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Chronic caffeine treatment protects against experimental autoimmune encephalomyelitis in mice: Therapeutic window and receptor subtype mechanism

Tan Wang [a], Na-na Xi [a], Yu Chen [a], Xiao-feng Shang [a], Qiang Hu [a], Jiang-fan Chen [a, b, **], Rong-yuan Zheng [a, *]

[a] Department of Neurology, The First Affiliated Hospital and Research Institute of Experimental Neurobiology, Wenzhou Medical University, PR China
[b] Department of Neurology, Boston University School of Medicine, USA

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ABSTRACT

Chronic treatment with caffeine, the most widely consumed psychoactive drug and a non-selective antagonist of adenosine receptors, can protection against myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS). In this study, we investigated the mechanism underlying caffeine-mediated neuroprotection against EAE by determining the effective therapeutic time-window of caffeine and the involvement of adenosine A_{2A} and A_1 receptor. We found that administration of caffeine during the effector phase (10 → 20 days post-immunization, d.p.i., corresponding to appearance of neurological deficits) but not the induction phase (0 → 10 d.p.i., before the appearance of ascending flaccid paralysis) significantly ameliorated EAE-induced neurobehavioral deficits, reduced the infiltration of inflammatory cells into the spinal cord and reduced the demyelination of spinal cord. Furthermore, genetic deletion of the A_{2A}R exacerbated MOG-induced brain damage and caffeine administering to A_{2A}R knockout mice reversed this EAE pathology by acting at non-A_{2A}R target. The protective effect of chronic caffeine treatment was associated with up-regulation of brain A_1R (but not A_{2A}R). The identification of the effective therapeutic window of caffeine at the effector phase and clarification of non-A_{2A}R target (likely A_1R) in caffeine action in EAE models advance the therapeutic prospective that chronic caffeine consumption may attenuate brain damage in MS.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease of the central nervous system (CNS) (Jadidi-Niaragh and Mirshafiey, 2011; Thompson et al., 2010), in which autoreactive myelin-specific T cells cause extensive brain damage, resulting in neurological deficits. Recent studies suggest that pathogenesis of MS and EAE may involve two distinct processes, initial neuro-inflammation and later degeneration (Lopez-Diego and Weiner, 2008; Neuhaus et al., 2003; Steinman, 2001). In mouse experimental autoimmune encephalomyelitis (EAE) model of MS induced by myelin oligodendrocyte glycoprotein (MOG), autoreactive CD4+ T cells are activated in the periphery organs during the induction phase of EAE and infiltrated into the brain. Proinflammatory cytokines and chemokines activate resident cells, recruit other immune cells and form the focal inflammatory lesion. CD4+ autoreactive T cells are thought to be the driving force at the initial neuroinflammatory phase. At the degenerative phase of the disease progression, multiple effector mechanisms may produce myelin sheath and axonal damage, including demyelination, axon and neuronal loss, accumulation of microglia and macrophages, anti-ganglioside antibodies and CD8+ CTL (Lopez-Diego and Weiner, 2008; Sospedra and Martin, 2005) and glutamate-mediated excitotoxicity of oligodendrocytes (Pitt et al., 2000; Sospedra and Martin, 2005; Werner et al., 2001).

Adenosine, which substantially derives from ATP breakdown via ecto-nucleotidase CD73 in cell surface (Augusto et al., 2013), serves as an autocrine- and paracrine-signaling molecule through its

* Corresponding author. Department of Neurology, The First Affiliated Hospital and Research Institute of Experimental Neurobiology, Wenzhou Medical University, Wenzhou, Zhejiang 325000, PR China.
** Corresponding author. Department of Neurology, Boston University School of Medicine, 715 Albany Street C329, Boston, MA 02118, USA.
E-mail addresses: chenjf@bu.edu (J.-f. Chen), zhengry1949@163.com (R.-y. Zheng).

