Genomic and Functional Changes Induced by the Activation of the Peripheral Cannabinoid Receptor CB2 in the Promyelocytic Cells HL-60

POSSIBLE INVOLVEMENT OF THE CB2 RECEPTOR IN CELL DIFFERENTIATION*

Jean-Marie Derocq‡, Omar Jbilo‡, Monsif Bouaboula, Michel Ségui, Christophe Clère, and Pierre Casellas§

From Sanofi-Synthelabo, 34184 Montpellier Cedex 04, France

The function of the peripheral cannabinoid receptor (CB2), which is mainly expressed on hematopoietic cells, remains an enigma. In an attempt to decipher its role, we used AffymetrixTM DNA chips to investigate the gene expression profile of the promyelocytic cells HL-60 transfected with the CB2 receptor and activated with the cannabinoid agonist CP 55,940. Agonist exposure of these cells led to an activation of a mitogen-activated protein kinase cascade and a receptor desensitization, indicating a functional coupling of the transfected receptors. At the genomic level, activation of the CB2 receptors induced an up-regulation of nine genes involved in cytokine synthesis, regulation of transcription, and cell differentiation. A majority of them are under the control of the transcription factor NF-κB, whose nuclear translocation was demonstrated. Many features of the transcriptional events, reported here for the first time, appeared to be related to an activation of a cell differentiation program, suggesting that CB2 receptors could play a role in the initialization of cell maturation. Moreover, we showed that CB2-activated wild-type HL-60 cells developed properties usually found in host defense effector cells such as an enhanced release of chemotactic cytokines and an increased motility, characteristic of more mature cells of the granulocytic-monocytic lineage.

Two G-protein-coupled receptors with seven transmembrane domains have been identified as cannabinoid receptors and are referred to as CB1 and CB2 (1–4). The central receptor CB1 is predominantly expressed in the brain, whereas the peripheral receptor CB2 is mainly expressed on cells of immune origin (5–7). In addition to psychotropic effects mediated by the central receptor, many reports have described a cannabinoid-induced modulation of immune functions, which could be attributed to the peripheral receptor subtype. With the exception of few examples reporting stimulating effects (8–10), most of the studies showed immunosuppressive properties of cannabinoids in very different areas of the immunity, including humoral and cellular responses as well as cytokine production (11–13). However many of these studies were conducted in the micromolar range higher than that required for receptor binding and often without controlling the specificity of the observed effects. As high concentrations of cannabinoids may induce nonspecific membrane perturbations (14), the question of the receptor specificity of the widespread suppressive effects reported so far is raised. Therefore, despite the characterization of the CB2 receptors on the immune cells (6), the identification of second messenger systems (15, 16), the finding of putative endogenous ligands (17–19), and the availability of specific antagonist ligands (20, 21), the function of CB2 receptors in the immune system is still elusive.

In the present paper, to better define the complex involvement of this receptor in the immune functions, we used the recently developed DNA microarray technology to study the gene expression profile induced through the specific activation of the CB2 receptor. In a preceding study (22), we show that a stimulation of the CB2 receptor in HL-60 cells was able to induce the expression of the two chemokines IL-8 and MCP-1. In this study, using DNA microarrays allowing the differential evaluation of thousands of genes at the same time, we went a step further by providing a more complete gene expression profile specifically induced via the CB2 receptors in HL-60 cells. As a source of hematopoietic cells and myelomonocytic committed progenitors, these cells constitute a relevant model for the analysis of cell differentiation and the study of development of the immune effector functions. Moreover these cells offer the advantage of expressing the CB2 receptors only, excluding CB1 interferences. To compensate for the low density of CB2 receptors and to amplify the cannabinoid-induced effects, the receptor was overexpressed by transfection.

Using this approach, we demonstrated for the first time that nanomolar concentrations of the cannabinoid ligand CP 55,940 induced the expression of factors mainly involved in gene transcription and cell differentiation processes as well as in the production of cytokines. Many of the genes shown to be regulated via the CB2 receptor in HL-60 cells have not been previously described as associated with the cannabinoid process. This specific gene expression profile led us to undertake functional studies that corroborated some of the genomic events shown mediated through the CB2 receptors and suggested a role for this receptor in inducing mechanisms of the cell differentiation.

