant, dose-dependent and long-lasting reduction of cortical lesion sizes due to eMCAO, when applied consecutively before, during and after eMCAO. In addition, severe motor deficits of animals suffering from eMCAO were significantly improved by KN38-7271. KN38-7271 remained effective, even if its application was delayed up to 6 h post eMCAO. Finally, we show that the endocannabinoid system assembles a comprehensive machinery to defend the brain against the devastating consequences of cerebral ischemia. In summary, this study underlines the therapeutic potential of CB1 and/or CB2 receptor agonists against neurodegenerative diseases or injuries involving acute or chronic imbalances of cerebral blood flow and energy consumption.

Key words: cerebral ischemia, endocannabinoid system, CB1 receptor, CB2 receptor, neuroprotection.

INTRODUCTION

The endocannabinoid system consists of CB1 and CB2 receptors, their endogenous ligands such as *N*-arachidonyl ethanolamide (anandamide) and 2-arachidonylglycerol (2AG) and enzymes responsible for the synthesis and degradation of endocannabinoids (Di Marzo, 2011; Mechoulam and Parker, 2012). CB1 receptors are expressed in the CNS, where they are predominantly located at pre-synaptic terminals. In contrast, CB2 receptors were originally claimed as peripheral CB receptors. This classification was based on its abundant expression in peripheral immune cells such as $CD4^+$ T cells, $CD8^+$ T cells, B cells, natural killer cells, monocytes and neutrophils (Derocq et al., 1995; Galiegue et al., 1995; Schatz et al., 1997; Carayon et al., 1998; McCoy et al., 1999; Buckley et al., 2000; Carlisle et al., 2002; Maresz et al., 2007; Dittel, 2008).

In addition, CB2 receptors have also been identified in resident immune cells of the CNS, i.e. in microglia cells (Carlisle et al., 2002; Klegeris et al., 2003; Walter et al., 2003; Beltramo et al., 2006; Maresz et al., 2007). Expression of CB2 receptors in microglia depends on its activation state (Carlisle et al., 2002; Walter et al., 2003; Stella, 2004; Maresz et al., 2007; Cabral et al., 2008; Pietr et al., 2009) and modulates microglial migration and infiltration into brain areas exposed to neuroinflammation and neuronal degeneration (Walter et al., 2003; Ashton et al., 2007; Fernandez-Ruiz et al., 2008; Miller and Stella, 2008; Price et al., 2009).

The extent of neuronal CB2 receptor expression in the peripheral and central nervous system remains a matter of controversial discussion (Atwood and Mackie, 2010). Several studies have suggested the presence of CB2 receptor in granule cells and/or Purkinje cells of rodent or human cerebellum (Skaper et al., 1996; Van Sickle et al., 2005; Ashton et al., 2006; Gong et al., 2006; Onaivi et al., 2008a,b). Furthermore, Van Sickle et al. (2005) and Gong et al. (2006) have reported of CB2 receptor mRNA and protein localization also in the brainstem. In addition, other studies have identified this

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Abbreviations: 2AG, 2-arachidonylglycerol; anandamide, *N*-arachidonyl ethanolamide; DAGL, diacylglycerol lipase; eMCAO, endothelin-induced transient occlusion of the middle cerebral artery; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; PBS, phosphate-buffered saline.

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cannabinoid receptor subtype in the hippocampal formation with predominately postsynaptic localization and associated with the rough endoplasmic reticulum (Gong et al., 2006; Brusco et al., 2008; Onaivi et al., 2008a,b). Despite distinct functional evidence, CB2 receptor expression in the cortex remains to be conclusively demonstrated (Atwood and Mackie, 2010). The endogenous CB1 and CB2 receptor agonists anandamide and 2AG are generated from membrane-derived lipid precursors in postsynaptic terminals (Mechoulam et al., 1995; Sugiura et al., 1995; Stella et al., 1997). Anandamide is released by the N-acylphosphatidyl-ethanolamine-specific phospholipase D, whereas 2AG originates from sn-1-Acyl-2-arachidonyl-glycerol by diacylglycerol lipase (DAGL). Both endocannabinoids are synthesized and released into the synaptic cleft upon postsynaptic depolarization. Activation of pre-synaptic CB1 receptor is coupled to the blockage of voltage-dependent Ca^{2+} channels and the activation of inwardly rectifying K^{+} channels (Daniel and Crepel, 2001), leading to neuronal hyperpolarization and inhibition of glutamate release (Schlicker and Kathmann, 2001). This mode of action constitutes a retrograde, perisynaptic feedback regulation of glutamate-dependent synaptic transmission, termed as depolarization-induced suppression of excitation (Di Marzo et al., 2004; Katona and Freund, 2008). Endocannabinoids are removed from their site of action by cellular reuptake. 2AG is then hydrolyzed by monoacylglycerol lipase (MAGL) and, to a lower extent, by fatty acid amide hydrolase (FAAH). Anandamide is degraded by FAAH and other lipases (Di Marzo, 2008, 2011; Mechoulam and Parker, 2012).

