FEBS 20613 FEBS Letters 433 (1998) 139–142

The endogenous cannabinoid anandamide potentiates interleukin-6 production by astrocytes infected with Theiler's murine encephalomyelitis virus by a receptor-mediated pathway

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Received 24 June 1998

Abstract Theiler's murine encephalomyelitis virus (TMEV) infection of a susceptible strain of mice results in virus persistence in the brain and chronic primary immune-mediated demyelination, which resembles multiple sclerosis. Recent attention has focused on the anti-inflammatory and immunosuppressive properties of interleukin-6, a pleiotropic cytokine involved in the regulation of immunological responses, acute phase protein production and hematopoiesis. Anandamide (arachidonoyl ethanolamine) is a natural brain constituent that binds a specific brain cannabinoid receptor. In this study we investigated whether anandamide can modify interleukin-6 production by primary cultures of murine brain cortical astrocytes infected with TMEV. Astrocytes from susceptible (SJL/J) and resistant (BALB/c) strains of mice infected with TMEV (10^5 PFU/well) increased IL-6 release over a period of 24 h. Anandamide caused an enhancement of the release of IL-6 by TMEV-infected astrocytes in a concentration-dependent manner (1–25 μM). Treatment of TMEV-infected astrocytes with 10 μM arachidonyl trifluoromethyl ketone, a potent inhibitor of the amidase that degrades anandamide, was found to potentiate the effects of anandamide on IL-6 release. A novel and selective cannabinoid receptor antagonist, SR 141617A, blocked the enhancing effects of anandamide on IL-6 release by TMEV-infected astrocytes, suggesting a cannabinoid receptor-mediated pathway. The physiological implications of these results are unknown, but may be related to the hypothesis of the protective effects of cannabinoids on neurological disorders like multiple sclerosis.

Key words: Anandamide; Cannabinoid; Interleukin-6; Theiler's virus; Astrocyte

1. Introduction

Susceptible mouse strains infected with Theiler's murine encephalomyelitis virus (TMEV), a picornavirus which has the mouse as its natural host, present lesions closely resembling those of multiple sclerosis (MS). The pathogenesis of demyelination in TMEV infection is considered to be dependent on the host immune response against the virus [1,2]. Astrocytes respond promptly to CNS injury and infection and are efficiently infected by TMEV [3,4]. Under such circumstances, astrocytes produce inflammatory mediators and several cytokines, including interleukin-6 [5]. Interleukin-6 (IL-6) is a pleiotropic cytokine involved in the regulation of inflammatory and immunological responses, acute phase protein production, and hematopoiesis. Increased IL-6 mRNA expression in blood and cerebrospinal fluid monocellular cells has been described in patients with MS [6]. Although initially considered to be a pro-inflammatory cytokine, recent findings suggest that IL-6 has many anti-inflammatory and immunosuppressive effects [7]. Within the CNS, IL-6 inhibits IFNγ/IL-1β- and IFNγ/LPS-induced synthesis of TNF-α in glial cells [8], and suppresses demyelination in a viral (TMEV) model of MS [9].

Cannabinoids, a class of biologically active compounds, are best known for their ability to alter CNS function and to produce immunosuppression. It is now recognized that cannabinoids exert their effects by both receptor- and non-receptor-mediated mechanisms. Two cannabinoid receptor subtypes have been identified. The first of these, designated CB1, is limited to neural tissue, including astrocytes [10], while the second, designated CB2, is found primarily within immune cells at peripheral sites [11]. Anandamide (arachidonyl ethanolamide) is a naturally occurring brain constituent that binds to CB1 receptors and is released after neuronal depolarization [12]. Anandamide biosynthesis is believed to be mediated by anandamide synthase by using arachidonic and ethanolamine as substrates in the brain [13] or phosphodiesterase-mediated cleavage of a novel phospholipid precursor, *N*-arachidonyl-phosphatidylethanolamine [14]. Anandamide behaves as a cannabimimetic compound and in vitro it stimulates receptor-mediated signal transduction that leads to the inhibition of forskolin-stimulated adenylate cyclase [15,16]. It is therefore likely that anandamide may act as an important regulatory molecule. However, the physiological role of anandamide has not yet been established.

