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Research Paper

A synthetic cannabinoid, CP55940, inhibits lipopolysaccharide-induced cytokine mRNA expression in a cannabinoid receptor-independent mechanism in rat cerebellar granule cells

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Abstract

Objectives The inflammatory response plays an important role in the pathogenesis of many diseases in the central nervous system. Cannabinoids exhibit diverse pharmacological actions including anti-inflammatory activity. In this study, we tried to elucidate possible effects of cannabinoids on lipopolysaccharide (LPS)-induced expression of inflammatory cytokine mRNAs in rat cerebellar granule cells.

Methods Inhibitory effects of cannabinoids on cytokine induction in cerebellar granule cells were determined by RT-PCR method.

Key findings In these cells, both mRNA and protein of cannabinoid receptor 1 (CB_1), but not CB_2, were expressed. LPS (1 μg/ml) produced a marked increase in the induction of inflammatory cytokines, including interleukin-1β, interleukin-6 and tumour necrosis factor-α. CP55940, a synthetic cannabinoid analogue, concentration-dependently inhibited inflammatory cytokine expression induced by LPS. On the other hand, the endocannabinoids 2-arachidonoylglycerol and anandamide were not able to inhibit this inflammatory response. Notably, a CB_1/CB_2 antagonist NESS0327 (3 μM) did not reverse the inhibition of cytokine mRNA expression induced by CP55940. GPR55, a putative novel cannabinoid receptor, mRNA was also expressed in cerebellar granule cells. Although it has been suggested that G_q associates with GPR55, cannabinoids including CP55940 did not promote phosphoinositide hydrolysis and consequent elevation of intracellular $Ca^{[2+]}$ concentration. Furthermore, a putative GPR55 antagonist, cannabidiol, also showed a similar inhibitory effect to that of CP55940.

Conclusions These results suggest that the synthetic cannabinoid CP55940 negatively modulates cytokine mRNA expression in cerebellar granule cells by a CB and GPR55 receptor-independent mechanism.

Keywords cannabinoid; cerebellar granule cells; CP55940; cytokine; inflammation

Introduction

Cannabinoids, which include the bioactive constituents of the marijuana plant *Cannabis sativa* and its synthetic or endogenous counterparts, modulate a range of central nervous system (CNS) functions and affect peripheral sites such as immune function and the cardiovascular system.[1,2] Several endogenous cannabinoid ligands have been isolated from the CNS, including anandamide[3] and 2-arachidonoylglycerol (2-AG).[4,5] To date, two classical cannabinoid receptors have been identified, namely cannabinoid receptor type 1 (CB_1)[6] and cannabinoid receptor type 2 (CB_2).[7] CB_1 is expressed mainly in the nervous system, especially the brain,[6] and has been assumed to play an important role in the attenuation of synaptic transmission.[2,8] Conversely, CB_2 is expressed mainly in various lymphoid tissues[7] and has been postulated to take part in the regulation of inflammatory reactions and immune responses.[2,8] Both cannabinoid receptors are coupled with pertussis toxin (PTX)-sensitive $G_{i/o}$-proteins.[2] One of the most widely studied cannabinoids is CP55940, a high-affinity synthetic ligand (K_i values of 0.58 nM and 0.68 nM for CB_1 and CB_2, respectively) with potent biological effects that are mediated through binding to both CB_1 and CB_2.[9] These receptors affect the activity of intracellular signalling molecules (e.g. inhibition of adenylyl cyclase, activation of mitogen-activated protein kinase, inhibition of voltage-dependent $Ca^{[2+]}$ channels, activation of K^+ channels, activation of focal adhesion kinase, activation of

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phospholipase A_2 and activation or inhibition of nitric oxide synthase).[10] In addition to CB_1 and CB_2 receptors, an orphan G-protein-coupled receptor, GPR55, was recently identified as a putative novel cannabinoid receptor[11,12] and it has been reported that CP55940 can bind and activate GPR55 (EC_{50} 5 nM).[11] It has been demonstrated that GPR55 activation by cannabinoids increases intracellular $Ca^{[2+]}$ concentration ($[Ca^{[2+]}]_i$) by activating signalling pathways quite distinct from CB_1 and CB_2.[13] However, there have been conflicting reports that CP55940 is not an agonist for GPR55[13,14] or is rather a competitive antagonist.[15,16] Despite the existence of these specific cannabinoid receptors, it is currently recognized that cannabinoids exert their effects through receptor-dependent or independent mechanisms.[17]

Cytokines, including interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α), play a crucial role in mediating inflammatory and immune responses and facilitate communication between immune and nervous systems.[18] Inflammation in the CNS is a key feature associated with neurodegenerative disorders, including multiple sclerosis, Alzheimer's disease and AIDS-related dementia.[19] There is substantial evidence from in-vitro studies that cannabinoid receptor agonists exert anti-inflammatory effects by inhibiting pro-inflammatory cytokine expression[20–23] or increasing anti-inflammatory cytokine release.[24] Pre-clinical[25] and clinical[26] studies also demonstrated the immunosuppressive effects of cannabinoid receptor activation. Furthermore, studies of the effects of cannabinoids on immune function have focused on responses in glial cells, such as astrocytes[21] and microglia,[20,22,27] and peripheral immune cells, such as T-cells[28] and macrophages,[29]; studies in primary cultured neurons are relatively lacking.

The purpose of this study is to determine the role of cannabinoids in the lipopolysaccharide (LPS)-induced cytokine expression in primary cultures of rat cerebellar granule cells (CGCs). Here, we show that a synthetic cannabinoid, CP55940, can negatively modulate inflammatory cytokine expression induced by LPS in CGCs. In addition, the inhibitory effect of CP55940 is not likely to be mediated by CB_1, CB_2 or GPR55 cannabinoid receptors and we propose the novel mechanism that CP55940 and cannabidiol suppress cytokine induction by their anti-oxidative activity.

Materials and Methods

Materials

CP55940, Ro106-9920, WIN55212-2, abnormal cannabidiol and cannabidiol were purchased from Tocris Bioscience (Ellisville, MO, USA). Anandamide was purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA). 2-AG was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). NESS0327, papain-latex, anti-CB_1 receptor antibody, Ro20-1724, lysophosphatidylinositol (LPI) and pyrrolidinethiocarbamate (PDTC) were purchased from Sigma-Aldrich (St Louis, MO, USA). Lipopolysaccharide from *Escherichia coli* and carbachol were purchased from Wako Pure Chemicals (Osaka, Japan). Neurobasal A and B-27 were purchased from Invitrogen (Grand Island, NY, USA). [^{3}H]Adenine and rat IL-1β ELISA kit were purchased from GE Healthcare Biosciences (Little Chalfont, UK). [^{14}C]cAMP was purchased from Moravek Biochemicals, Inc (Brea, CA, USA). SYBR Premix Ex Taq (Perfect Real Time) was purchased from Takara Bio, Inc. (Otsu, Japan). [^{3}H]Inositol (23.4 Ci/mmol) was purchased from DuPont/NEM (Boston, MA, USA). The anion exchange column (AG1X8) was purchased from Bio-Rad Laboratories (Hercules, CA, USA). All other chemicals were of reagent grade or the highest quality available.

