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MODULATION OF THE BALANCE BETWEEN CANNABINOID CB_1 AND CB_2 RECEPTOR ACTIVATION DURING CEREBRAL ISCHEMIC/REPERFUSION INJURY

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Abstract—Cannabinoid receptor activation has been shown to modulate both neurotransmission (CB_1) and neuroinflammatory (CB_2) responses. There are conflicting reports in the literature describing the influence of cannabinoid receptor activation on ischemic/reperfusion injury. The goal of this study was to evaluate how changing the balance between CB_1 and CB_2 activation following cerebral ischemia influences outcome. CB_1 and CB_2 expression were tested at different times after transient middle cerebral artery occlusion (MCAO) in mice by real-time RT-PCR. Animals subjected to 1 h MCAO were randomly assigned to receive different treatments: a CB_1 antagonist, a CB_2 antagonist, a CB_2 agonist, a CB_1 antagonist plus CB_2 agonist, a CB_2 antagonist plus CB_2 agonist or an equal volume of vehicle as control. Cerebral blood flow was continuously monitored during ischemia; cerebral infarction and neurological deficit were tested 24 h after MCAO. Cerebral CB_1 and CB_2 mRNA expression undertook dynamic changes during cerebral ischemia. The selective CB_1 antagonist significantly decreased cerebral infarction by 47%; the selective CB_2 antagonist increased infarction by 26% after 1 h MCAO followed by 23 h reperfusion in mice. The most striking changes were obtained by combining a CB_1 antagonist with a CB_2 agonist. This combination elevated the cerebral blood flow during ischemia and reduced infarction by 75%. In conclusion, during cerebral ischemia/reperfusion injury, inhibition of CB_1 receptor activation is protective while inhibition of CB_2 receptor activation is detrimental. The greatest degree of neuroprotection was obtained by combining an inhibitor of CB_1 activation with an exogenous CB_2 agonist. © 2008 Published by Elsevier Ltd on behalf of IBRO.

Key words: endogenous cannabinoids, cerebral ischemia/reperfusion injury, cerebral blood flow, inflammatory responses.

The endocannabinoid system refers to two major types of cannabinoid receptors (termed CB_1 and CB_2), the endogenous ligands for those receptors and specific enzymes responsible for their degradation and inactivation (Rodriguez de Fonseca et al., 2005). The CB_1 receptor is primarily expressed in the CNS, exhibiting a presynaptic location and playing a prominent role in synaptic neurotransmission (Pazos et al., 2005; Rodriguez de Fonseca et al., 2005). The CB_2 receptor is expressed predominantly by cells of the immune system, such as lymphocytes and neutrophils, but is also expressed on resident inflammatory cells within the CNS. CB_2 stimulation has been shown to have immunomodulatory properties, such as decreasing the activity of antigen presenting cells (APC) and downregulating cytokine (IFN-γ and TNF-α) production during inflammatory responses (Berdyshev, 2000; Walter and Stella, 2004; Klein and Cabral, 2006; Lombard et al., 2007).

A number of investigations have shown that CB_2 receptor activation has anti-inflammatory therapeutic potential in various CNS diseases, such as multiple sclerosis, traumatic brain injury and Alzheimer's disease (Grundy et al., 2001; Molina-Holgado et al., 2002; Croxford, 2003; Ni et al., 2004; Ramirez et al., 2005). Because inflammatory responses have been shown to be important contributors to secondary injury following cerebral ischemia; the CB_2 receptor has been investigated as a potential therapeutic target in stroke. It was demonstrated that selective activation of CB_2 receptor attenuated cerebral ischemia/reperfusion injury in mice which was associated with decreased leukocyte/endothelial cell interactions (Zhang et al., 2007). The CB_1 receptor has also been studied in cerebral ischemia/reperfusion injury (Nagayama et al., 1999; Jin et al., 2000; Parmentier-Batteur et al., 2002; Hayakawa et al., 2004). However, to date there are few studies focusing on the roles of CB_1 and CB_2 activation by endogenous cannabinoids in cerebral ischemic injury. In this investigation, we evaluated how ischemia/reperfusion injury influences cannabinoid receptor expression and how modification of the balance between cannabinoid CB_1 receptor (CB_1R) and cannabinoid CB_2 receptor (CB_2R) ac-

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Abbreviations: CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotid artery; MCAO, middle cerebral artery occlusion; MCAO/R, middle cerebral artery occlusion and reperfusion; rCBF, regional cerebral blood flow; TTC, triphenyltetrazolium chloride; 2-AG, 2-arachidonoylglycerol.

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tivation by endogenous and exogenous cannabinoids influences outcome after stroke.

EXPERIMENTAL PROCEDURES

Animals and surgical procedures

The cerebral ischemia/reperfusion studies were carried out in 8-week-old male C57BL/6 mice (weighing 23–27 g; Taconic, Hudson, NY, USA) and conducted in accordance with the guidelines approved by the Institutional Animal Care and Use Committee at Temple University and followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering.

Middle cerebral artery occlusion and reperfusion (MCAO/R)

The animals were anesthetized by i.p. injection of a mixture of ketamine (100 mg/ml) –xylazine (20 mg/kg) (1:1) at a dose of 1 ml/kg. Body and cerebral temperature were maintained at 37±5 °C by a heating lamp and heating pad. MCAO was achieved by the intraluminal filament method (Hata et al., 1998). Briefly, a midline neck incision was made under an operating microscope. The right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were isolated. The ECA was ligated with 6-0 silk suture distal from the ICA–ECA branch and then cut distal from the ligated point. Another 6-0 silk suture was tied loosely around ECA close to the origin at the CCA. A blunted 5-0 monofilament nylon suture coated with poly-L-lysine (0.1% in deionized water, Sigma Inc., St. Louis, MO, USA) (Belayev et al., 1999) was introduced through a small incision in ECA and advanced into the circle of Willis, finally occluding the origin of the middle cerebral artery. The silk suture around the ECA stump was tied tightly to prevent bleeding and secured with a silk suture. The nylon suture was removed after 60 min occlusion and the ECA was permanently tied. Reperfusion was confirmed when pulsations were again observed in ICA.