We have previously reported that anandamide inhibits nitric oxide and TNF-α production by astrocytes stimulated with LPS or infected with TMEV [17]. These results suggest that astrocytes are possible target cells for immunomodulatory activities of anandamide. In the present study we attempted to investigate the effects of anandamide on IL-6 production by TMEV-infected astrocytes and whether anandamide acts by a cannabinoid receptor pathway.

2. Materials and methods

2.1. Mice

One day postnatal SJL/J (Jackson Laboratories) and BALB/c mice pathogen free were obtained from the Central Animal Laboratory of the Cajal Institute (CSIC, Madrid, Spain). Animal care procedures were accordance with the guidelines set by the European Community Council Directives (86/609/EEC).

2.2. Virus

TMEV strain Daniel (DA) generously gifted by Dr. Raymon P. Roos (Department of Neurology, University of Chicago, IL) was used. The DA strain of TMEV was plaque purified and passaged four times in baby hamster kidney cells (BHK-21). Virus was titrated

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PII: S0014-5793(98)00851-5

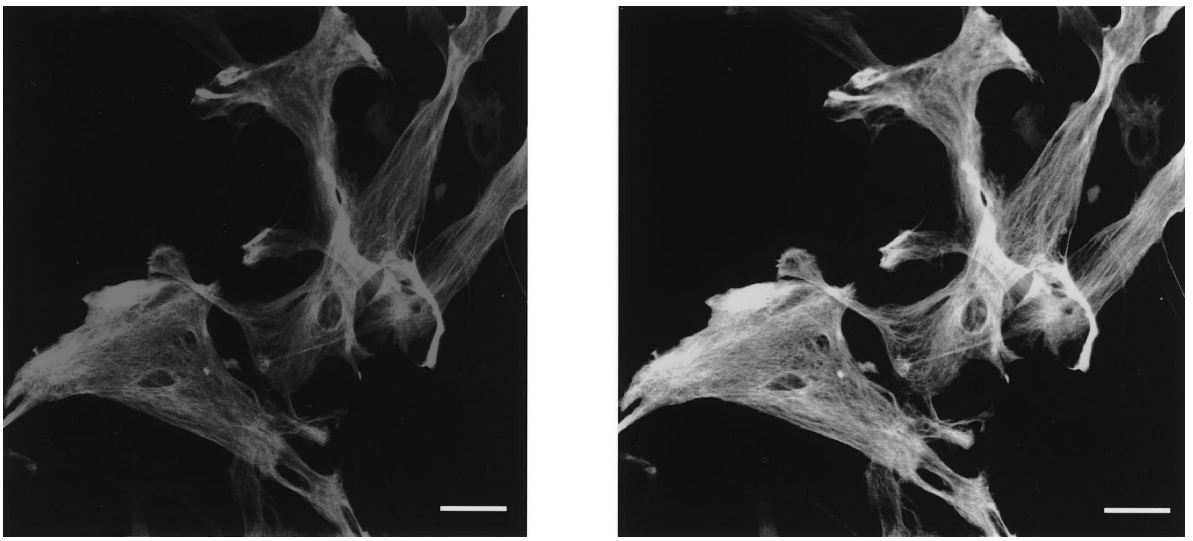

Fig. 1. Immunofluorescence staining of astrocyte cells in culture with anti-GFAP, using confocal microscopy (Leica 500M).

and stored in aliquots at −70°. Virus was used at 1×10^5 plaque-forming units (PFU)/ml.