EXPERIMENTAL PROCEDURES

Reagents—The cannabinoid agonist CP 55,940 and the CB2 receptor antagonist SR 144528 were synthesized at the chemistry department of

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‡ These authors contributed equally to this work.
§ To whom correspondence should be addressed: Sanofi-Synthelabo, 371 rue du Prof. Joseph Blayac, 34184 Montpellier Cedex 04, France. Tel.: 33-467-10-62-90; Fax: 33-467-10-60-00; E-mail: pierre.casellas@sanofi-synthelabo.com.
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Sanofi-Synthelabo (Montpellier, France) (21). Anandamide and 2-arachidonyl glycerol were purchased from Sigma-Aldrich. The anti-phosphorylated MAP kinase rabbit antibody was purchased from New England Biolabs (Beverly, MA), and the antibodies to IκB-α and p65 and p50 NF-κB were from Santa-Cruz Biotechnology (Santa-Cruz, CA). The 4P anti-CB2 rabbit antibody was produced at Sanofi-Synthelabo (16).

Cell Culture and Treatment—Cell lines were grown at 37 °C in humidified 5% CO₂ in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal calf serum, 25 mM Hepes, 2.5 mM sodium pyruvate, and 20 μg/ml gentamycin. The human promyelocytic cell line HL-60 was purchased from ATCC (American Type Culture Collection). The stably transfected HL-60 cell line with CB2 receptors (CB2-HL-60) was produced as follows. Cells were transfected with the recombinant plasmid pCDNA3-CB2 by electroporation and then maintained in the presence of 600 μg/ml G418. Cells were screened 4 weeks later for CB2 receptor expression by flow cytometry using an anti-CB2 receptor polyclonal antibody (23). For RNA extraction, cells were grown in culture medium with 0.5% fetal calf serum (fetal calf serum) 24 h before treatment with 10 nM CP 55,940 or 200 nM SR 144528 for different time periods.

Biotinylated Probes Preparation and Hybridization on Microarray—Affymetrix huGene FL™ arrays (Santa Clara, CA) containing 8500 genes were used for mRNA expression profiling. Poly(A⁺) RNA was isolated from 10⁸ cells using the Fast Track 2.0 Kit (Invitrogen NV, Breda, the Netherlands). Double-stranded cDNA was prepared using Life Technologies superscript choice system and an oligo(dT)₂₄-anchored T₇ primer. Biotinylated RNA was synthesized using the T7 megascript system (Ambion, Inc. Texas) with biotin-11-CTP and biotin-16-UTP for 4 h at 37 °C. In vitro transcription products were purified using microspin S200 high-resolution columns (Amersham Pharmacia Biotech). Biotinylated RNA was then treated for 35 min at 94 °C in a buffer composed of 200 mM Tris acetate, pH 8.1, 500 mM potassium acetate, 2.5 mM sodium pyruvate, and 20 μg/ml gentamycin. The human promyelocytic cell line HL-60 was purchased from ATCC (American Type Culture Collection). The stably transfected HL-60 cell line with CB2 receptors (CB2-HL-60) was produced as follows. Cells were transfected with the recombinant plasmid pCDNA3-CB2 by electroporation and then maintained in the presence of 600 μg/ml G418. Cells were screened 4 weeks later for CB2 receptor expression by flow cytometry using an anti-CB2 receptor polyclonal antibody (23). For RNA extraction, cells were grown in culture medium with 0.5% fetal calf serum (fetal calf serum) 24 h before treatment with 10 nM CP 55,940 or 200 nM SR 144528 for different time periods.

Electrophoretic Mobility Shift Assay—A typical EMSA consists of two major steps: 1) the preparation of nuclear extracts from the cells, and 2) the analysis of DNA-protein complexes.

RESULTS

For migration assay, 5 × 10⁵ undifferentiated or differentiated wt HL-60 cells in 100 μl of the above-mentioned medium were added to the top chamber of a 6.5-mm diameter, 3-μm pore polycarbonate Transwell™ culture insert (Costar, Cambridge, MA) and incubated in duplicate for 4 h at 37 °C with 600 μl of the indicated concentrations of CP 55,940 located in the bottom chamber. The absolute number of migrated cells was determined by comparing the number of analyzed cells and beads with the amount of beads initially added in the bottom well. Basal migration was evaluated by scoring the number of cells that migrated in the absence of CP 55,940. Results expressed as migration index were calculated as the ratio between CP 55,940-induced migration and basal migration. RESULTS

