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Anandamide suppresses nitric oxide and TNF-α responses to Theiler's virus or endotoxin in astrocytes

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ASTROCYTES are an important cell population in the CNS, involved in cytokine homeostasis and participating in a variety of important physiological and pathological processes. In the present study we showed that primary cultures of neonatal mouse cortical astrocytes stimulated with lipopolysaccharide (Balb/c mice strain, LPS: 1 μg/ml, 18 h) or Theiler's virus, TMEV (SJL/J mice strain, TMEV: 10^5 PFU/well, 24 h) released an increased amount of nitrites (NO_2^-) and tumour necrosis factor-α (TNF-α) into the culture medium. Exogenous cannabinoids are known to modulate the function of immune cells. Anandamide, an endogenous ligand for the cannabinoid receptor, blocked the release of NO_2^- and TNF-α induced by LPS in a dose-dependent manner. In TMEV-stimulated astrocytes anandamide also suppressed, in a dose-related manner, the stimulatory effects of TMEV on both NO_2^- and TNF-α. It is suggested that anandamide exerts an immunoregulatory role in the CNS. These results could have important implications in the modulation of immunological and inflammatory processes by cannabinoid agents.

Key words: Anandamide; Astrocyte cultures; Lipopolysaccharide (LPS); Nitric oxide; Theiler's virus; Tumour necrosis factor-α

Introduction

Cannabinoids exert a variety of modulatory effects on the host response to immune activation and inflammation.[1–3] Δ^9-Tetrahydroxannabinol (Δ^9-THC), the major psychoactive component of *Cannabis sativa L*, has been reported to act as an immunosuppressive agent. Δ^9-THC induces alterations in the peripheral cytokine network[4,5] and also reduces nitric oxide (NO) production by macrophages.[6] The actions of Δ^9-THC are mediated by G-protein-coupled receptors[7] expressed in different cell types, including neurones and astrocytes.[8,9] Recently, the identification of an endogenous ligand, anandamide,[10] which binds and activates the central cannabinoid receptor CB1, has enormously extended the interest in the field of cannabinoid research. Anandamide (arachidonylethanolamide), a brain arachidonate derivative, has been reported to act as a transcellular cannabinimetic messenger in the CNS[11] by mediating specific effects in astrocytes.[12] Astrocytes, astrocytoma cell lines and astrocytoma tumours express CB1 receptors,[9] and primary astrocytes in culture take up and degrade anandamide to a degree similar to neurones.[8] At present, the mechanism of action of anandamide in astrocytes remains unclear, but the possibility exists that it may act as an immunomodulatory agent, as it has been suggested in the periphery.[13]

Intracerebral infection of susceptible mice strains with the Theiler's murine encephalomyelitis virus (TMEV), a member of the cardiovirus subfamily of the *Picornaviridiae*, results in an immune-mediated demyelinating disease considered as an animal model for multiple sclerosis (MS).[14] Cortical-enriched astrocyte cultures from susceptible mice (SJL/J mice strain) are efficiently infected with TMEV and produce several cytokines in response.[15] *In vitro*, astrocytes also respond to lipopolysaccharide (LPS) by inducing several cytokines and inflammatory mediators such as NO and prostaglandins.[16–18] Nitric oxide (NO) and tumour necrosis factor-α (TNF-α) are involved in various physiopathological conditions in the CNS. Increased expression of iNOS has been described in experimental autoimmune encephalomyelitis (EAE),[19] MS[20,21] and viral or bacterial infections.[22] In addition, studies in patients with MS have revealed the presence of TNF-α in specific brain lesions.[23]

The recent demonstration of anandamide binding to CB1 receptors in astrocytes raised important questions about the potential physiological and pathogenic significance of endogenous cannabinoids in mediating neuroimmunomodulation. Because NO and TNF-α play important roles in the CNS, we evaluated the effects of anandamide on the LPS or TMEV-induced production of NO and TNF-α in mouse primary astrocyte cultures.