2.3. Astrocyte cultures

Primary astrocyte cultures were prepared from the cerebral cortex from 1-day-old postnatal SJL/J or BALB/c mice, as previously described in detail [18], and grown in T150 flasks for at least 14 days in Dulbecco's modified Eagle's medium (DMEM) plus 10% heat-inactivated fetal calf serum (Whittaker, France), 20 mM glutamine (ICN Biomedicals, California) and gentamicin with the medium being changed twice weekly. On reaching confluence (2 weeks) the cells were shaken at 240 rpm overnight at 37°C to dislodge cells adhering to the astrocyte layer (primarily oligodendrocytes and microglia). The media were replaced and the cells were allowed to recover for 2–3 days before experiments. To visualize glial fibrillary acidic protein (GFAP), cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Afterwards, the cells were washed three times with PBS and blocked for 15 min in PBS containing 0.2% BSA, 5% goat serum and 0,2% Triton X-100. Monoclonal anti-GFAP (1:500; Sigma) was diluted in the same solution and was applied for 45 min. The second antibody Texas red donkey anti-mouse (Jackson Immunochemicals) was diluted (1:100) and applied under the same conditions. The resulting cultures consisted of $>95\%$ astrocytes as determined by GFAP staining and $<3\%$ of cells were positive for the microglia marker Mac-1 (Fig. 1). For stimulation, astrocyte monolayers were incubated with the virus for 1 h at 37°C in serum-free DMEM plus 0.1% BSA. Following incubation the cells were washed and placed in new medium. On the basis of previous studies, we selected a dose of 10^5 PFU/ml. The variations in IL-6 release over a period of 24 h after TMEV infection led us to choose a post-infection period of 24 h for the experiments with anandamide. Anandamide (RBI, Natick, MA) at doses of 1, 10 and 25 μM, was added prior to the TMEV infection and then maintained throughout the post-infection period. The effect of an inhibitor of anandamide degradation [19], arachidonyl trifluoromethyl ketone (Sigma Chemical, St. Louis, MO), was studied by adding it, together with anandamide. The possible mediation of cannabinoid receptors was studied by using the selective CRB1 antagonist, SR 141716A (*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl) 4-methyl-1H-pyrazole-3-carboxamide hydrochloride), a generous gift of Sanofi Research [20].

2.4. IL-6 assay

The presence of IL-6 in the harvested supernatants from astrocyte cultures was investigated using enzyme-linked immunoadsorbent assay (Intertest-6X mouse ELISA kit, Genzyme, Cambridge, MA). The detection limit was 8.8 pg/ml. Coefficients of intra- and inter-assay variation were 3.1 and 8.7% respectively. There was no detectable crossreactivity with other cytokines.

2.5. Statistics

The data are presented as mean ± S.E.M. of 3–4 independent determinations and each experiment was carried out in triplicate. Comparisons were analyzed using one-way analysis of variance (ANOVA) followed by the posteriori Student-Newman-Keuls *t*-test. Statistical significance was established at $P<0.05$.

3. Results

We initially examined the effect of TMEV infection on IL-6 generation by astrocytes from both susceptible (SJL/J) and resistant (BALB/c) strains of mice over a period of 24 h. By phase contrast microscopy, cultures of infected astrocytes maintained the same cell density and appearance as non-infected cultures, showing no cytopathic effect (not shown). As illustrated in Fig. 2, cultured astrocytes derived from both strains of mice were able to produce IL-6 following TMEV infection. The pattern of IL-6 production was identical in the two strains of mice, showing the higher levels at 24 h post-infection. Therefore, in the following experiments with anandamide we only used TMEV-infected astrocytes from SJL/J mice and an interval of post-infection time of 24 h.

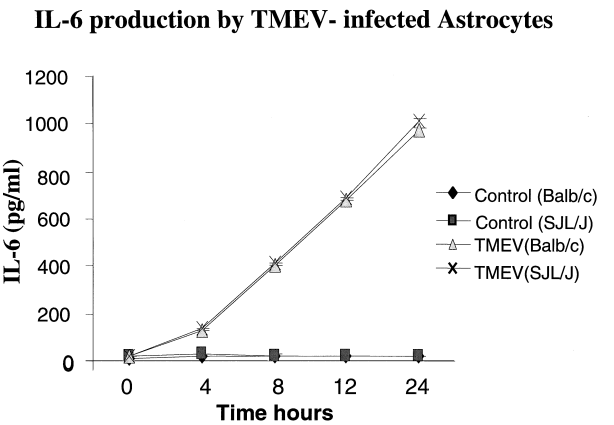

Fig. 2. Time course of IL-6 release by murine TMEV-infected astrocytes (10^5 PFU/well), derived from susceptible (SJL/J) and resistant (BALB/c) mouse strains. Results represent the mean ± S.E.M. of a representative experiment done in triplicate.