Preparation of primary cultured cerebellar granule cells

Primary cultures of rat CGCs were prepared from 7-day-old postnatal Sprague–Dawley rats (Charles-River Japan, Yokohama, Japan) as described previously.[30] Briefly, cerebellums were dissociated with 200 U papain-latex (Sigma-Aldrich) in dissociation medium (composition in mM: 81.8 Na_2SO_4, 30 K_2SO_4, 0.25 $CaCl_2$, 8 HEPES, 20 glucose, 12 $MgCl_2$, pH 7.5), then the cells were plated at 2×10^6 cells/well (6-well plate), 1×10^6 cells/well (12-well plate) or 0.5×10^6 cells/well (24-well plate) and maintained in Neurobasal A (Invitrogen) containing 25 mM KCl, 1% L-glutamine, 2% B-27, 50 µg/ml streptomycin and 50 U/ml penicillin at 37°C in humidified 95% air/5% CO_2. All plates were coated with poly-D-lysine (100 µg/ml) (Sigma-Aldrich). Cytosine arabinoside was added on DIV 1 at a final concentration of 5 µM to prevent the proliferation of mitotic cells, such as glial cells, and those cells were not observed as assessed through morphologic inspection. CGCs were used for experiments on DIV 4–7.

Reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA was isolated from CGCs using the TRI Reagent (Sigma-Aldrich), according to the manufacturer's protocol. Total RNA (1 µg) was reverse-transcribed using ReverTraAce (Toyobo, Osaka, Japan) and the oligo (dT) primer. Primer sequences used were as follows: CB_1 receptor sense primer 5′-ATA AGA GGA TCG TCA CCA GG-3′ and antisense primer 5′-AGT TCA GCA GGC AGA GCA TA-3′ (498 bp product); CB_2 receptor sense primer 5′-AAG CCC TCG TAC CTG TTC AT-3′ and antisense primer 5′-AGG CAC AGC ATG GAG CAG AA-3′ (668 bp product); β-actin sense primer 5′-AGG GAA ATC GTG CGT GAC AT-3′ and antisense primer 5′-TCC TGC TTG CTG ATC CAC AT-3′ (467 bp product); TNF-α sense primer 5′-ACT GAA CTT CGG GGT GAT TG-3′ and antisense primer 5′-GTG GGT GAG GAG CAC GTA GT-3′ (319 bp product); IL-6 sense primer 5′-GAG GAG ACT TCA CAG AGG AT-3′ and antisense primer 5′-TCC TTA GCC ACT CCT TCT GT-3′ (465 bp product); IL-1β sense primer 5′-CAG GAA GGC AGT GTC ACT CA-3 and antisense primer 5′-GGG ATT TTG TCG TTG CTT GT-3′ (339 bp product); GPR55 sense primer 5′-CTC CCT CCC ATT CAA GAT GA-3′ and antisense primer 5′-AAG ATC TCC AGG GGG AAG AA-3′ (342 bp product). PCR products were separated by electrophoresis through an agarose gel and stained with ethidium bromide. An image of each gel was digitally captured using FASIII (Toyobo). For the quantitation of mRNA expression, real-time PCR was carried out in a 20 µl solution containing SYBER Premix Ex Taq (10 µl), RT template (3 µl), water (6 µl) and primers (1 µl) using the DNA engine Opticon

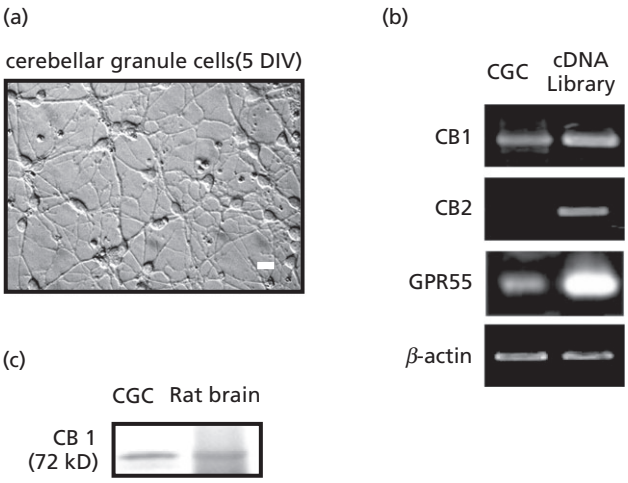

Figure 1 Expression of CB_1 receptors and GPR55 in rat cerebellar granule cells (CGCs). (a) Picture of CGC cultures at DIV5. Cells dissociated from 7-day-old rat cerebellums were grown on poly-D-lysine-coated plastic plates (bar = 10 μm). (b) The total RNA was extracted from CGCs, and RT-PCR was performed using a primer mixture corresponding to rat CB_1, CB_2 or rat GPR55. β-Actin was used as a control gene. (c) CB_1 protein expression in CGCs and brain lysate was determined by Western blotting.

System (MJ Research, Waltham, MA, USA). We did not observe any changes in β-actin mRNA expression following LPS treatment only (data not shown). The amount of each PCR product was normalized to that of β-actin, and expressed as a percentage change relative to the LPS treatment.

Measurement of interleukin-1β by enzyme-linked immunosorbent assay (ELISA)

CGCs were seeded at 1.0×10^6 (cells/well) in a 24-well plate and grown for 4 days. CGCs were treated with LPS in the presence of appropriate concentrations of CP55940, and the incubation medium was collected after centrifugation to remove floating cells. Detection was performed by incubating the plates, coated with anti-rat IL-1β antibody in the presence of a biotinylated anti-rat IL-1β antibody and streptavidin-linked peroxidase conjugate. After incubation, tetramethylbenzidine was used as substrate to quantify the amount of bound conjugate by colorimetric measurement (SUNRISE; TECAN Group Ltd, Maennedorf, Switzerland).

Western blotting

CGCs were lysed in Laemmli buffer (75 mM Tris-HCl, 2% SDS, 10% glycerol, 3% 2-mercaptoethanol, 0.003% bromophenol blue) and separated by electrophoresis (applied voltage, 80 V/gel) on a 10% polyacrylamide gel. Proteins were transferred onto a polyvinylidene difluoride membrane (GE Healthcare Biosciences) using a semi-dry blotting method. The blots were incubated with anti-CB_1 receptor antibody (1 : 250 dilution) overnight at 4°C. After several washes, the blots were incubated with secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG antibody). Blots were developed using enhanced chemiluminescence on Hyperfilm (Hyperfilm ECL; GE Healthcare Biosciences).

Measurement of cyclic adenosine monophosphate (cAMP) levels

Intracellular cAMP levels were measured using the following protocol. CGCs on 24 well-plates were labelled with 2 μCi/ml of [^{3}H]adenine (23 Ci/mmol), which was added to the growth medium for 3 h, then the labelled cells were washed twice with NeurobasalA-10 mM HEPES (37°C, pH 7.4). The cells were pretreated with selective CB_1/CB_2 receptor antagonist, NESS0327 (3 μM) for 15 min in the DMEM-HEPES containing phosphodiesterase inhibitor, Ro20-1724 (100 μM). Then, after incubation with CP55940 for 15 min, the cells were further stimulated with forskolin for 15 min. After aspirating the incubation buffer, the reactions were terminated by adding 0.4 ml of 2.5% $HClO_4$ containing [^{14}C]cAMP. Acid-extracts were mixed with 1/10 volume of 4.2 M KOH to neutralize the acid, forming potassium perchlorate as a precipitate. [^{3}H]cAMP in the supernatant was separated by Dowex 50W-X8/alumina double columns, and the radioactivity was quantified by liquid scintillation counting. After normalizing the column elution efficiency by measuring the radioactivity of [^{14}C]cAMP, the increase in [^{3}H]cAMP levels was expressed as the percentage of the control (no drug).