Besides retrograde inhibition of glutamate-dependent synaptic transmission, the cannabinoid-induced suppression of excessive glutamate release may provide another, potentially fundamental function, i.e. to counteract excitotoxicity and its deleterious consequences on demand (Nagayama et al., 1999; Marsicano et al., 2003; Di Marzo et al., 2004; Katona and Freund, 2008). The efficacy of endogenous neuroprotection might be further increased by the additional involvement of CB2 receptors and the mediation of anti-inflammatory effects for instance after a stroke or traumatic brain injury (Eljaschewitsch et al., 2006; Wolf et al., 2008; Stella, 2010). Cerebral inflammation potentiates neuronal degeneration spreading from the infarct zone through the penumbra. Consequently, CB1 and CB2 receptor activation may affect neuronal cell death and survival efficiently and in a synergistic manner.

In the present study, we studied the localization of CB1 and CB2 receptors in the post ischemic rat brain. Cell type-specific distribution patterns of both receptor subtypes were analyzed at predefined time points after eMCAO. Furthermore, we have investigated the effects of a high-affinity CB1/CB2 receptor agonist on eMCAO-dependent cerebral lesion volumes and motor behavior deficits. This study suggests the endocannabinoid system as efficient endogenous protector of brain cells against cerebral ischemia.

EXPERIMENTAL PROCEDURES

Endothelin-1, phosphate-buffered saline (PBS), Triton X-100, thionine blue, Dimethyl sulfoxide, (2-Hydroxypropyl)-β-cyclodextrin were purchased from Sigma (Steinheim, Germany). Chloral hydrate was from VWR (Darmstadt, Germany). Sodium chloride, sucrose and RHC-80267 were obtained from Merck (Darmstadt, Germany), ethanol and citric acid from Roth (Karlsruhe, Germany). KN38-7271 was from Bayer Healthcare (Leverkusen, Germany), ACEA, JWH133 and MAFP were from Biotrend (Switzerland). URB597, URB602 and RHC-80267 were purchased from Alexis Biochemicals (Switzerland).

Animals

All protocols for animal experiments described in this study are performed according to the German Tierschutzgesetz from 1998. Male Sprague–Dawley rats (8 weeks old, 250–280 g) were obtained from Harlan Laboratories (Eystrup, Germany). The animals were maintained under constant environmental conditions with ambient temperature of 20 ± 2 °C and relative humidity of 50% and were housed with a 12-h light–dark cycle. Food and water were given *ad libitum*. Experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). The study was approved by the authorities of the State of Saxony-Anhalt (*Landesverwaltungsamt*) and performed according to institutional guidelines.

eMCAO and drug application

The occlusion of the middle cerebral artery by unilateral microinjection of endothelin-1 adjacent to the blood vessel was modified based on the procedure published by Sharkey and Butcher (1995). During the surgical procedure, the body temperature was kept at 37 °C using a thermostatically controlled heating blanket. 4% halothane in a mixture of nitrous oxide/oxygen (ratio 70:30) and 2% halothane given via a nose cone were used for the induction and the maintenance of anesthesia, respectively. The rats were placed in a Kopf stereotaxic frame and a burr hole (1 mm diameter) was drilled into the skull (coordinates: anterior 0.9 mm from bregma, lateral 5.2 mm to satura sagittalis). A 29-gauge steel cannula was inserted stereotaxically 7.5 mm below the dura, adjacent to the rat middle cerebral artery according to the rat brain atlas of Paxinos and Watson (2005). MCA occlusion was performed by the injection of 1.25 nmol endothelin-1 in 5 µl PBS (0.1 M, pH 7.4) over 3 min. Subsequently, the cannula remained in place for further 3 min and was then slowly withdrawn. In sham-operated animals, PBS was injected instead of endothelin-1.

The following drugs were used to modulate the endocannabinoid system: ACEA, a highly selective CB1 receptor agonist (Hillard et al., 1999), JWH133, a potent CB2 receptor- selective agonist (Huffman et al., 1999), MAFP, a potent, irreversible inhibitor of FAAH (De Petrocellis et al., 1997; Deutsch et al., 1997), URB597, a selective and irreversible blocker of FAAH not affecting MAGL (Kathuria et al., 2002; Mor et al., 2004; Puente et al., 2011), URB602, a selective inhibitor of MAGL with no inhibitory effect on FAAH at doses up to 100 µM (Tarzia et al., 2003; Hohmann et al., 2005) and RHC 80267, an inhibitor of DAGL (Sutherland and Amin, 1982). URB597 enhanced short-term depression in the extended amygdala in rats (Puente et al., 2011). Blockage of endocannabinoid synthesis by RHC 80267 has been shown to abolish cannabinoid-dependent short-term depression in the extended amygdale (Puente et al., 2011) and in mouse cerebellar cortex (Szabo et al., 2006). The latter effect has been suggested to be mediated by a RHC 8026-induced decrease of endocannabinoid levels.