Differential changes in GPR55 during microglial cell activation

Maciej Pietr¹, Ewa Kozela¹, Rivka Levy¹, Neta Rimmerman¹, Yi Hsing Lin¹,b,c, Nephi Stella¹,b,c, Zvi Vogel a,d,*, Ana Juknâte

¹ Neurobiology Department, Weizmann Institute of Science, Rehovot 76100, Israel
² Department of Pharmacology, University of Washington, Seattle, WA, USA
³ Department of Psychiatry and Behavioral Sciences, University of Washington, Seattle, WA, USA
⁴ The Dr. Miriam and Sheldon G. Adelson Center for the Biology of Addictive Diseases, Sackler Faculty of Medicine, Tel Aviv University, Israel

ABSTRACT

We examined how lipopolysaccharide (LPS) and interferon gamma (IFN-γ), known to differentially activate microglia, affect the expression of G protein-coupled receptor 55 (GPR55), a novel cannabinoid receptor. We found that GPR55 mRNA is significantly expressed in both primary mouse microglia and the BV-2 mouse microglial cell line, and that LPS down-regulates this message. Conversely, IFN-γ slightly decreases GPR55 mRNA in primary microglia, while it upregulates this message in BV-2 cells. Moreover, the GPR55 agonist, lysophosphatidylinositol, increases ERK phosphorylation in BV-2 stimulated with IFN-γ, in correlation with the increased amount of GPR55 mRNA. Remarkably, these stimuli-induced changes in GPR55 expression are similar to those observed with CB2-R, suggesting that both receptors might be involved in neuroinflammation and that their expression is concomitantly controlled by the state of microglial activation.

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1. Introduction

To date, two cannabinoid receptors belonging to the G protein-coupled receptor family have been identified. These include the CB1 receptor (CB1-R), mostly located in neural cells, and the CB2 receptor (CB2-R), predominantly expressed by immune cells but also present in the CNS during neuroinflammatory states [1]. However, there is growing evidence that a number of pharmacological and physiological effects produced by cannabinoids cannot be fully explained by the actions of these two cannabinoid receptor subtypes [2–5]. Recently, an orphan G protein-coupled receptor 55 (GPR55) cloned several years ago [6], has emerged as a candidate for mediating some of the non-CB1/non-CB2 receptor subtypes [2–5]. Several cannabinoids were shown to possess anti-inflammatory activities in various in vivo and in vitro inflammatory models [1,14]. These effects are mostly due to cannabinoid binding to the CB2–R, which is abundantly expressed by macrophages, dendritic cells and B cells [15]. In several cases the CB1-R has also been implicated [1]. However, some of the anti-inflammatory actions of cannabinoids are independent of CB1-R and CB2-R. Specifically, studies using CB1–R−/− and CB2–R−/− mice support the existence of additional cannabinoid targets in immune cells [14]. Thus, numerous cannabinoids were shown to inhibit lipopolysaccharide (LPS)-induced mRNA expression of several inflammatory cytokines in rat microglia and this effect was not blocked by either CB1-R or CB2-R antagonists [16]. Finally, studies on neuropathic and inflammatory pain employing GPR55−/− mice indicate that GPR55 regulates the production of various cytokines, thus resulting in blunted inflammatory mechanical hyperalgesia [17].

Microglial cells are the resident innate immune cells in the brain, and are responsible for controlling CNS infections and cells. Furthermore, various species of L-α-lysophosphatidylinositol (LPI) including the arachidonoyl, stearoyl and palmitoyl species elicit fast Ca2+ transients, as well as rapid phosphorylation of the extracellular signal-regulated kinase (ERK) [13]. Thus, there is broad agreement that LPI is a GPR55 agonist that could act as its endogenous ligand [5,8,13].

Abbreviations: FCS, fetal calf serum; GPR55, G protein-coupled receptor 55; IFN-γ, interferon gamma; LPI, lysophosphatidylinositol; LPS, lipopolysaccharide; qPCR, quantitative real time polymerase chain reaction
* Corresponding author; Address: Neurobiology Department, Weizmann Institute of Science, Rehovot 76100, Israel. Fax: +972 8 9344131.
E-mail address: zvi.vogel@weizmann.ac.il (Z. Vogel).

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promoting tissue repair [18]. However, overactivation of microglial cells has been implicated as an active contributor to the development of several neurodegenerative diseases, including multiple sclerosis and Alzheimer’s diseases [19,20]. Microglial cells can be differentially activated by distinct stimuli that couple to independent signaling pathways, such as the bacterial inflammatory endotoxin LPS and interferon gamma (IFN-γ) [21].