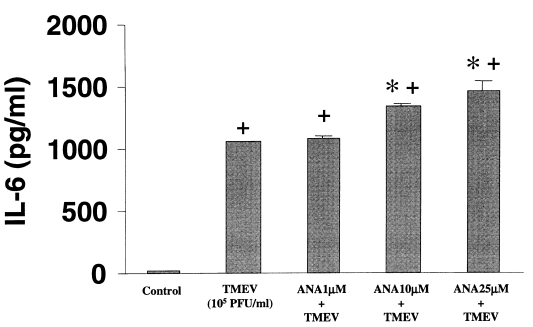

Fig. 3. Effect of different doses of anandamide on IL-6 release by TMEV-infected astrocytes. Murine astrocytes from a susceptible strain of mice (SJL/J) were infected with 10^5 PFU/well and the supernatants for IL-6 assay were collected 24 h post-infection. Results are the mean ± S.E.M. of six experiments. Statistical differences: $^{*}P < 0.001$ vs. TMEV; $^{+}P < 0.01$ vs. anandamide 1 μM; $^{++}P < 0.05$ vs. anandamide 10 μM.

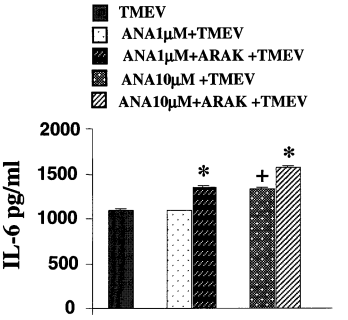

Fig. 4. Effect of the presence of the inhibitor of amidase, arachidonyl trifluoromethyl ketone (ARAK), at a dose of 10 μM on IL-6 release by TMEV-infected astrocytes treated with 1 and 10 μM anandamide. Results are the means ± S.E.M. of six experiments. Statistical differences: $^{*}P < 0.001$ vs. anandamide 1 μM or 10 μM; $^{+}P < 0.001$ vs. TMEV.

Treatment with anandamide caused a concentration-dependent increase in the release of IL-6 by TMEV-infected astrocytes (Fig. 3). ANOVA of the results revealed a significant effect of the treatment ($F_{4,30}$=745.277; $P < 0.0000$), and a posteriori test indicated a significant effect among the three doses of anandamide employed ($P < 0.01$ and $P < 0.05$, respectively). At the highest concentration of anandamide tested, IL-6 release was increased by 38.52% over IL-6 production by TMEV-infected astrocytes. The stimulation of IL-6 release by anandamide was confirmed using an inhibitor of anandamide degradation. Anandamide is rapidly metabolized by amidases to arachidonic acid and ethanol amine [18]. Treatment of TMEV-infected astrocytes with 10 μM arachidonyl trifluoromethyl ketone, an inhibitor of amidases, was found to potentiate significantly the release of IL-6 by anandamide 1 μM ($P < 0.01$) and 10 μM ($P < 0.01$) (Fig. 4). This observation supports a direct signalling role of anandamide.

The actions of cannabinoids are thought to be mediated, in part, by specific receptors that are expressed on the surface of responsive cells. A significant question is therefore whether anandamide exerts its effects on IL-6 production by binding to specific receptors in astrocytes. To examine this question we used a selective antagonist of brain CBR1 receptors, SR141617A. Under these conditions, the presence of 1 μM SR141617A blocked the potentiating effect of 10 μM anandamide on IL-6 release by TMEV-infected astrocytes (Table 1), suggesting a receptor-mediated action of anandamide.

Table 1
Blockade by SR141617A, a selective antagonist of brain cannabinoid receptors, of the effects of anandamide (ANA) on IL-6 release by TMEV-infected astrocyte

	IL-6 (pg/ml)
Control	25.86 ± 1.104
TMEV (10^5 PFU/ml)	1041.83 ± 13.09***
ANA (10 μM)+TMEV	1499 ± 8.521**
SR141617A (1 μM)+ANA (10 μM)+TMEV	903.33 ± 50.841^{+}
SR141617A (1 μM)	48.33 ± 19.30

Results are the mean ± S.E.M. of four experiments. $^{***}P < 0.0001$ vs. control; $^{**}P < 0.01$ vs. TMEV; $^{+}P < 0.01$ vs. ANA+TMEV.