Measurement of intracellular calcium concentration ($[Ca^{[2+]}]_i$)

$[Ca^{[2+]}]_i$ levels were measured by monitoring the intensity of Fura2 fluorescence. CGCs were washed with modified Tyrode's solution (composition in mM: 137 NaCl, 2.7 KCl, 1.0 $MgCl_2$, 0.18 $CaCl_2$, 10 HEPES, 5.6 glucose, pH 7.4). Then CGCs were loaded with 1 μM Fura2/AM for 30 min at 37°C. After buffer washing, the cells were stimulated with drugs diluted in buffer containing 1 mM $Ca.^{[2+]}$ $[Ca^{[2+]}]_i$ was measured using a $Ca^{[2+]}$ imaging system (Aquacosmos 2.0; Hamamatsu Photonics, Hamamatsu, Japan) and fluorescence microscope (Olympus IX70; Olympus, Tokyo, Japan). Fura2 fluorescence at 510 nm was monitored with excitation at 340 and 380 nm, and expressed as a ratio (340/380 nm).

Measurement of total inositol phosphates

The accumulation of total inositol phosphates was measured as follows: CGCs cultivated in 12-well plates were incubated in medium containing [^{3}H]inositol (2 μCi/ml) overnight. After washing twice with serum-free EMEM-HEPES buffer (pH 7.4), the cells were preincubated in EMEM-HEPES containing LiCl (10 mM) for 10 min, before incubating with drugs for additional 10 min. The reaction was terminated by aspirating the medium and replacing it with 1 ml of ice-cold 5% trichloroacetic acid. Trichloroacetic acid extracts were washed three times with diethylether to remove all traces of trichloroacetic acid and then were incubated at 47°C for 30 min to remove all traces of diethylether. Total [^{3}H] inositol phosphates were separated in an anion exchange column (AG-1X8, formate form, 100–200 mesh), and radioactivity was quantified by liquid scintillation counting as previously described.[31]

Statistical analysis

Data are expressed as the mean ± SEM Significant differences were analysed using Mann–Whitney's *U*-test,

Dunnett's or Tukey–Kramer's multiple comparison tests. The level of significance was set at $P < 0.05$.

Results

It has been reported that majority of the cannabinoids, including CP55940, bind to CB_1 and CB_2 receptors.[32] Hence, the mRNA expression of these receptors was examined in rat CGCs (Figure 1a) using RT-PCR method (Figure 1b). Although CB_2 mRNA expression was not detected in our model, the expression of CB_1 receptor mRNA was observed in rat CGCs (Figure 1b). In addition, it was confirmed that CB_1 protein was expressed in CGCs (Figure 1c). An orphan G-protein-coupled receptor, GPR55, was recently identified as a putative novel cannabinoid receptor that binds to certain cannabinoids including CP55940.[11] For this reason, we examined the expression of GPR55 by RT-PCR and we showed that GPR55 mRNA was detected in rat CGCs (Figure 1b).

To determine whether cytokine expression could be affected by CP55940, we examined mRNA expression of typical inflammatory cytokines including IL-1β, IL-6 and TNF-α. CGCs were pretreated with CP55940 (0.3–10 μM) for 15 min, then stimulated for additional 4 h with LPS (1 μg/ml). CP55940 concentration-dependently inhibited LPS-induced cytokine mRNA expression (Figure 2). IL-1β protein secretion by LPS (1 μg/ml) was also blocked by CP55940 in a concentration-dependent manner (data not shown).

Since CP55940 inhibited the LPS-induced cytokine expression concentration dependently, we examined whether this suppression of cytokine expression in CGCs was observed in response to the endocannabinoids anandamide

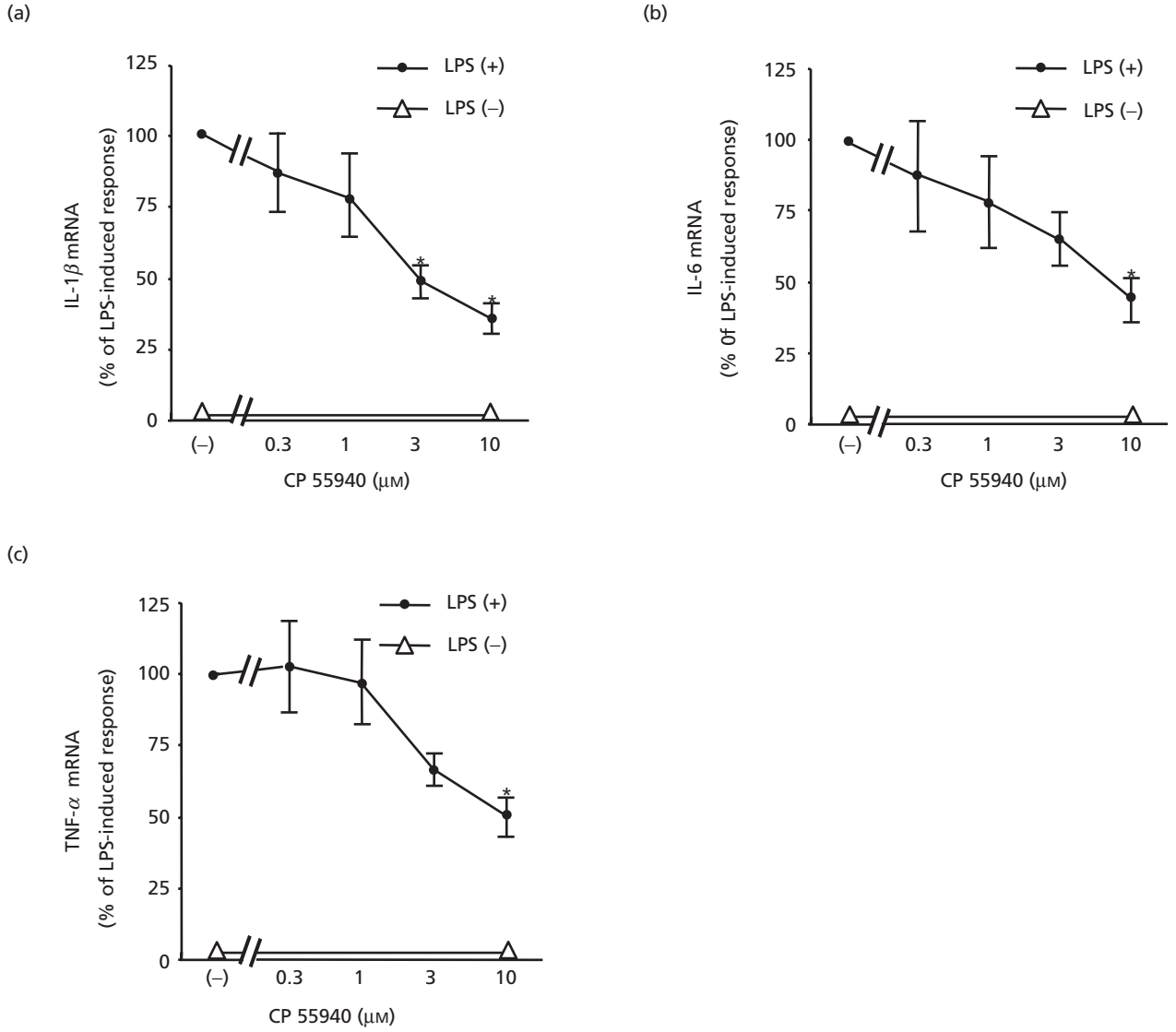

Figure 2 CP55940 inhibits cytokine mRNA expression. Rat cerebellar granule cells (CGCs) were pretreated with the indicated concentrations of CP55940 for 15 min before stimulation with 1 μg/ml lipopolysaccharide for 4 h. The mRNA levels of IL-1β (a), IL-6 (b) and TNF-α (c) were determined by RT-PCR. The data represent the means ± SEM from 3–7 experiments. *$P < 0.05$ vs control.