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receptors (namely A_1, A_{2A}, A_{2B} and A_3 subtypes). Several studies including ours have demonstrated that A_1 and A_{2A} receptors modulate neuroinflammation in EAE and MS (Du and Xie, 2012; Mills et al., 2008, 2012a, 2012b; Tsutsui et al., 2004; Yao et al., 2012). The A_1 receptor (A_1R) is down-regulated in peripheral blood cells of MS (Johnston et al., 2001; Mayne et al., 1999), while the A_{2A} receptors ($A_{2A}R$) is up-regulated in MS patient's lymphocytes (Vincenzi et al., 2013) and in brain of secondary progressive MS (Rissanen et al., 2013). Extracellular adenosine acting at the A_1R and $A_{2A}R$ provide neuroprotection against MOG-induced brain damage as revealed by A_1R and $A_{2A}R$ knockouts (Tsutsui et al., 2004; Yao et al., 2012). However, modulation of EAE pathology by adenosine may be complex since genetic deletion of CD73 (with reduced extracellular adenosine) also displayed a reduced brain damage in EAE model (Mills et al., 2008). Importantly, caffeine, a non-selective antagonist of adenosine receptors, has been consistently shown to attenuate MOG-induced EAE in mice (Chen et al., 2010; Mills et al., 2008; Tsutsui et al., 2004). This raises an exciting possibility that caffeine, at the concentration attained by regular consumption of ~70% human population, may modify MS disease course. Given its public health implication, it is critical to understand the mechanism whereby caffeine exerts its therapeutic effect in EAE model of MS.

In present study, we addressed two mechanistic questions of caffeine action in EAE model: i) given the complex and distinct disease courses of MS, we examined whether caffeine specifically influence the induction (i.e. initial inflammation) or effector (later degenerative) phases of EAE to exert its neuroprotective effect; ii) given the complex pharmacological targets of caffeine action, we combined caffeine treatment with $A_{2A}R$ knockout (KO) model to determine whether caffeine acts at the $A_{2A}R$ to modulate brain inflammation and neurodegeneration.

2. Materials and methods

2.1. Animals

C57BL/6 mice were obtained from the Laboratory Animal Center of Wenzhou Medical University, China. The $A_{2A}R$ KO mice and WT littermates mice were generated and characterized as described previously (Chen et al., 1999; Yao et al., 2012; Yu et al., 2008), and the parent mice were provided by Dr. Jiang-fan Chen at the Department of Neurology, Boston University School of Medicine, USA. All animal procedures in present study were conducted in the procedures approved by the Institutional Animals Care and Use Committee at Wenzhou Medical University, which adhered to the NIH Guide for the Care and Use of Laboratory Animals.

2.2. Induction of EAE

EAE was induced as we described previously (Li et al., 2012; Yao et al., 2012). Briefly, female mice (aged 8–10 weeks) were immunized with MOG_{35-55} (AC scientific, China) emulsified in incomplete Freund's adjuvant (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 8 mg/ml mycobacterium tuberculosis H37Ra (MOG_{35-55}-CFA). Mice were subcutaneously injected in the flanks with 200 μg MOG_{35-55}-CFA. Furthermore, pertussis toxin (200 ng, Sigma–Aldrich) was injected intraperitoneally immediately and 48 h after immunization. Clinical EAE scores and body weights were evaluated daily.

2.3. Caffeine treatment and groups

In order to determine the effective therapeutic time-window for caffeine-mediated protection against EAE, C57BL/6 mice were randomly divided into six groups according to their immunizing inductor (CFA or MOG_{35-55}) and caffeine treatment schedule: (1) water-CFA group ($n = 6$):Mice had free access to drinking water during the experiment and were immunized with CFA as the control. (2) Water-EAE group ($n = 10$): Mice had free access to drinking water before and after immunization with MOG_{35-55}. (3) CAF (−10 → 0)-EAE group ($n = 8$): Mice were pretreated with caffeine 30 mg/kg per day in their drinking water from 10 days prior to immunization with MOG_{35-55} to the day of immunization (day 0). (4) CAF (0 → 10)-EAE group ($n = 8$): Mice were treated with caffeine 30 mg/kg per day in their drinking water from the day of immunization with MOG_{35-55} to day 10 after immunization, corresponding to the initial inflammation phase in MS. (5) CAF (10 → 20)-EAE group ($n = 8$): Mice were treated with caffeine 30 mg/kg per day in their drinking water from day 10 to day 20 after immunization, corresponding to the later degenerative phase. (6) CAF (−10 → 20)-EAE group ($n = 10$): Mice were pretreated with caffeine 30 mg/kg per day in their drinking water from 10 days prior to immunization with MOG_{35-55} to the day 20 after immunization when the mice were sacrificed.