While the presence of functional CB2-R in microglia is under debate, CB2-R expression by microglia in culture is well accepted [22–24]. The level of CB2-R expression can be up- or downregulated by certain pathogens and cytokines [22,24–26]. Exposure to LPS decreases the expression of CB2-R at both mRNA and protein levels [22]. Conversely, IFN-γ alone does not significantly affect CB2-R expression in primed microglia [22,26]. However, treatment of the microglial cell line BV-2 with this cytokine significantly upregulates CB2-R expression [28]. It is therefore of interest to study the regulation of GPR55 expression and its functionality when microglia are activated by these two different stimuli.

In this study, we report that primary mouse microglia and the BV-2 microglial cell line express GPR55, and that this receptor is differentially modulated by microglial activation. In parallel to CB2-R, GPR55 mRNA expression is downregulated by LPS while GPR55 mRNA is upregulated by IFN-γ activation, but only in BV-2 cells and not in microglial cells in primary culture.

2. Materials and methods

2.1. Materials

LPS from Escherichia coli serotype 055:B5 was from Sigma (St. Louis, MO, USA); L-α-LPI from soybean was from Avanti Polar Lipids Inc. (Alabama, AL, USA). It contains primarily the palmitoyl and stearoyl LPI molecular species. Primers for mouse CB1-R (NM_009807), for mouse CB2-R (NM_009924) and for the mouse homolog of GPR55 (Mm_Gm218; NM_001033290) were designed by Qiagen (Hilden, Germany). The following primers were used for mouse β-actin, forward: GAAATCGTGCGT and reverse: AGGAAGGCTGGAAAAGAGCCT; and for mouse β-microglobulin, forward: ATGGGAAACCGCAACATCTG and reverse: CAGTCTCAGTGGGTTGAAT.

2.2. Cell cultures

Mouse microglia in primary culture were prepared as previously described [29]. The BV-2 cell line, kindly provided by Prof. E.J. Choi (Korea University, Seoul, Korea), was cultured in high glucose DMEM (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 5% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin under a humidified 5% CO2 atmosphere at 37 °C. The N18TG2 mouse neuroblastoma cell line was grown as described [4].

2.3. Isolation of total RNA and reverse transcription

RNA was extracted using the Versagene RNA purification kit (Gentra, Minneapolis, MN, USA) and RNA samples were reverse transcribed using the QuantiTect Reverse Transcription Kit from Qiagen (Hilden, Germany) including DNase treatment of contaminating genomic DNA.

2.4. Quantitative real time polymerase chain reaction (qPCR)

Expression of CB2-R and GPR55 mRNAs were determined by qPCR, using β-actin or β-microglobulin as normalizing genes, as previously described [30]. Normal and mock reversed transcribed samples (in the absence of reverse transcriptase), as well as no template controls (total mix without cDNA) were run for each of the examined mRNA’s. qPCR reactions were subjected to an initial step of 15 min at 95 °C to activate the HotStar Taq DNA polymerase, followed by 40 cycles consisting of 15 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C. Fluorescence was measured at the end of each elongation step. Efficiency and the threshold cycle value Ct (calculated from the exponential phase of each PCR sample) were obtained using the Rotor-Gene software (Corbett Life Sciences, Australia). Amounts of mRNAs were calculated and expressed in relative units of SYBR Green fluorescence, using the Pfaffl mathematical model [31].

2.5. ERK phosphorylation

BV-2 cells were stimulated with recombinant IFN-γ (Peprotech, Rocky Hill, NY, USA) for 18 h under low serum conditions (0.1% FCS). Cells were then incubated for 10 min with LPI and lysed in RIPA buffer as described [32]. Protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). Proteins in lysates were separated by 8% SDS–PAGE, electrophoretically transferred to nitrocellulose membranes and immunoblotted with anti pERK or anti ERK1/2 from Sigma. Blots were developed with horseradish peroxidase-conjugated antibodies (Jackson Immuno-Research Laboratories, West Grove, PA, USA) and visualized using the super-signal-enhanced chemiluminescence assay (Pierce, Rockford, IL, USA).

2.6. Data analysis

Data were collected from three or more independent experiments and are expressed as mean ± S.E.M. The statistical analysis of the data was accomplished with one- or two-way factor analysis of variance (ANOVA), followed by Bonferroni post hoc multiple comparison test.

3. Results

3.1. Microglial cells express GPR55 receptors

In our first set of experiments, we measured the level of CB1 and CB2 receptor mRNA by qPCR analysis. We found a very low level of CB2-R mRNA in both primary microglia (Ct of 36) and BV-2 cells (Ct of 34; Fig. 1A). Indeed, when comparing these levels to the level of CB2-R mRNA present in the murine neuroblastoma cell line N18TG2 (which is known to contain high levels of CB2-R; [33]) we found that the neuroblastoma cell line (Ct of 26) expresses approximately 10 000-fold higher levels than microglia. It is worth noting that in BV-2 cells this very small amount of CB2-R mRNA was not significantly affected by either LPS or IFN-γ treatments (data not shown).