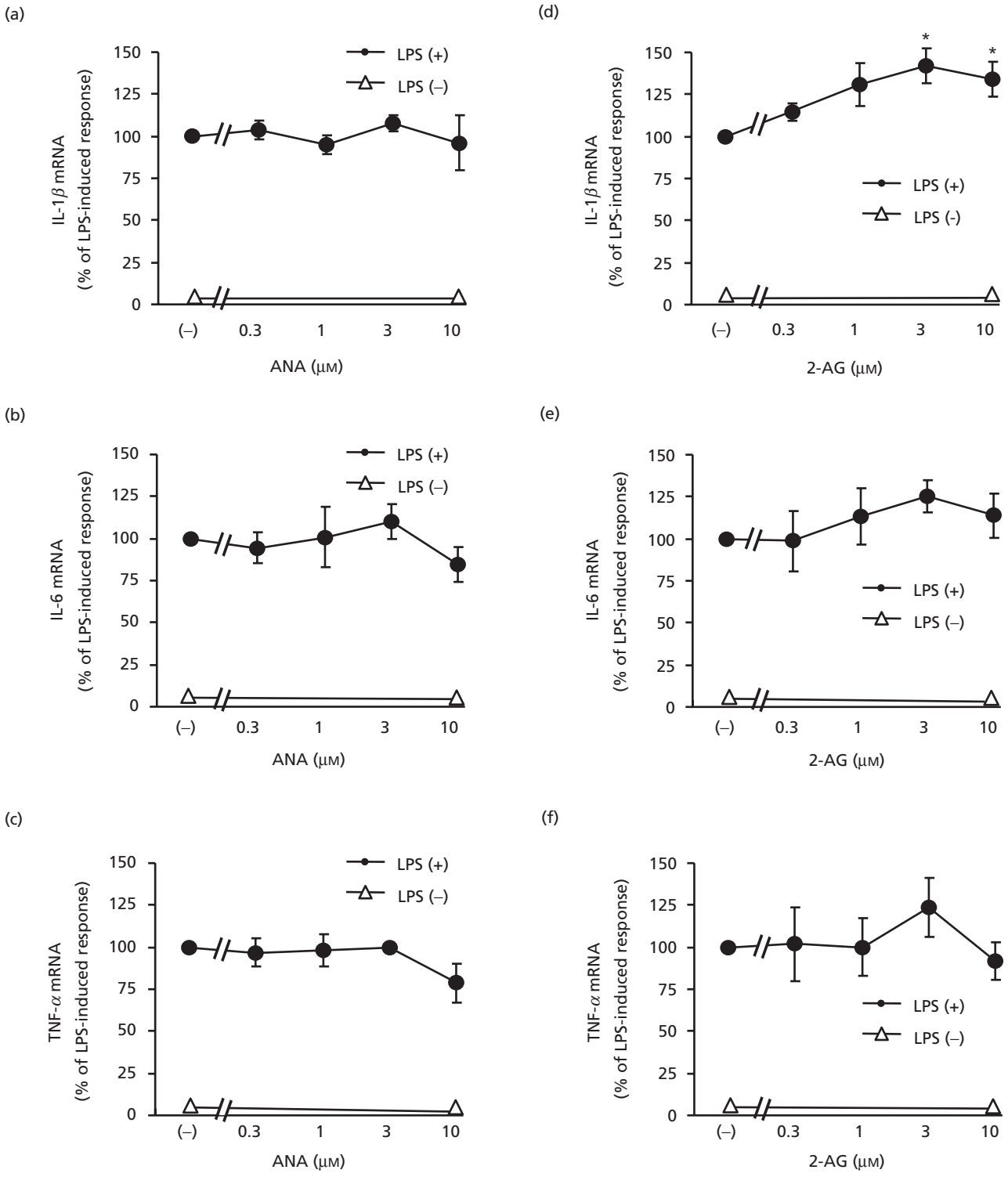

Figure 3 Endocannabinoids do not inhibit the lipopolysaccharide (LPS)-induced mRNA expression of cytokines. Rat cerebellar granule cells (CGCs) were pretreated with the indicated concentrations of anandamide (ANA) and 2-AG for 15 min before stimulation with 1 μg/ml LPS for 4 h. The mRNA levels of IL-1β (a, d), IL-6 (b, e) and TNF-α (c, f) were determined by RT-PCR. The data represent the means ± SEM from 3–6 experiments. *$P < 0.05$ vs control.

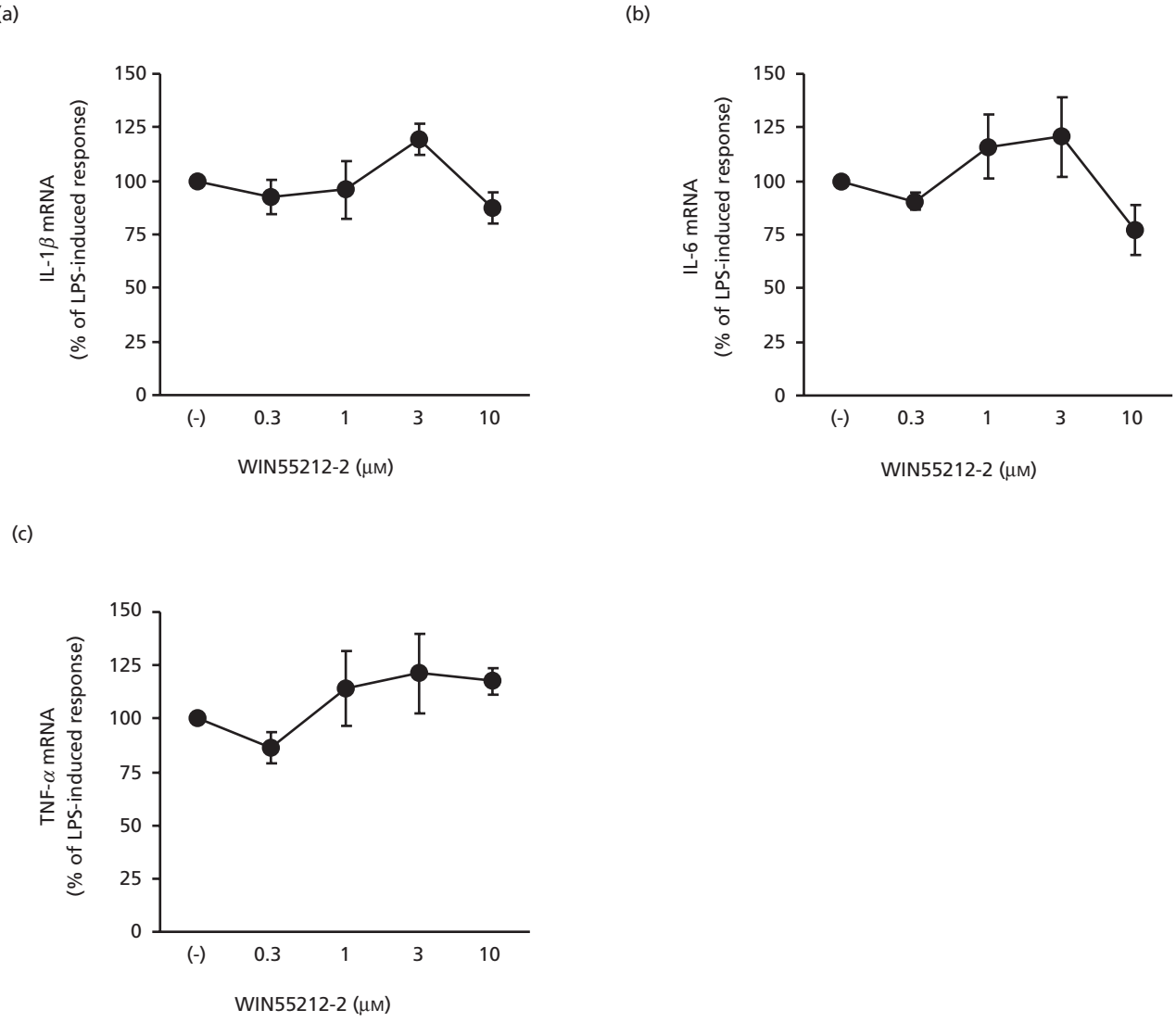

Figure 4 Synthetic cannabinoid WIN55212-2 does not inhibit lipopolysaccharide (LPS)-induced cytokine mRNA expression. Rat cerebellar granule cells (CGCs) were pretreated with the indicated concentrations of WIN55212-2 for 15 min before stimulation with 1 μg/ml LPS for 4 h. The mRNA levels of IL-1β (a), IL-6 (b) and TNF-α (c) were determined by RT-PCR. The data represent the means ± SEM from 6 experiments.