C57BL/6 $A_{2A}R$ wild-type (WT) and KO mice (female, aged 8–10 weeks) were divided into four groups: (1) $A_{2A}R$ WT-water group ($n = 6$): $A_{2A}R$ WT mice had free access to drinking water before and after immunization with MOG_{35-55}. (2) $A_{2A}R$ WT-caffeine group ($n = 6$): $A_{2A}R$ WT mice were treated with caffeine 30 mg/kg per day in their drinking water from 10 days before immunization with MOG_{35-55} to 20 days after immunization. (3) $A_{2A}R$ KO-water group ($n = 6$): $A_{2A}R$ knockout mice had free access to drinking water before and after immunization with MOG_{35-55}. (4) $A_{2A}R$ KO-caffeine group ($n = 6$): $A_{2A}R$ KO-Mice were treated with caffeine 30 mg/kg per day in their drinking water from 10 days before immunization with MOG_{35-55} to 20 days after immunization.

Mice with caffeine intervention were housed individually and the drinking water consumed was checked daily to ensure the dosage was accurate. The caffeinated water was changed three times per week. On average, each mouse drank 4 ml water per day. Caffeine was added to the water at 0.15 mg/ml; therefore, individual mouse received an average daily dose of 0.6 mg caffeine, which corresponds to a dose of 30 mg/kg based on a body weight of 20 g.

2.4. Behavioral evaluation of EAE

The day of MOG_{35-55}-CFA immunization was regarded as day 0 d.p.i. Neurological deficits were scored twice a day for each mouse according to the previously established criteria (Weaver et al., 2005): Tail: 0, no signs; 1, half paralyzed tail; 2, fully paralyzed tail; Limbs: 0, no signs; 1, weak or altered gait; 2, paresis; 3, fully paralyzed limb. Each of the hind and forelimbs were assessed separately. Thus, a fully paralyzed quadriplegic animal would attain a score of 14 and mortality equates to a score of 15.

2.5. Histological analyses

At the 20 d.p.i., the animals were sacrificed and the spinal cords were dissected out for processing by routine paraffin-embedding.

Then H&E staining was performed to investigate the inflammatory cells infiltration. The histological evaluation was performed as described before (Okuda et al., 1999). Semi-quantitative histological evaluation based on the severity of inflammation was performed using the following scale: 0 = no inflammation; 1 = cellular infiltrates only in the perivascular areas and meninges; 2 = mild cellular infiltrates in the parenchyma (1–10 cells/section); 3 = moderate cellular infiltrates in the parenchyma (11–100 cells/section); 4 = marked cellular infiltrates in the parenchyma (>100 cells/section). Sections from the cervical, thoracic and lumbar level of the spinal cord of all mice were evaluated by two observers in a blind fashion. At least nine sections (cervical, thoracic and lumbar were three section, respectively.) from all animals in each group were quantified. We observed four areas of each section (400×) and evaluated the severity of inflammation.

We used Luxol Fast Blue method to examine myelination in the brain followed the protocol described previously (Kim et al., 2006). Briefly, brain sections were incubated with Luxol Fast Blue solution at 56 °C overnight after a defatting and anhydration. After a rinse with 95% ethanol and distilled water to remove excess stain, the slides were differentiated in lithium carbonate solution for 30 s and then in 70% ethylalcohol for 30 s. The slides were then rinsed in distilled water. Differentiation was verified under microscope to ensure that myelin was sharply stained. Sections were the mounted for examination under a light microscope. The color intensities of LFB from the groups were measured using Image J.

2.6. RT-PCR detection

Total RNA was isolated from cerebral cortex (50–100 mg) using Trizol, according to the manufacturer's instructions (Invitrogen, USA). RNA was quantitated by spectrophotometer (Beckman, LA, USA) to ensure that the ratio of the absorbance at 260 nm and 280 nm wavelength (A260/A280) was between 1.8 and 2.0. Equivalent RNA was used for the synthesis of complementary DNA (TaKaRa Bio Inc., RT-PCR Kit (AMV), China). PCR amplification was carried out in triplicate using a SYBR Green kit (TaKaRa Bio Inc., BcaBESTTMRNA PCR Kit, China). The primer sequences used for PCR analysis in this study were described in Supplemental Table 1. PCR products were separated by electrophoresis through a 2.0% agarose gel and visualized by ethidium bromide staining. The relative abundance of the target gene was normalized to β-actin.

2.7. Statistical analyses

The SPSS16.0 statistical program was used for all statistical analysis. All data are presented as mean ± SEM unless otherwise stated. Mouse EAE neurologic behavioral deficit scores and histopathologic scores (with two factors of treatment and post-immunization day) were assessed by two-way ANOVA accompanied with LSD post-hoc comparisons and Mann–Whitney *U*-tests. Comparisons between multiple groups with single treatment factor were analyzed by one-way ANOVA. $P < 0.05$ was considered significant.