4. Discussion

In the present study we show that anandamide exhibits immunomodulatory activity in astrocytes as demonstrated by the enhancement of IL-6 production in response to TMEV infection. Numerous studies suggest that the host immune response plays a primary role in the initiation of events leading to myelin destruction in the TMEV model of MS [2,3]. In this model, the main reservoir of virus in the CNS is infected macrophages, but also astrocytes contain viral antigen [3]. The presence of both IL-6 and IL-6 mRNA in the CNS of intracranially TMEV-infected mice has been described in previous studies [5,21]. Elevated levels of IL-6 in the CNS of EAE diseased animals but not in their spleens are suggestive of a local production of IL-6 within the brain [22]. Here, we show that murine astrocytes from both susceptible and resistant strains of mice are able to synthesize IL-6, indicating, in agreement with other studies [3], that IL-6 seems not to be involved in determining susceptibility/resistance to the development of demyelinating disease.

Exogenous cannabinoids are now known to exert immunomodulatory activity. In our study, the endogenous cannabinoid anandamide was found to potentiate IL-6 production by TMEV-infected astrocytes in a dose-dependent manner. This effect can be attributed to anandamide per se, because experiments performed in the presence of an inhibitor of anandamide degradation showed a potentiation of the effects of anandamide on IL-6 production. The fact that SR141717A, a potent and selective antagonist of the brain cannabinoid receptor, blocked the enhancing effects of anandamide on IL-6 release suggests the possibility of a cannabinoid receptor-mediated pathway. The lack of activity of anandamide on spleen cells [23] and the fact that anandamide has only been identified in neuronal tissue preparations may be indicative that anandamide is a neural-specific cannabinoid agonist. However, there are studies showing that anandamide is able to inhibit cytokine release, including IL-6, by human mononuclear cells [24]. Furthermore, a second endogenous cannabinoid ligand, 2-arachidonyl glycerol, isolated from the intestinal tract was shown to inhibit long-term potentiation [25]. It seems, therefore, that endogenous cannabinoids are able to

interact with cannabinoid receptors at both the peripheral and the central level.

The finding that anandamide activates the release of IL-6 by TMEV-infected astrocytes is consistent with the hypothesis that anandamide may be a physiologically significant immunoregulatory molecule. This is in line with our previous results in which anandamide suppressed TNF-α and nitric oxide responses by stimulated astrocytes [16]. An action of cannabinoids in astroglial cells is supported by the presence of the CB1 receptor in astrocytes and astrocytoma cells [26]. IL-6 is a multifunctional cytokine that is produced in a variety of inflammatory conditions in vivo. In the CNS, IL-6 has multiple effects on neural cells, and may function in an autocrine manner as glioblastoma, astrocytoma cell lines and primary astrocyte cultures express specific high affinity receptors for IL-6 [27]. Although overproduction of IL-6 in brain of transgenic mice resulted in important deficits, tremors and astrogliosis [28], astrocytes secrete nerve growth factor in response to IL-6 [29]. Increasing evidence pointed to the neuroprotective role of IL-6 [7]. Although the full range of the anti-inflammatory effects mediated by IL-6 has not been explored in the brain, IL-6 inhibits IFNγ/IL-1β-induced synthesis of TNF-α in glial cells at either the post-transcriptional and/or the post-translational level. The role of IL-6 in MS is unclear. The intrathecal production of IL-6 in acute meningo-encephalitis has been suggested to have desirable effects such as enhancement of antiviral antibodies and to promote neural repair [29]. Interestingly, in the MS model induced by TMEV, administration of human recombinant IL-6 reduced demyelination and inflammation in the spinal cord [9]. IL-6 synthesis activation may therefore represent an important response of viral-infected astrocytes to anandamide. There are earlier studies showing beneficial effects of the administration of exogenous cannabinoids such as Δ^9-tetrahydrocannabinol on demyelination in several experimental models of MS [30]. A possible protective role of anandamide in MS disease needs to be clarified in further studies.

Acknowledgements: This study was supported by grants from the DGICYT (PB94/0098) and Fundación Salud 2000. We thank C. García-Andrés for her technical assistance. We are indebted to Dr. Raymond R. Roos for providing the TMEV. We also thank Sanofi Research for generously providing SR141617A.

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