and 2-AG. After CGCs were exposed to anandamide (0.3–10 μM) and 2-AG (0.3–10 μM) for 15 min, the cells were treated for 4 h with LPS (1 μg/ml), and total RNA was harvested for RT-PCR. Unlike CP55940, 2-AG and anandamide were not able to inhibit the expression of the examined inflammatory cytokines (Figure 3), although LPS-induced IL-1β mRNA expression was increased by 3 μM and 10 μM of 2-AG (Figure 3d). The application of 10 μM of 2-AG and anandamide without LPS did not affect expression of cytokines. In addition, we examined the effect of another synthetic cannabinoid, WIN55212-2, on LPS-induced cytokine expression. As a result, WIN55212-2 (0.3–10 μM) also failed to inhibit cytokine expression in response to LPS (Figure 4).

Next, we examined whether the inhibitory effect of CP55940 on LPS-induced cytokine expression in CGCs was mediated by the CB_1 receptor. For this purpose, cells were treated with NESS0327, a potent CB_1 and CB_2 receptor antagonist.[33,34] NESS0327 (3 μM) did not reverse the inhibition of cytokine mRNA expression induced by CP55940 (Figure 5a–c), suggesting that CP55940 inhibited LPS-induced cytokine mRNA expression in a manner independent of CB_1 and CB_2. We also examined the effects of CP55940 (10 μM) and NESS0327 (3 μM) on the elevation of intracellular cAMP levels induced by forskolin (10 μM), an adenylyl cyclase activator. CP55940 inhibited the forskolin-mediated increase in intracellular cAMP levels, and this inhibitory effect was completely reversed by NESS0327 (Figure 5d).

In addition to CB_1 and CB_2, GPR55 has been identified as a putative novel cannabinoid receptor.[11,13,35] It was demonstrated that LPI was a potent GPR55 ligand using HEK293 cells stably overexpressing GPR55.[14] Moreover, it has been shown that LPI induced ERK1/2 phosphorylation and elevation of intracellular $Ca^{[2+]}$ is mediated by GPR55.[14] However,

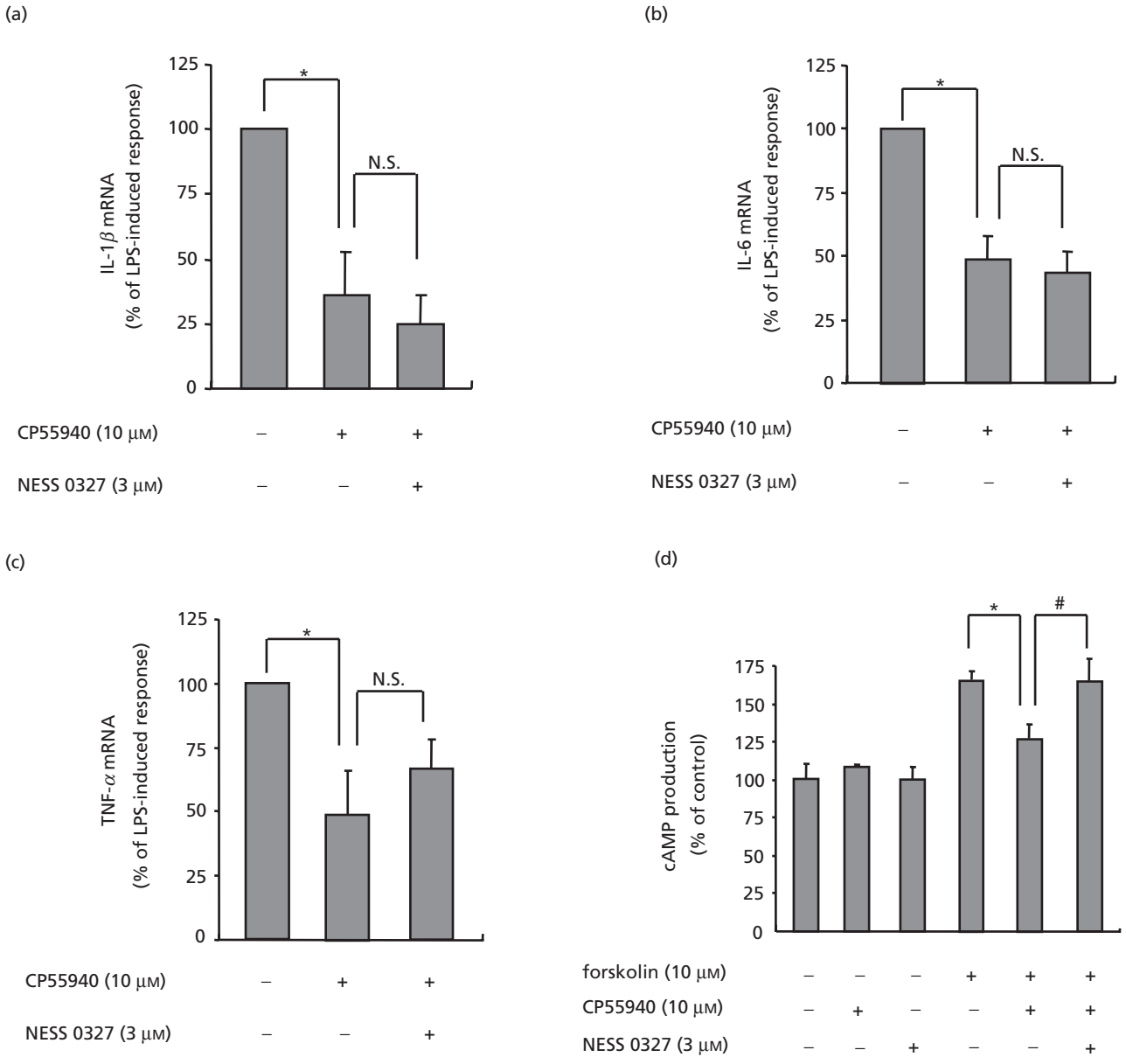

Figure 5 Inhibition of the lipopolysaccharide (LPS)-induced cytokine mRNA expression by CP55940 is not through the CB_1 receptor. Rat cerebellar granule cells (CGCs) were pretreated with 3 μM NESS0327 for 15 min and then incubated with or without 10 μM CP55940 for 15 min before further stimulation with 1 μg/ml LPS for 4 h. The mRNA levels of IL-1β (a), IL-6 (b) and TNF-α (c) were examined by RT-PCR. The data represent the means ± SEM from 5 experiments. *$P < 0.05$ vs control. N.S., not statistically significant. (d) CGCs (DIV5) were labelled with 2 μCi/ml [^{3}H]adenine for 2 h. After the cells (DIV5) were pretreated with 3 μM NESS0327 for 15 min, the cells were incubated with or without 10 μM CP55940 for 15 min before further stimulation with 10 μM forskolin for 15 min in the present of 100 μM Ro20-1724. cAMP production was expressed as percentage of control. The data represent the means ± SEM from 3 experiments. *$P < 0.05$, forskolin alone vs forskolin plus CP55940. #$P < 0.05$ forskolin plus CP55940 vs forskolin plus CP55940 and NESS0327.