In contrast to the very low abundance of CB2-R, we found that both CB2-R and GPR55 mRNAs were present at relatively high level in unstimulated primary and in BV-2 microglial cells (Fig. 1B and C). Specifically, qPCR analysis of CB2-R mRNA in BV-2 and primary microglia resulted in Ct’s of 25.5 and 27, respectively, and of GPR55 mRNA in Ct’s of 28 and 31.5, respectively (Fig. 1B and C). Note that the levels of GPR55 mRNA are only 8- and 30-fold lower than those found for CB2-R mRNA.

3.2. LPS and IFN-γ modulate GPR55 mRNA expression in primary microglial cells

Microglia cells were treated with LPS (100 ng/ml) for 8 h or IFN-γ (200 U/ml) for 18 h. Total RNA was extracted, reverse-transcribed and the cDNA subjected to qPCR analysis. The mRNA for β-micro-
globulin, which was not affected by either of the treatments, was used for data normalization.

These treatments significantly affected the amount of GPR55 mRNA \( F(2, 8) = 62.2; P < 0.01 \). Specifically, LPS reduced GPR55 mRNA levels by 87% \( (P < 0.01) \), whereas IFN-\( \gamma \) induced a lower, but significant, reduction of 45% in GPR55 mRNA \( (P < 0.01) \); Fig. 2). Remarkably, the expression of CB2-R mRNA was concomitantly affected by both treatments \( F(2, 8) = 109.8; P < 0.01 \), since LPS reduced CB2-R mRNA expression by 90% \( (P < 0.05) \) and IFN-\( \gamma \) by 25% \( (P < 0.05) \); Fig. 2).

3.3. LPS downregulates and IFN-\( \gamma \) upregulates GPR55 mRNA expression levels in BV-2 cells

We then compared the regulation of GPR55 and CB2-R mRNA expression in BV-2 cells after treatment with LPS and IFN-\( \gamma \). The cells were treated with either LPS (1, 10 and 100 ng/ml) for 4 and 8 h or IFN-\( \gamma \) (50, 100 and 200 U/ml) for 8 and 18 h. The amount of GPR55 mRNA was markedly downregulated after 4 and 8 h of LPS treatment. This downregulation was dose- \( F(1, 27) = 132; P < 0.01 \) but not time-dependent \( F(1, 27) = 0.09; P = 0.8 \).
Again, a concomittant downregulation of CB2-R was found since 1 ng/ml LPS reduced this receptor’s mRNA by 62% at 4 h and at 8 h and 100 ng/ml LPS reduced its mRNA by 89% \( (P<0.01; \text{Fig. 3A}) \). Note that the LPS-induced downregulation of GPR55 mRNA was already maximal after 4 h of treatment. These results prompted us to study the time dependency of LPS-induced downregulation of mRNA for both receptors in more detail. Fig. 3B shows the expression of CB2-R and GPR55 mRNAs as a function of time in the presence of 10 ng/ml LPS. It shows that the reduction in CB2-R mRNA expression is faster than that of GPR55, with a 50% decrease in CB2-R mRNA expression after 1 h, while more than 2 h were needed to reach the same reduction in GPR55 mRNA levels.

Conversely, the addition of IFN-γ resulted in a dose- \( [F(3, 27) = 21.4; P<0.01] \) and time-dependent \( [F(1, 27) = 5.5; P<0.05] \) upregulation of the expression of GPR55 mRNA. This upregulation reached a 3-fold increase after 8 h treatment \( (P<0.01) \) and 4.5-fold increase after 18 h treatment \( (P<0.01) \) with 200 U/ml IFN-γ (Fig. 4). The expression of CB2-R mRNA was similarly upregulated in a dose- \( [F(3, 31) = 36; P<0.01] \) and time-dependent \( [F(1, 31) = 41; P<0.01] \) manner and reached a 4-fold increase \( (P<0.01) \) after 8 h and a 6-fold increase \( (P<0.01) \) after 18 h with 200 U/ml IFN-γ.