Materials and Methods

Astrocyte cultures: Primary astrocyte cultures were prepared from the cerebral cortex of 1-day-old neonatal mice (BALB/c or SJL/J Cajal Institute, Madrid, Spain) as described in detail previously and grown in T150 flasks for at least 14 days in Dulbecco's modified Eagles medium (DMEM) and 10% heat-inactivated fetal calf serum (Whittaker, France), 20 mM glutamine (INC Biomedicals, CA) and gentamicin with the medium changed twice per week. Astrocytes cultures were maintained at 37ºC in a 5% CO_2 atmosphere. On reaching confluency (2 weeks) the cells were shaken at 240 r.p.m. overnight at 37ºC to dislodge cells adhering to the astrocyte layer (primarily oligodendrocytes and microglia). The medium was removed from the wells and cultures washed with Hanks' balanced salts (HBSS) modified solution (Sigma, St. Louis, MO). The media were replaced and the cells were allowed to recover for 2–3 days before experiments. The resulting cultures consist of > 95% astrocytes as determined by glial fibrillary acidic protein (GFAP)-positive cells and < 2% microglia based on Mac-1 staining (data not shown). All experiments were conducted in accordance with European Communities Council directive 86/609/EEC.

LPS treatment: LPS was added to astrocyte cultures from Balb/c mice at a dose of 1 μg/ml for a period of 18 h. In the experiments with anandamide, different doses (10, 50 and 100 μM) were added at the beginning of the incubation and maintained throughout the incubation period.

Virus preparation and cell culture inoculation: TMEV from a DA strain was kindly donated by Dr R. P. Roos, Department of Neurology, University of Chicago, IL. The DA strain of Theiler's virus was plaque purified four times on baby hamster kidney cells (BHK-21). Virus was titrated and stocks of 1×10^8 plaque-forming units (pfu)/ml were stored at –80ºC. TMEV was maintained at 4ºC until use, prepared in culture media and used at 1×10^5 pfu/well. Astrocytes obtained from the susceptible mouse strain SJL/J responded to TMEV infection; however, cells from non-susceptible mice strain BALB/c did not respond to TMEV stimulation. After reaching confluence, cells were washed twice with 2 ml DMEM containing 0.1% bovine serum albumine (BSA) and infected with dilutions of TMEV in DMEM plus 0.1% BSA. Infection was allowed to take place for 1 h at 37ºC in a 5% CO_2 atmosphere, and supernatants were harvested for NO_2^- and TNF-α assays 24 h post-infection. Anandamide, at doses of 10, 25 or 50 μM, was added previously to the TMEV infection and maintained throughout the 24 h post-infection period.

Nitrite assay: The production of NO was assessed by measurement of NO_2^- (an oxidation product of NO) accumulation in the cell-free supernatants. Following the incubation (18 h for astrocytes from Balb/c mice or 24 h post-infection for astrocytes from SJL/J mice) a 100 μl aliquot of the culture medium was mixed with 100 μl Griess reagent (1% sulphanilamide, 0.1% *N*-(1-naphthyl)ethylenediamine dihydrocloride, 2.5% H_3PO_4), a colourimetric indicator for the presence of NO_2^-.[25] The samples were incubated in the dark at room temperature for 10 min. The absorbance was measured at 570 nm in a microplate reader (TiterTek MultiSkan MCC). Fresh culture medium served as the blank in all experiments. Solutions of sodium nitrite ($NaNO_2$) diluted in culture medium were used as standards. The protein content was determined by a BCA Protein Assay Reagent (Pierce, IL) with bovine serum albumin as the standard.

Measurement of TNF-α: The release of TNF-α to the cultured supernatants was performed using enzyme-linked immunoadsorbent assay (Factor-Test-X Mouse TNF-α ELISA kit, Genzyme, Cambridge, MA). The detection limit was 15 pg/ml. Interassay variation was 6% and the antibodies in the kit showed no detectable cross-reactivity with other cytokines.

Materials: LPS (*Escherichia coli*, serotype 026:06B), bovine serum albumin, sulphanilamide, *N*-(1-naphthyl)ethylenediamine dihydrocloride, H_3PO_4 and $NaNO_2$ were all obtained from Sigma Chemical Co. (St. Louis, MO). Anandamide was purchased from RBI (Natick, MA).