conflicting results also have been reported that CP55940 is not an agonist for GPR55[13,14] or is rather a competitive antagonist.[15,16] In CGCs, GPR55 mRNA was detected (Figure 1b) therefore it was assumed that GPR55 protein is also expressed in CGCs and that the $G_{q/11}$-mediated phosphoinositide hydrolysis is promoted upon stimulation with a cannabinoid such as CP55940, which binds to GPR55. We measured the phosphoinositide hydrolysis by stimulating CGCs with cannabinoids and carbachol, a muscarinic receptor agonist, as a positive control. Although carbachol (100 μM, 10 min) promoted phosphoinositide hydrolysis, any of the employed cannabinoids (10 μM each, 10 min) produced significant effects in this parameter (Figure 6a). To investigate $G_{q/11}$-mediated signalling in CGCs in more detail, we measured $[Ca^{[2+]}]_i$ in CGCs. However, CP55940 (10 μM) did not significantly increase $[Ca^{[2+]}]_i$ (Figure 6b).

Next, we further examined whether the inhibitory effect of CP55940 on LPS-induced cytokine expression was mediated

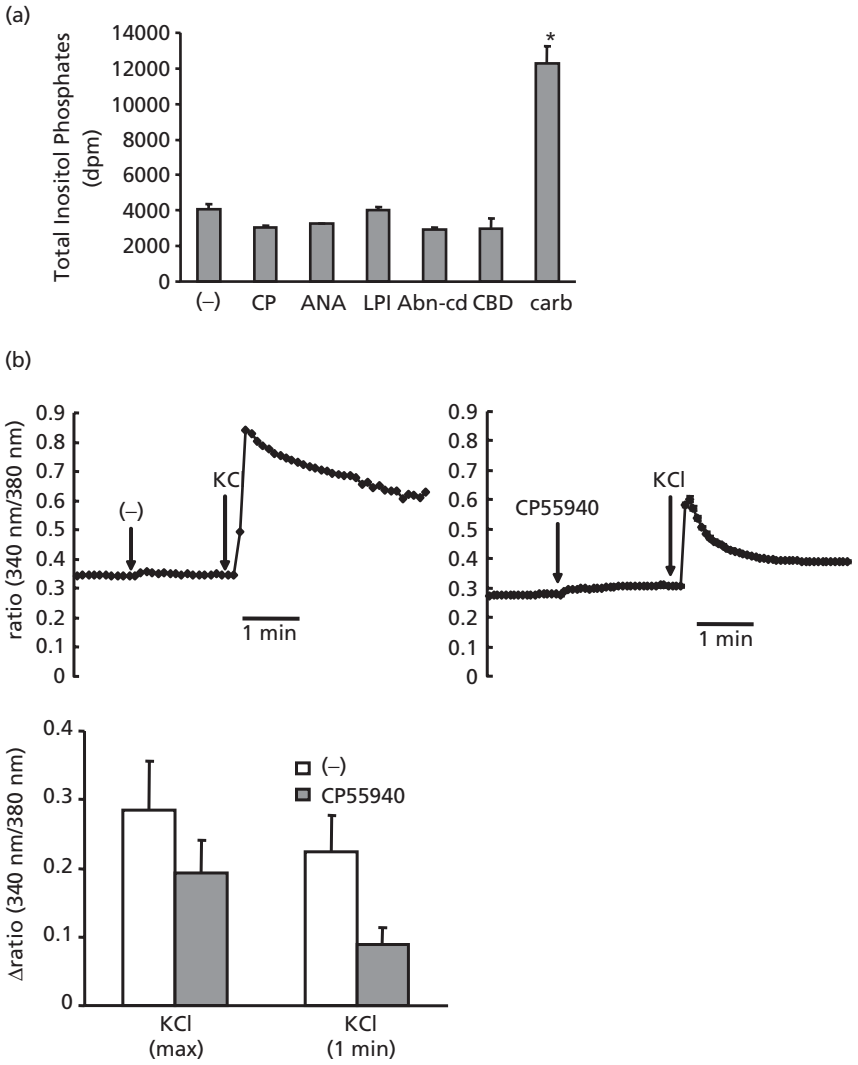

Figure 6 Functional GPR55 protein is not expressed in rat cerebellar granule cells (CGCs). (a) After labelling with 2 μCi/ml [^{3}H]inositol for 24 h, CGCs were treated with various cannabinoids (10 μM each) or carbachol (100 μM) for 10 min. Then, total inositol phosphates were measured as described in Materials and Methods. CP, CP55940; ANA, anandamide; LPI, lysophosphatidylinositol; Abn-cd, abnormal cannabidiol; CBD, cannabidiol; carb, carbachol. (b) After the cells loaded with Fura-2/AM were preincubated in the presence or absence of CP55940 (10 μM) at 37°C for 2 min, these cells were treated with KCl (25 mM). (Upper) A typical trace of 340 nm/380 nm ($[Ca^{[2+]}]_i$). (Lower) ratio of 340 nm/380 nm. The data represent the means ± SEM from 3–4 experiments. *$P < 0.05$ vs control.

by GPR55. For this purpose, cells were treated with cannabidiol, a GPR55 antagonist.[11] Unexpectedly, cannabidiol (50 μM) alone inhibited LPS-induced cytokine mRNA expression (Figure 7). Because LPS-induced cytokine expression is mediated by reactive oxygen species[36] and both CP55940 and cannabidiol show anti-oxidative activity,[37,38] we investigated the anti-oxidative effects on LPS-induced cytokine expression. The cells were treated with PDTC, an anti-oxidative agent. PDTC (100 μM) inhibited LPS-induced cytokine expression (Figure 8).

Discussion

We found that a synthetic cannabinoid, CP55940, negatively modulated cytokine mRNA expression in CGCs via a receptor-independent mechanism and this possibly resulted from its anti-oxidative activity.

We investigated the expression of CB receptors in rat CGCs using the RT-PCR and Western blotting methods. This study clearly showed CB_1 receptors, but not CB_2 receptors, were expressed in CGCs (Figure 1b and 1c). This result was consistent with previous reports that CB_1 is highly expressed in the CNS, including the cerebellum, but CB_2 is dominantly expressed in the immune system.[39] Moreover, this study has shown for the first time that CP55940 modulates LPS-induced cytokine expression in CGCs.

LPS induced a marked induction of inflammatory cytokines including IL-1β, IL-6 and TNF-α in CGCs. Addition of CP55940 caused a concentration-dependent inhibition of the expression of these inflammatory cytokines (Figure 2).