Regional cerebral blood flow (rCBF)

A laserPro Blood Perfusion Monitor (TSI Inc., Shoreview, MN, USA) was used to monitor and record rCBF prior to ischemia, during MCAO/R. A 1 mm diameter microfiber laser-Doppler probe was attached to the skull 4 mm lateral and 2 mm posterior of Bregma. The MCAO was considered adequate if rCBF showed a sharp drop to 25% of baseline (pre-ischemia) level, otherwise, animals were excluded (Tsuchiya et al., 2003).

Injection of cannabinoid receptor agonist and antagonists in MCAO/R

The CB_1 antagonist (SR141716) and CB_2 antagonist (SR144528) were dissolved in a DMSO:Cremophor:saline mixed solution (1:1:18). The antagonists (5 mg/kg or 20 mg/kg) or an equal volume of vehicle was administered 1 h before MCAO i.p. The CB_2 agonist (O-1966) was dissolved in a pure ethanol:emulphor:saline mixed solution at 1:1:18. The CB_2 agonists (1 mg/kg) or an equal volume of vehicle was administered as an i.v. injection into the jugular vein or intraperitoneally in a separate experimental group 1 h before MCAO.

Real time PT-PCR

CB1 and CB2 expression was detected by the SYBR Green–based real time RT-PCR technique. Animals were killed and transcardially perfused with cold PBS to remove the blood from vessels. Total RNA was isolated from brain specimens at 1, 3, 6 or 24 h after MCAO by using Ultraspec reagent (Biotecx Laboratories, Houston, TX, USA). Normal brain was used as sham. cDNA was prepared by reverse transcription. The 20 μl (total volume) of the PCR mixture consists of 4 μl diluted cDNA, 10 μl SYBR Green–containing PCR master mixture (2×) and 150 μM of each primer. The CB1 and CB2 primers for real-time RT-PCR were designed by using the Primer Express software from Applied Biosystems (Foster City, CA, USA), and are as follows: CB_1 sense: 5′-TGA AGT CGA TCT TAG ACG GCC-3′ and antisense: 5′-GTG GTG ATG GTA CGG AAG GTA-3′; CB_2 sense: 5′-TGA ATG AGC AGA CCG ACA GG-3′ and antisense: 5′-AGA GAT GTT TGC TGG GTG GC-3′; β-actin sense: 5′-TCC ACC ACC ACA GCT GAG AGG-3′, and antisense: 5′-CAG CTT CTC TTT GAT GTC ACG-3′. Real time RT-PCR was performed using the Stratagene Mx3005P (Stratagene Corporation, La Jolla, CA, USA), and the cycling conditions used were 95 °C for 15 s, 60 °C for 1 min, for 40 cycles, followed by a melting point determination or dissociation curves. The expression level of each gene is indicated by the cycle numbers needed for the cDNA to be amplified to reach a threshold. The amount of DNA is calculated from the cycle numbers by using standard curves and the results are normalized to the housekeeping gene β-actin from the same sample.

Infarct volume assessment

The animals were killed with an overdose of pentobarbital (200 mg/kg i.p.) 24 h after MCAO and the brains were removed, and chilled on ice for 10 min to slightly harden the tissue. Five 2 mm coronal sections were cut using a mouse brain matrix (Zivic Laboratory, Pittsburg, PA, USA).The brain sections were placed in 2% triphenyltetrazolium chloride (TTC) (Sigma Inc.) dissolved in saline and stained for 20 min at 37 °C in the dark. The brain sections were then fixed in 4% paraformaldehyde at 4 °C for 24 h and the anterior and caudal face of each section was scanned by a flatbed color scanner (Microtek Inc., Carson, CA, USA). The resulting images were captured as JPEG files and analyzed with NIH image software. The hemispheric infarct volumes were corrected for brain edema/swelling: the hemispheric infarct volume in each section was calculated by subtracting the area of normal, TTC-stained tissue in the hemisphere ipsilateral to the ligation from the contralateral nonischemic area to generate the infarct fraction (%), as described by Swanson et al. (1990) and Lin et al. (1993).

Neurological function evaluation

The severity of neurological deficits was evaluated 24 h after ischemic insult using a five-point deficit score (0=normal motor function; 1=flexion of torso and of contralateral forelimb upon lifting of the animal by tail; 2=circling to the contralateral side but normal posture at rest; 3=leaning to contralateral side at rest; and 4=no spontaneous motor activity) (Hata et al., 1998).

Statistical analysis

Bonferroni's test after one-way ANOVA was used for analyzing differences in infarct volume, neurological score and average of rCBF. The mRNA expression of CB1 and CB2 was analyzed by two-way ANOVA (times, hemispheres) followed by Bonferroni's test. Data were presented as means±S.E.M. A statistically significant difference was assumed at $P<0.05$.

RESULTS

CB_1 and CB_2 mRNA expression in brain during MCAO

There were no differences in CB_1 mRNA expression in the non-ischemic hemisphere compared with normal control at 1, 3, 6 and 24 h after MCAO. CB_1 expression in the