Studies on the Receptor Functionality—The functional coupling of the CB2 receptor in transfected HL-60 was investigated by Western blots at two different levels. Phosphorylation of G-protein-coupled receptors is a key event in their dynamic regulation-desensitization after agonist exposure. Therefore, we first studied the phosphorylation status of the CB2 receptor by immunodetection with a phosphorylation state-specific antibody. Using the specific anti-human CB2 receptor antibody 4P, which only recognizes the non-phosphorylated form of the receptor at the cytoxyl-terminal region (16), we were able to show for the first time in HL-60 cells that stimulation of this receptor with 50 nM CP 55,940 led to a clear, time-dependent attenuation of the 4P signal, which was maximal after 15 min, indicating a strong agonist-induced phosphorylation of the receptor (Fig. 1, upper). Second, we showed that activated CB2

Cytokine Measurements and Cell Migration Assay—Undifferentiated CB2-transfected or wild type (wt) HL-60 cells were incubated at 37 °C in a 5% CO₂ atmosphere at 1 × 10⁵/ml with the indicated concentrations of CP 55,940 in RPMI medium supplemented with 0.5% fetal calf serum. The supernatants were collected and levels of IL-8, MCP-1, TNF-α, or MIP-1β were determined using enzyme-linked immunosorbent assay kits from R&D Systems (Abingdon, UK). Some similar experiments were also carried out on HL-60 cells differentiated by a 4-day treatment with 1.5% dimethyl sulfoxide (Me₂SO). When needed, the CB2 receptor antagonist SR 144528 was added to the cells 30 min before the addition of CP 55,940. The data shown are means of duplicate wells, and experiments were done at least twice. In some experiments results were expressed as % increase of chemokine production as follows.

% increase = (Cytokine in treated sample – cytokine in control) / Cytokine in control × 100

(Eq. 1)

For migration assay, 5 × 10⁵ undifferentiated or differentiated wt HL-60 cells in 100 μl of the above-mentioned medium were added to the top chamber of a 6.5-mm diameter, 3-μm pore polycarbonate Transwell™ culture insert (Costar, Cambridge, MA) and incubated in duplicate for 4 h at 37 °C with 600 μl of the indicated concentrations of CP 55,940 located in the bottom chamber. The absolute number of migrated cells was determined by comparing the number of analyzed cells and beads with the amount of beads initially added in the bottom well. Basal migration was evaluated by scoring the number of cells that migrated in the absence of CP 55,940. Results expressed as migration index were calculated as the ratio between CP 55,940-induced migration and basal migration.
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Gene expression changes were reproducibly observed in CB2-HL-60 cells and displayed greater than a 2-fold change in their expression. These genomic modulations were induced after stimulation of the CB2 receptor through the CB2 receptor superfamily. Its expression correlates with the lymphoid response and provides a strong basis to further investigate the cannabinoid-induced genomic modulation in this model.

**DNA Microarray Analysis**—The identification of the regulated genes following CB2 receptor stimulation was performed by a comparative analysis of the data obtained from experiments in which the olonucleotide array hybridized with the sample treated with 10 nM CP 55,940 for 1 h with those obtained from the control array. As shown in Table I, 10 genes were modulated after stimulation of the CB2 receptor and displayed greater than a 2-fold change in their expression. These genomic modulations were reproducibly observed in three different experiments.

The first group of genes shown to be up-regulated encodes inflammatory mediators and a related regulating factor. Three chemokines (MCP-1, IL-8, and MIP-1β) and one cytokine (TNF-α) genes were consistently up-regulated. Previously, in conditions similar to those reported here and using a cDNA expression array spotted with 588 different cDNAs, we had already reported an up-regulation of IL-8 and MCP-1 genes (22). Here, we showed that the gene expression of two other inflammatory cytokines was also enhanced, indicating that stimulation of CB2 receptors promotes HL-60 cells to produce effectors involved in the regulation of immune responses and inflammation. Furthermore, the TNF-α-responsive gene A20 was also shown to be up-regulated. It encodes a zinc finger protein whose expression protects cells from TNF-α cytotoxicity by interacting with TRAF2, one of the adapter proteins that associate with the cytoplasmic death domain of the TNF receptor superfamily. Its expression correlates with the lymphocyte activation and monocyte differentiation and is under the control of the transcription factor NF-κB (27, 28).