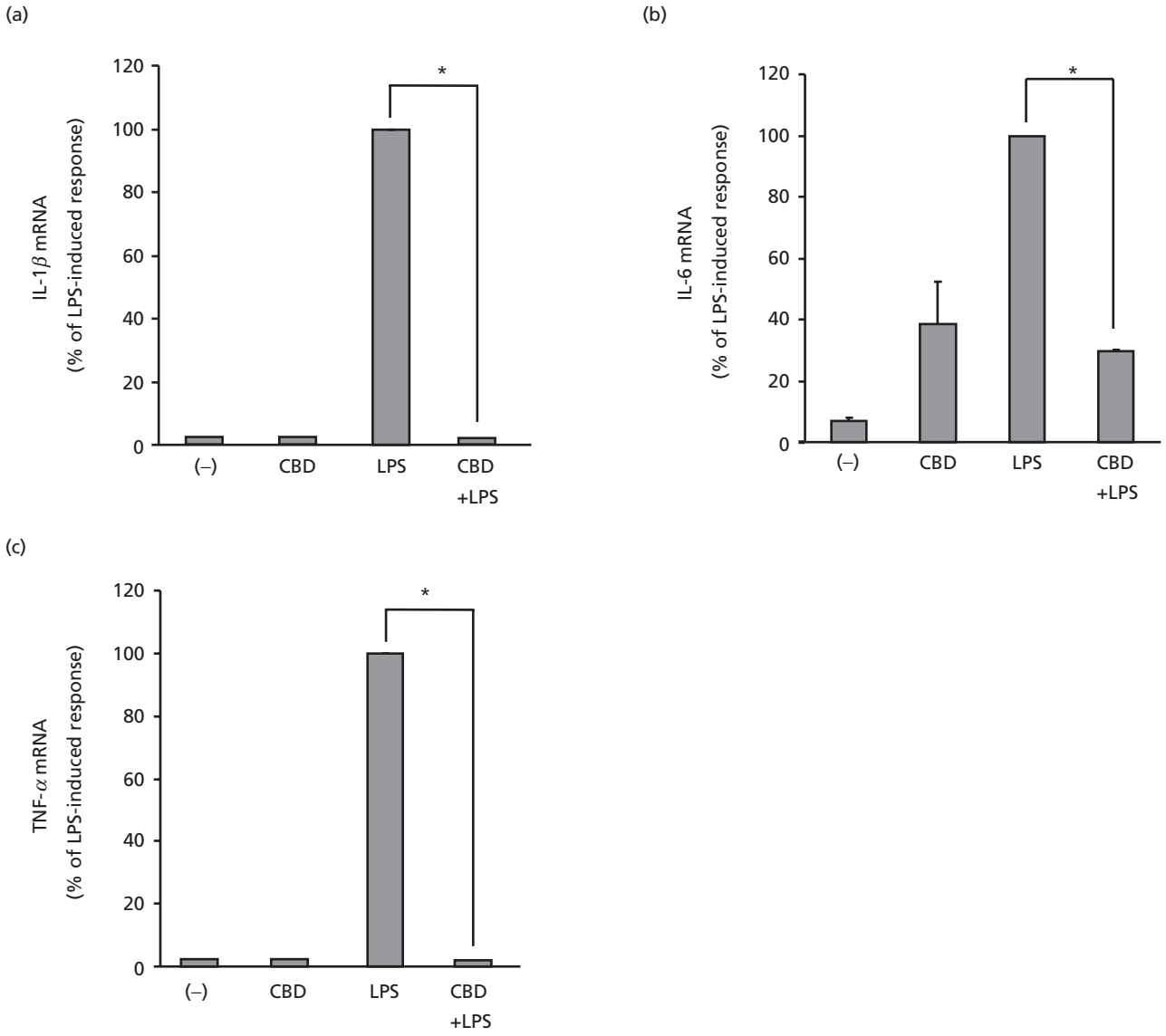

Figure 7 GPR55 antagonist inhibits lipopolysaccharide (LPS)-induced cytokine mRNA expression. After pretreatment with cannabidiol (CBD) (50 μM) for 30 min, rat cerebellar granule cells (CGCs) were stimulated with LPS (3 μg/ml) for 4 h. The mRNA levels of IL-1β (a), IL-6 (b), and TNF-α (c) were examined by RT-PCR. The data represent the means ± SEM from 3 experiments. *$P < 0.05$ vs control.

Notably, unlike CP55940, endocannabinoids including anandamide and 2-AG were not able to inhibit the inflammatory cytokine expression (Figure 3). In this study, although anandamide did not affect the LPS-induced cytokine expression, 2-AG (3 μM and 10 μM) enhanced the LPS-induced IL-1β mRNA expression (Figure 3). This result suggests that 2-AG rather promoted the inflammatory responses. It has been widely recognized that arachidonic acid is involved in inflammatory cascades, and 2-AG can rapidly be metabolized to arachidonic acid,[40] which can be a trigger for inflammatory responses. Indeed, 2-AG induced ear swelling, and this swelling was completely blocked by cannabinoids in mice.[41] Moreover, another synthetic cannabinoid, WIN55212-2, did not inhibit the inflammatory cytokine expression (Figure 4a–c). Unlike CP55940 and △9-tetrahydrocannabinol (THC), WIN55212-2 is an aminoalkylindole[42] and is structurally dissimilar. Because CB_1 or CB_2 agonists from a different class to CP55940 did not show the effect, the hypothesis that CB_1 or CB_2 does not mediate inhibition of cytokine expression has been strengthened.

In addition, we examined whether the inhibitory effect of CP55940 on the LPS-induced cytokine expression in CGCs was through the CB receptor by treating cells with NESS0327, a CB receptor antagonist. NESS0327 did not reverse the inhibition of cytokine mRNA expression by CP55940 (Figure 5a–c). Moreover, NESS0327 alone did not affect cytokine expression (data not shown). Because CP55940 also activates CB_2 in addition to CB_1, the possibility was raised that CB_2 is involved in the effect of CP55940. However, the CB_2 receptor was not expressed in CGCs determined by RT-PCR method (Figure 1b). Also, although NESS0327 (3 μM) that we used in this study blocks both