Statistical analysis: Data are presented as mean ± s.e.m. of 4–7 independent determinations in triplicate within each experiment. Comparisons were analysed by using one-way analysis of variance (ANOVA) followed by the a posteriori Student–Newman–Keuls *t*-test. A value of $p < 0.05$ was considered significant.

Results

Effect of anandamide on NO_2^- accumulation in astrocytes stimulated with LPS or TMEV: The effect of LPS alone or in combination with anandamide on NO_2^- production by Balb/c astrocytes is shown in Fig. 1 (left panel). ANOVA of the results showed a significant effect of the treatments ($F(6,39) = 5,654$;

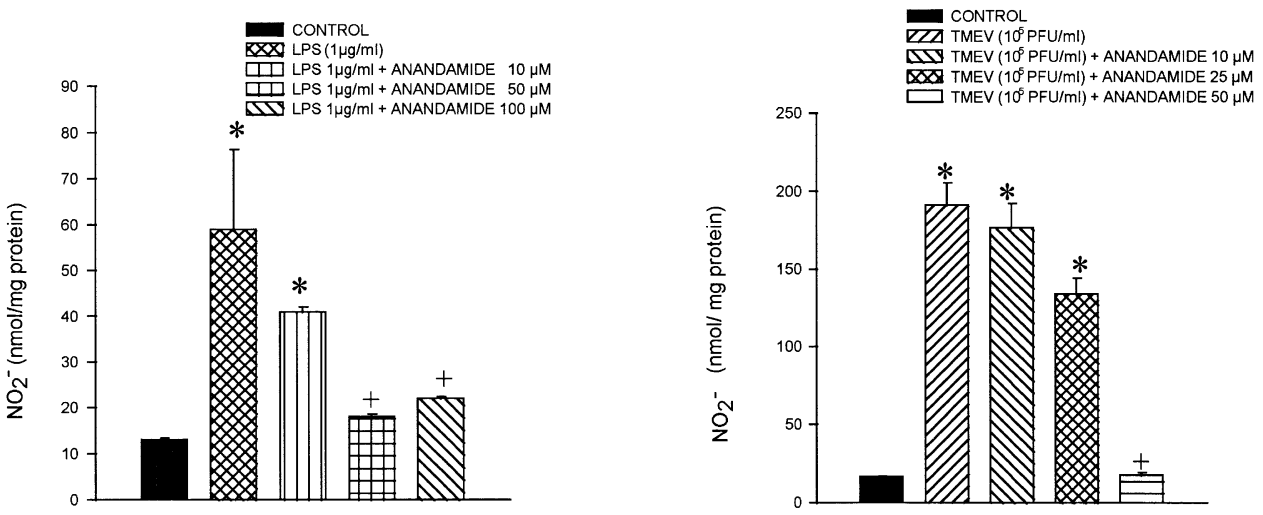

FIG 1. (Left). Effects of LPS on astrocyte NO_2^- production. Primary Balb/c mouse astrocytes were treated with medium alone (control) or medium containing LPS (1 μg/ml) in the absence or presence of anandamide. Supernatants were harvested and assay for NO_2^-. Data are mean ± s.e.m. of values from triplicate wells and are representative of seven separate experiments. *$p < 0.001$ *vs* control; +$p < 0.01$ *vs* LPS. (Right). Effects of TMEV on astrocyte NO_2^- production. Primary SJL/J mouse astrocytes were treated with medium alone (control) or medium containing TMEV (10^5 pfu/well) in the absence or presence of anandamide. Supernatants were harvested and assayed for NO_2^-. Data are mean ± s.e.m. of values from triplicate wells and are representative of four separate experiments. *$p < 0.001$ *vs* control; +$p < 0.01$ *vs* TMEV and TMEV + anandamide (10 μM and 25 μM).