### 3.4. LPI induces a dose-dependent phosphorylation of ERK1/2 in GPR55-upregulated BV-2 cells

BV-2 cells were incubated for 18 h in the presence or absence of 200 U/ml IFN-γ. LPI was then added for 10 min at 1, 5 and 10 µM, and the level of ERK1/2 phosphorylation determined by Western blotting analysis. Fig. 5 shows that LPI stimulates ERK phosphorylation. While in the absence of IFN-γ the stimulation by LPI was relatively small, in IFN-γ-stimulated cells LPI induced a marked dose-dependent phosphorylation of ERK. Both ERK1 and ERK2 were similarly phosphorylated. It is interesting to note that IFN-γ by itself (in the absence of LPI) also induced ERK1/2 phosphorylation, as previously shown in RAW 264.7 cells [34]. These effects of IFN-γ and LPI were significant since two-way ANOVA analysis of these data resulted in \( F(1, 27) = 10.12; P<0.01 \) for IFN-γ and \( F(3, 23) = 62.1; P<0.01 \) for LPI dose. Moreover, IFN-γ treatment and LPI dose showed a significant statistical interaction \( F(3, 27) = 3.11; P=0.05 \).

In a parallel experiment, BV-2 cells were incubated for 4 h in the presence or absence of 100 ng/ml LPS, followed by 10 min incubation with or without LPI. However, LPS by itself induced a very

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(Fig. 3A), with GPR55 mRNA levels reduced by as much as 92% \( (P<0.01) \) at 8 h with 100 ng/ml LPS. A lower, but significant, reduction of 70% in GPR55 mRNA was observed with concentrations as low as 1 ng/ml of LPS after either 4 h or 8 h incubation \( (P<0.01) \).
strong phosphorylation of ERK1/2, which prevented the determination of the effect of reducing GPR55 expression on ERK activity (data not shown).

4. Discussion

Recent studies show that various cannabinoids applied to GPR55-transfected HEK293 cells stimulate GTP\(\gamma\)S binding as well as increase the release of calcium from IP\(_3\) receptor-dependent stores [10,12]. These results led to the notion that GPR55 is a novel cannabinoid receptor subtype, even though it is only 13.5% identical to CB\(_2\)-R and 14.4% to CB\(_2\)-R [7]. GPR55 is expressed in many mammalian tissues including several brain regions [6,7], however its physiological roles are largely unknown.

Here we show that GPR55 mRNA is expressed in both mouse primary microglia cells and in the murine microglial cell line BV-2, and confirm the presence of CB\(_2\)-R mRNAs in these cells. Moreover, we found that their pattern of regulation is very similar. In addition, we demonstrate that following IFN-\(\gamma\)-treatment, the effect of the GPR55 agonist, LPI, on ERK phosphorylation is increased. This is in correlation with the increased amount of GPR55 mRNA in BV-2 cells that we found. This differential modulation of CB\(_2\)-R and GPR55 expressions by various stimuli during BV-2 microglial activation suggests that similar to CB\(_2\)-R, GPR55 could be involved in modulating inflammatory signaling and that in turn BV-2 cell activation might be affected by both receptors. Indeed, it has been recently reported that GPR55 signaling influences the regulation of certain cytokines that contribute to hyperalgesia [17].

There is increasing evidence that the CB\(_2\)-R in microglia plays a functional role in CNS immunity and anti-inflammatory effects [24,27]. In agreement with previous reports, we show that various stimuli affect CB\(_2\)-R mRNA expression in microglia [22,26]. The fact that treatment of microglial cells with IFN-\(\gamma\) or LPS leads to different states of activation and to different properties of the activated cells agrees with the "in vitro model of multi-step activation". According to this model, microglial cells reach the primed state (antigen presentation) in response to IFN-\(\gamma\) treatment, which is followed by the fully activated state (cytotoxic stage) after exposure to LPS [22,27]. We found that contrary to the significant upregulation in the expression of CB\(_2\)-R and GPR55 in BV-2 cells, in primary microglial cells IFN-\(\gamma\) treatment leads to a small, but significant, downregulation of the mRNA levels for both receptors. This observation is similar to previous results on the regulation of CB\(_2\)-R mRNA by IFN-\(\gamma\) [22,26,28]. This difference between BV-2 and primary microglia could be due to the fact that the two preparations reflect different states of microglial activation. Microglial cells in primary cultures are intrinsically activated or "primed" because of the procedure involved in transferring these cells into culture [35] and IFN-\(\gamma\) increases the expression of CB\(_2\)-R only in combination with GM-CSF [22,26]. Whereas in BV-2 cells, which intrinsically exhibit high rates of proliferation, activation with IFN-\(\gamma\) is sufficient to increase the expression levels of both CB\(_2\)-R and GPR55 without the need of co-stimulation by the growth factor GM-CSF.

Our results show that CB\(_2\)-R mRNA in microglia cells is present at relatively low levels, and this independently of the states of cell activation. This result is in agreement with the finding that CB\(_2\)-R was not detected in the murine macrophage-like cell line RAW264.7 regardless of cell activation state and was found in neonatal rat microglia at very low levels [22,23].

In conclusion, our results show that GPR55 is expressed by microglia and suggest that this novel cannabinoid receptor may participate in regulating inflammatory responses depending on the microglial specific activation state.

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