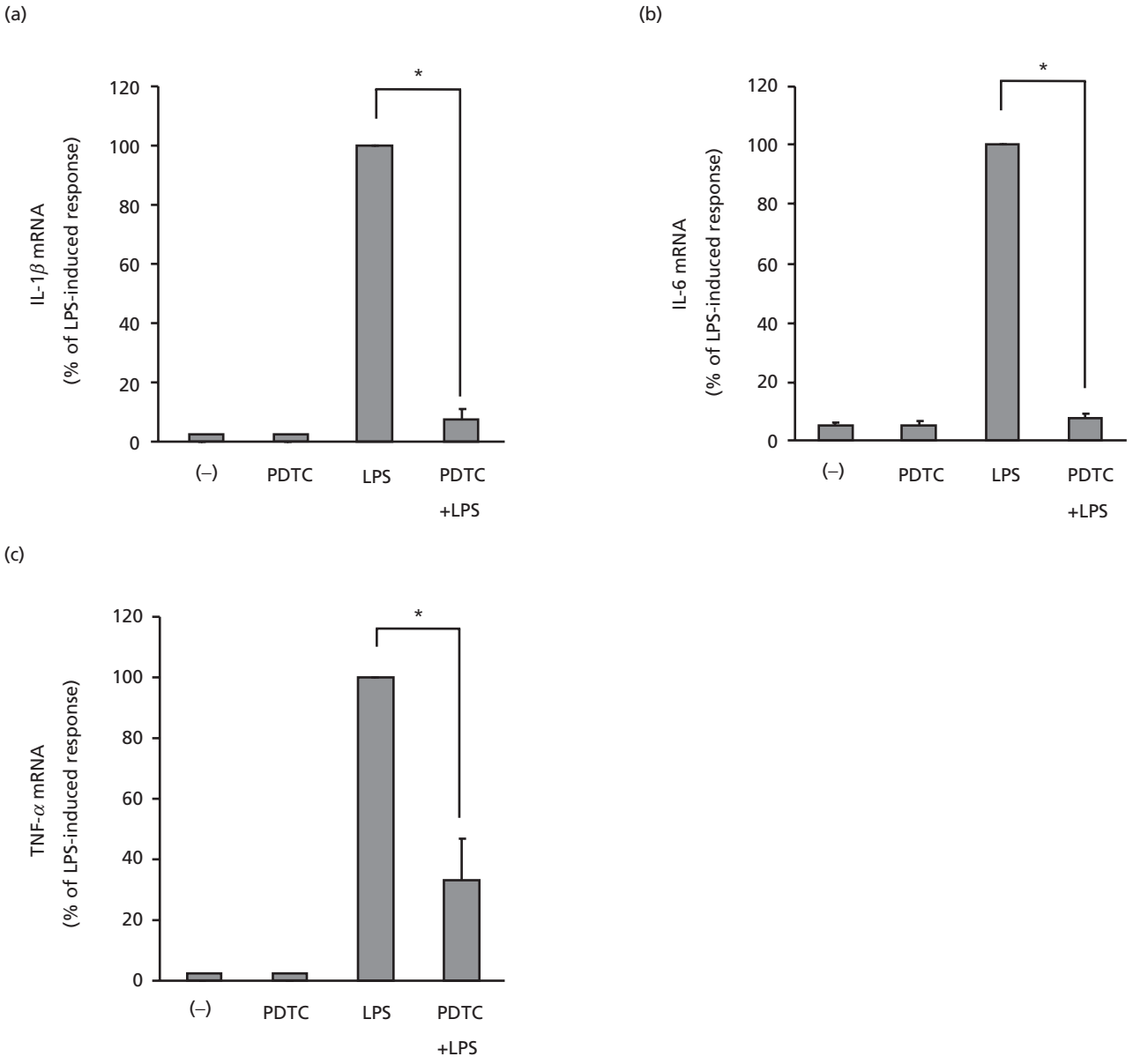

Figure 8 Pyrrolidinethiocarbamate (PDTC) inhibits lipopolysaccharide (LPS)-induced cytokine expression. After pretreatment with PDTC (100 μM) for 1 h, rat cerebellar granule cells (CGCs) were stimulated with LPS (3 μg/ml) for 4 h. The mRNA levels of IL-1β (a), IL-6 (b) and TNF-α (c) were examined by RT-PCR. The data represent the means ± SEM from 5 experiments. *$P < 0.05$ vs control.

CB_1 and CB_2,[33,34] it did not reverse the effect of CP55940 (Figure 5). It is widely known that CB_1 receptors are coupled with the $G_{i/o}$ family of G proteins, through which they control cAMP production by inhibiting adenylyl cyclase activity.[43] We examined the effect of CP55940 and NESS0327 on the elevation of intracellular cAMP levels induced by forskolin, an adenylyl cyclase activator. CP55940 inhibited the forskolin-elevated intracellular cAMP levels, and this inhibitory effect was completely reversed by NESS0327 (Figure 5d), indicating NESS0327 blocked CB_1 and CB_2 efficiently. Because a CB_1 and CB_2 antagonist did not reverse the inhibitory effect of CP55940 and other CB_1 agonists, including anandamide, 2-AG and WIN55212-2, did not suppress cytokine induction, we concluded that CB_1 receptors were not involved in the inhibition of the LPS-induced cytokine induction.

Alternatively, CP55940 may have bound to unidentified CB receptors. It has long been discussed that unidentified CB receptors exist besides the CB_1 and CB_2 receptors, and one of these receptors is GPR55.[11,13,35] It was clearly demonstrated that GPR55 was activated by cannabinoid ligands including Δ^9-THC, CP55940, 2-AG, anandamide and LPI.[11,14] Moreover, it has been shown that LPI induced ERK1/2 phosphorylation, elevation of intracellular $Ca^{[2+]}$ and RhoA activation through GPR55.[14,15] These studies suggest that GPR55 is coupling with the $G_{q/11}$ and G_{13}. In contrast, conflicting results

also have been reported that CP55940 is not an agonist for GPR55[13,14] or is rather a competitive antagonist.[15,16] For this reason, we examined the expression of GPR55 by RT-PCR in rat CGCs (Figure 1b). In addition, we measured the phosphoinositide hydrolysis by stimulating CGCs with cannabinoids and carbachol, a muscarinic receptor agonist, as a positive control. Although carbachol promoted phosphoinositide hydrolysis, no cannabinoids did (Figure 6a). To investigate $G_{q/11}$-mediated signalling in CGCs in more detail, we measured $[Ca^{[2+]}]_i$ in CGCs, but CP55940 did not increase $[Ca^{[2+]}]_i$ (Figure 6b). These results suggest at least three possibilities: (1) functional GPR55 protein is not expressed in CGCs; (2) GPR55 is not functionally coupled with $G_{q/11}$ signaling in CGCs or (3) CP55940 is not a GPR55 agonist. Thus, it is suggested that GPR55 is not involved in inhibition of the LPS-induced cytokine expression by CP55940. In addition, we carried out further experiments using cannabidiol, a putative GPR55 antagonist[11] in CGCs, and because cannabidiol itself clearly blocked LPS-induced cytokine expression in CGCs (Figure 7), it was impossible to determine whether GPR55 is involved in inhibition of cytokine expression by CP55940 using cannibidiol. As another possibility, TRPV1, a non-selective ion channel, is a target of certain cannabinoids.[44] However, since it has been confirmed that CP55940 does not bind to TRPV1,[45] the possibility of the involvement of this channel is excluded.

There is a possibility that CP55940 inhibits nuclear factor (NF)-κB activation as a part of mechanism of inhibition of cytokine expression action by CP55940. For instance, it has been reported that anandamide inhibits LPS-induced NF-κB activation and controls the production of IL-6, IL-8, and MCP-1 in human gum fibroblasts.[46] In our preliminary experiments, the LPS-induced cytokine expression in CGCs was inhibited by Ro106-9920, which blocks NF-κB activation (Chiba *et al*., unpublished observation). LPS-induced NF-κB activation involved reactive oxygen species,[36] and it is assumed that the drugs that have an anti-oxidative effect, such as PDTC, can inhibit the activation of NF-κB.[47] Indeed, CP55940 has anti-oxidative activity but not anandamide.[38] Furthermore, cannabidiol, which mimicked CP55940 in this study, also showed an anti-oxidative activity.[37] To investigate the anti-oxidative effects on LPS-induced cytokine expression, cells were treated with PDTC, an anti-oxidative agent. PDTC (100 μM) inhibited LPS-induced cytokine expression (Figure 8), suggesting the anti-oxidative effects as a part of mechanism of inhibition of cytokine expression by CP55940. Therefore, in this study, it is supposed that CP55940 inhibits the cytokine expression by suppressing the reactive oxygen species-dependent activation of NF-κB.

Conclusion

Our results show that the synthetic cannabinoid, CP55940, inhibits the LPS-induced cytokine mRNA expression in CGCs, while endocannabinoids are not able to inhibit the cytokine expression. Furthermore, the inhibitory effect of CP55940 on the LPS-induced inflammatory cytokine expression does not involve CB_1, CB_2 or GPR55. It has been thought that cannabinoids could be useful as anticancer agents.[48] Furthermore, in the CNS, pro-inflammatory cytokine production has been associated with neurodegenerative disorders, including multiple sclerosis, Alzheimer's disease and AIDS-related dementia. Elucidation of the fundamental physiology of neuro–immune interactions by cannabinoids and the immunosuppressive properties of cannabinoids will provide information to promote the development of these compounds for the treatment of neuro-inflammatory disorders.

Acknowledgements

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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