$p < 0.0002$). Incubation of primary cultures of neonatal Balb/c astrocytes with LPS (1 μg/ml) for 18 h led to NO production ($p < 0.001$), as measured by increased levels of nitrites in the harvested supernatants. The basal release of NO_2^- in the control wells was very low and close to the sensitivity limit of the assay. Astrocytes incubated with anandamide alone (10, 50 and 100 μM) showed no significant variation in the amount of NO_2^- released to the culture medium (data not shown). No effect on astroglial cell viability was observed after any anandamide treatment, as assessed by the tryplan blue exclusion method. When astrocytes were exposed to LPS, the presence of anandamide reduced NO_2^- levels in the supernatants in a dose-related manner, although the dose of 50 μM was as effective as 100 μM (LPS + anandamide 10 μM: ns; LPS + anandamide 50 μM: $p < 0.010$; LPS + anandamide 100 μM: $p < 0.010$).

The effect of anandamide on nitrite accumulation by TMEV-infected astrocytes of the SJL/J strain is shown in Fig. 1 (right panel). ANOVA of the results revealed a significant effect of the treatments ($F(3,43) = 76.33$; $p < 0.0000$). When SJL/J astrocytes were infected with TMEV (10^5 pfu/well), the magnitude of NO_2^- production increased dramatically in the culture medium ($p < 0.001$). We then added anandamide at doses of 10, 25 or 50 μM to test its potential to reduce nitrite release to the culture medium. Treatment with anandamide at the doses of 25 and 50 μM reduced the accumulation of NO_2^- (TMEV + anandamide 25 μM: $p < 0.010$; TMEV + anandamide 50 μM: $p < 0.001$). The dose of 50 μM was the most effective in reducing NO_2^- accumulation after TMEV infection, and almost completely abolished the ability of TMEV to induced nitrite release from astrocytes.

Effect of anandamide on TNF-α release from astrocytes stimulated with LPS or TMEV: Effect of LPS alone or in combination with anandamide upon TNF-α release from Balb/c astrocytes is shown in Table 1. ANOVA of the results revealed a significant effect of the treatments ($F(3,24) = 16.848$; $p < 0.0001$). LPS significantly stimulated TNF-α release ($p < 0.01$) compared with basal secretion. Pretreatment with anandamide reduced the effect of LPS on TNF-α release (LPS + anandamide 10 μM: 16%; LPS + anandamide 50 μM: 43%).

The effect of TMEV alone or in combination with anandamide on TNF-α production is shown in Fig. 2. ANOVA of the results revealed a significant effect of the treatments ($F(3,23) = 29.71$; $p < 0.0001$). Inoculation of primary cultures of SJL/J astrocytes with TMEV (10^5 pfu/well) produced a significant

Table 1. Effects of anandamide on TNF-α release by astrocytes stimulated with LPS

Treatment	TNF-α release (pg/ml)
Control	46.57 ± 2.91
LPS	849.60 ± 86.3***
LPS + anandamide 10 μM	713.66 ± 63.7***
LPS + anandamide 50 μM	484.20 ± 45.0***+

Data expressed as mean ± s.e.m. of 4–6 independent experiments in triplicate. ***$p < 0.001$ *vs* control; +$p < 0.01$ *vs* LPS alone or LPS + anandamide 10 μM.