A second major set of strongly up-regulated genes (fold change >3) was the group of genes encoding proteins involved in the transcription machinery and cell cycling. The immediate early gene junB is a member of the transcription factor complex AP-1 (29). It plays, like other products of jun and fos genes, an important role in the transcription of genes involved in cell cycling and differentiation processes (30, 31). Aldolase C is one of the four glycolytic enzymes described so far with dual functions, the first being a role in the glycolytic pathway and the second related to DNA binding properties. Thus, aldolase C is reported to take part as a nuclear factor in the stabilization of DNA synthesis and be involved in the transcription of genes associated with cell growth and differentiation (32). BTG2 is an antiproliferative p53-dependent component of the DNA damage cellular pathway with a major role in growth control and differentiation (33, 34). The gene IκB-α (Mad-3) that displayed an almost 4-fold enhancement encodes a cytoplasmic protein that regulates the activity of the ubiquitous transcription factor NF-κB. Under unstimulated conditions, NF-κB is quiescent in the cytoplasm in combination with IκB and is activated for nuclear entry and DNA binding after dissociation from IκB as a response to different stimuli (35). The IκB-α modulation was confirmed at a protein level by Western blots (not shown), which revealed a biphasic regulation consisting of a degradation followed by a re-expression of the protein within 2 h of cannabinoid treatment. This latter phase, which requires de novo synthesis of IκB-α after the cytoplasmic degradation of the preformed protein, is consistent with the up-regulation of the corresponding gene mentioned above. The IκB modulation, demonstrated at both genomic and protein levels, was therefore a strong indication of an involvement of the NF-κB complex in the transduction machinery following CB2 receptor activation. This important notion was directly confirmed by an electrophoretic mobility shift assay (Fig. 2), which clearly showed in the nuclear extract of cannabinoid-treated cells an enhanced level of the retarded band of NF-κB. The specificity of the binding of NF-κB to its consensus DNA sequence was demonstrated by the supershift band generated with anti-NF-κB antibodies. Therefore, we demonstrated at different levels that NF-κB was involved in the transcriptional program elicited via CB2 receptor stimulation. Moreover, this finding was consistent with the observed up-regulation of genes whose transcription requires a mobilization of the NF-κB axis such as IκB itself, the A20 protein, and the cytokines mentioned above (36–39). Finally, metallothionein was the only gene shown to be down-regulated. It encodes a metal-binding protein involved in the defense against heavy metal-induced injury (40).

**Northern Blot Studies**—The accuracy and the specificity of the modulation in gene expression observed in the DNA chip analysis was confirmed by conventional Northern blot experiments performed with a subset of inducible genes. These included IκB-α (Mad-3), TNF-α, IL-8, MCP-1, and MIP-1β. Cells were incubated at different periods of time with 10 nM CP 55,940. As shown in Fig. 3, IκB, TNF-α, and MIP-1β transcription peaked at 1 h, whereas 3 h were required for a maximum expression of MCP-1 and IL-8 transcripts. When 200 nM CB2-antagonist SR 144528 was used at the maximum peak level

| Table I | Cannabinoid-induced modulation of genes in CB2-HL-60 |
|---------|------------------------------------------|
| Genes involved in cytokine production and regulation | M28130 | +2.3 |
| IL-8 | HDR4069 | +2.8 |
| MCP-1 | HT4339 | +2.8 |
| MIP-1β | M99293 | +4.7 |
| TNF-α | X02910 | +2.8 |
| A20 | M59465 | +2.2 |
| Genes involved in transcription process and cell cycling | X51345 | +3.5 |
| Jun B | X05196 | +3.4 |
| Aldolase C | U27649 | +3.5 |
| BTG2 | M69043 | +3.9 |
| Miscellaneous | Metallothionein | V00594 | −1.7 |

**Fig. 1.** Effect of CP 55,940-induced CB2 activation on receptor phosphorylation and MAP kinase activation. CB2-HL-60 cells were treated with 50 nM CP 55,940 for various time periods. After cell lysis, 50 μg of protein extract were analyzed in Western blots for monitoring the CB2 phosphorylation status (upper) using the 4P anti-CB2 antibody that recognizes only the non-phosphorylated form of the receptor and for evaluating MAP kinase (MAPK) phosphorylation (lower) using an anti-phosphorylated MAP kinase isofrom antibody.
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Fig. 2. CP 55,940-induced binding of NF-κB to its consensus sequence. Nuclear proteins from control or CP 55,940-treated CB2-HL-60 cells were analyzed for NF-κB DNA binding activity by electrophoretic mobility shift assay as described under “Experimental Procedures.” Lanes 1 and 2, nuclear extract from untreated CB2-HL-60 cells. Lane 3, nuclear extract from cells treated with 50 nM CP 55,940 for 45 min. Lanes 4 and 5, specificity binding controls: lane 4, mobility shift competition assay in the presence of a 30-fold excess of unlabeled probe; lane 5, binding reaction performed after a 30-min preincubation of nuclear extracts with 0.5 μg of anti-p65 and 0.5 μg of anti-p50 NF-κB rabbit antiserum.