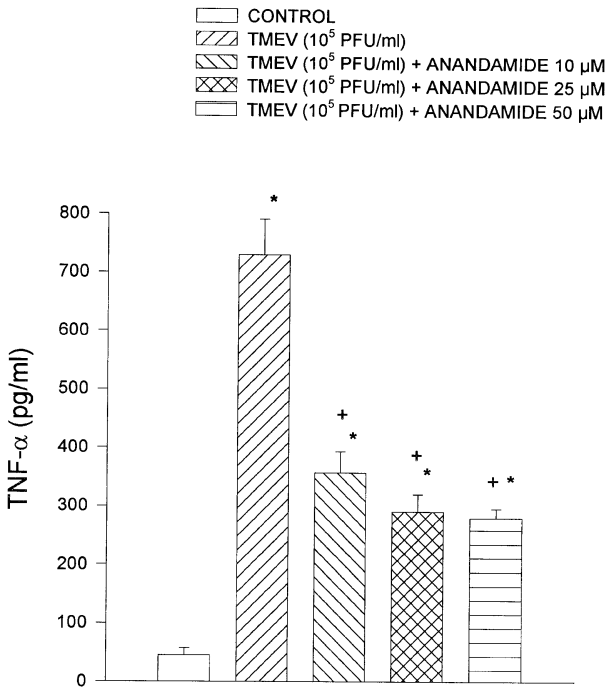

FIG. 2. Effects of TMEV on astrocyte TNF-α production. Primary SJL/J mouse astrocytes were treated with medium alone (control) or medium containing TMEV (10^5 pfu/well) in the absence or presence of anandamide. Supernatants were harvested and assayed for TNF-α. Data are mean ± s.e.m. of values from triplicate wells and are representative of four separate experiments. *$p < 0.01$ *vs* control; +$p < 0.01$ *vs* TMEV.

increase of TNF-α release ($p < 0.01$). Treatment of TMEV-infected astrocytes with anandamide at different doses (10, 25 and 50 μM) dose-dependently inhibited the release of TNF-α in the cell free medium culture (TMEV + anandamide 10 μM: $p < 0.01$; TMEV + anandamide 25 μM: $p < 0.01$; TMEV + anandamide 50 μM: $p < 0.01$).

Discussion

This study describes for the first time the ability of anandamide, an endogenous cannabinoid receptor agonist, to modify astrocyte responsiveness to endotoxin treatment (LPS) or TMEV infection. Stimulation of primary astrocyte cultures with LPS or TMEV strongly increased the production of important CNS signaling mediators such as NO and TNF-α. Anandamide treatment inhibited the LPS or TMEV-induced production of NO and TNF-α by astrocytes, in a dose-dependent manner. Both NO and TNF-α have been identified in neurological diseases such as MS[20,22] and EAE.[19] Over-induction of NO synthase has been frequently associated with brain pathology and research during the past years suggests that NO may contribute to the pathogenesis of the above disorders.[21,25] Administration of inhibitors of NO synthase has been shown to reduce brain inflammation in mice with EAE.[26] In addition, TNF-α has been postulated to be an important effector in immune-mediated demyelination.[27]

The results of the present study are consistent with reports that Δ^9-THC, an exogenous ligand for the cannabinoid receptor, impairs nitrite release by LPS-stimulated macrophage and mastocytoma cell cultures.[6] Binding of anandamide to CB1 receptors in astrocytes[8] represents one of the putative mechanisms by which this compound could induce NO suppression. Although the signal transduction pathway of anandamide on astrocytes is unknown, the involvement of the nuclear transcription factor NF-κB, has been considered critical for Δ^9-THC induced inhibition of iNOS in LPS-stimulated macrophages.[28] This transcription factor acts as regulator of many genes related to immune and inflammatory responses, including iNOS.[29]

It is also interesting that Δ^9-THC had inhibitory effects on the LPS or LPS+IFNγ-induced secretion of TNF-α in different types of cells, including macrophage cell lines such as RAW264.7.[3,4] The mechanisms responsible for these effects have not been not established, but in LPS-stimulated peritoneal macrophages the suppression of TNF-α release has been associated with the inhibition of tyrosine phosphorylation (p77 and p82) by Δ^9-THC.[3]

Reduction of NO and TNF-α production by astrocytes following anandamide treatment may contribute to the recovery in diseases such as EAE or MS, emphasizing the importance of the endogenous cannabinoids as modulators of immune reactivity in the brain. Exogenous cannabinoids ameliorate EAE,[1,2] but the central mechanisms involved are unknown. This study presents the first evidence for a role of anandamide in the modulation of TNF-α and NO production by stimulated-astrocytes. The mechanisms by which anandamide exerts the above effects in astrocytes require further investigation.

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General Summary
Anandamide, an endogenous ligand for central cannabinoid receptors, markedly attenuated *in vitro* astrocyte responsiveness to endotoxin treatment or viral (Theiler's murine encephalomyelitis virus; TMEV) infection. Our data show that the stimulated production of nitric oxide (NO) and tumour necrosis factor-α (TNF-α) following lipopolysaccharide or TMEV exposure is suppressed by anandamide treatment. Because NO and TNF-α are involved in various physiopathological changes in the CNS, the inhibitory effects of anandamide may have physiological relevance in neurological disorders with an immunological component.