Fig. 3. Validation of the DNA chip data by conventional Northern blots. For each of the indicated genes, Northern blots (left) are represented together with the corresponding probe set taken from the hybridized oligonucleotide microarrays (right). For Northern blots, 20 μg of total RNA were purified from CB2-HL-60 cells treated for 1, 3, and 6 h with 10 nM CP 55,940 or with solvent. The receptor specificity was evaluated for each gene at the corresponding maximum peak level (1 h for IL-8, TNF-α, and MIP-1β and 3 h for MCP-1 and IL-8) by pretreating the cells with 200 nM SR 144528 with or without 10 nM CP 55,940. Northern blots were then hybridized with the indicated cDNA probes. The corresponding probe sets shown in this figure were taken from the DNA chip experiments and correspond for each gene to the unstimulated (C) and to the CP 55,940-stimulated sample (CP). Each gene is represented by 20 oligonucleotide pairs. One member of each pair corresponds to a perfectly matched sequence. The other pair contains a single base mismatch.

concomitantly with CP 55,940, a blockade of the expression of all these transcripts was observed, clearly indicating a specific CB2 receptor-mediated process. The decrease in the basal level of the mRNA expression in the presence of SR 144528 alone confirmed the inverse agonist properties of this compound (41).

Effect of CB2 Receptor Activation on Cytokine Release and Cell Motility—A major set of genes shown to be remarkably enhanced in the DNA micro-array analysis was the group of genes encoding the chemotactic and inflammatory cytokines: IL-8, MCP-1, MIP-1β, and TNF-α. To determine whether this transcriptional up-regulation was also evidenced at the protein level, we evaluated the respective release of these mediators in the culture supernatants of HL-60 cells stimulated for 24 h with CP 55,940. Fig. 4 shows the concentration-dependent effect of CP 55,940 on the production of MIP-1β (upper) and TNF-α (lower) in culture supernatants of wt (A and C) and CB2 (B and D) HL-60 cells. A significant increase was already observed at 1 nM that reached a maximum between 10 and 100 nM for both cytokines in wt and CB2-HL-60, similar to what was previously observed for MCP-1 and IL-8 (22). Table II summarizes the effect induced by the optimal concentration of 100 nM CP 55,940 on the release of the 4 cytokines, which was increased by 50% and more in both wt and transfected cells. The CB2 receptor specificity of the effect on cytokine production was ascertained by the inhibition of the observed effect after co-treatment with the CB2 antagonist SR 144528, as exemplified for IL-8 in wt HL60 (Fig. 5A) or MCP-1 in CB2-HL60 (Fig. 5B). Contrary to the potent synthetic ligand CP 55,940, neither anandamide nor 2-arachidonyleglycerol, two putative endogenous ligands (17–19), were able to modulate the production of these cytokines (not shown). Our data clearly indicated that the cytokine gene transcription observed with the DNA chips was followed by an effective translation program and was, moreover, not restricted to the transfected cells. Since the range of CP 55,940-induced cytokine stimulation was very similar and specific in both CB2-transfected and wt HL-60 cells, these latter were used in the following experiments.

The cannabinoid-mediated up-regulation of inflammatory cytokines at both transcriptional and translational levels would likely indicate a maturational change. HL-60 undergoing maturation under the effects of differentiating agents displayed cytoskeletal rearrangements underlying cell migration (42, 43). We therefore evaluated the impact of a CB2 receptor stimulation on the motility of HL-60 cells using the Transwell™ migration chambers. As shown in Fig. 6A, the presence of CP 55,940 in the lower chamber induced a very significant increase in the migration of the undifferentiated HL-60 cells located in the upper compartment. This effect was optimal around 10 nM CP 55,940, with a migration index of 2.3, and followed a bell-shaped dose-effect curve. In the presence of the CB2 antagonist SR 144528, cell migration was totally abolished, clearly indicating a specific receptor-mediated process. When CP 55,940 was added in both chambers, a similar in-
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TABLE II

| Cytokines | wt HL-60 | CP-induced | Stimulation index | wt HL-60 | CP-induced | Stimulation index |
|-----------|----------|------------|-------------------|----------|------------|-------------------|
| Basal     |          |            |                   | Basal    |            |                   |
| IL-8      | 631 ± 21 | 950 ± 41   | 1.51              | 1978 ± 72| 3400 ± 115| 1.71              |
| MCP-1     | 302 ± 25 | 592 ± 45   | 1.82              | 1895 ± 102| 2850 ± 55 | 1.50              |
| MIP-1αβ   | 280 ± 11 | 460 ± 25   | 1.64              | 150 ± 8  | 310 ± 30   | 1.95              |
| TNF-α     | 5.3 ± 0.2| 8.1 ± 0.5  | 1.53              | 8.2 ± 0.6| 14 ± 1.3   | 1.71              |

FIG. 5. Receptor specificity of the CP 55,940-induced effect on chemokine production. The CP 55,940-induced modulation of IL-8 production in wt HL-60 (A) or MCP-1 production in CB2-HL-60 (B) was evaluated after a 24 h treatment with 100 nM CP 55,940 in the presence of the indicated concentrations of the CB2 antagonist SR 144528. The lower dashed line indicates the basal production, and the upper dashed line indicates the chemokine production induced by CP 55,940 alone.

FIG. 6. Effect of CP 55,940 on cell migration. A, wt HL-60 cells were preincubated 30 min at 37 °C with medium (○) or with 1 μM CB2-antagonist SR 144528 (△) and then added to the top chambers of a 3 μm polycarbonate Transwell™ membrane. Migration across the membrane was evaluated as indicated under “Experimental Procedures” after a 4-h incubation with the indicated concentrations of CP 55,940 located in the bottom chambers. B, distinction between chemotaxis and chemokinesis was performed by adding the same concentration of CP 55,940 (3 nM) in the upper and lower chambers.

crease in the cell migration was obtained (Fig. 6B), indicating an enhanced cell locomotion rather than a true directional migration and suggesting that CP 55,940 was rather chemokinetic than chemotactic. We next compared the effect of CP 55,940 on undifferentiated and MeSO4-differentiated HL-60 cells. Surprisingly, differentiated HL-60 cells became much less sensitive to the effect of CP 55,940. This was demonstrated by an abolition of the CP 55,940-induced MCP-1 production compared with the undifferentiated counterparts and a much lower impact on cell migration at both the range of migrated cells and the optimal concentration of CP 55,940 required (Fig. 7, A and B, respectively). A completely inverse pattern was observed on the chemotactic effect induced by C5a (Fig. 7C); the undifferentiated HL-60 cells that were unresponsive to C5a became highly sensitive to its chemotactic effect after differentiation. This is in line with the fact that chemotactic receptors such as C5a receptors are dramatically up-regulated in HL-60 during the maturation process induced by differentiating agents such as MeSO4 (42). Surprisingly, we observed an inverse phenomenon for the CB2 receptors since a dramatic decrease in CB2 receptor mRNA expression occurred during the course of the MeSO4-induced differentiation (Fig. 8). The loss of sensitivity of the differentiated cells to the cannabinoid agonist was therefore likely a consequence of the down-regulation of the expressed CB2 receptors.

DISCUSSION

Although some studies suggested that the CB2 receptor is specifically involved in the cannabinoid-induced immunomodulating effects (6–8, 21), many reports dealing with immunomodulatory effects of cannabinoids have little meaning since most of the data reported were obtained using ligand concentrations outside the physiological range and probably via receptor-independent pathways (44). Therefore, the exact function of this receptor is still a matter of conjecture. The recently described cDNA microarray or DNA chip technology allows expression monitoring of thousands of genes simultaneously and provides a framework for identifying genes as well as changes in their activity (45). This technology is particularly well suited for analyzing the gene expression profile induced by the activation of receptors whose function is still poorly understood, as is the case for the peripheral cannabinoid receptor CB2 expressed on immune cells.

Using this powerful tool, we investigated here the effect of nanomolar concentrations of a specific cannabinoid ligand on the transcriptional program induced in the promyelocytic cell line HL-60. This cell line, which lacks central CB1 receptors, made it possible to focus specifically on the peripheral receptor subtype CB2, which was further overexpressed by transfection in order to amplify an eventual cannabinoid-induced effect on gene modulation. In addition, this cell line, which is a derivative of committed progenitors of the granulocytic/monocytic lineage (46), also gave us the opportunity to study a possible influence on both cell differentiation and development of the immune functions.

In a previous study using a similar approach based on hybridization on arrays containing 588 human cDNAs, we already reported a specific CB2-mediated up-regulation of two genes encoding MCP-1 and IL-8 chemokines (22). In this report, using the recently developed microarrays spotted with several thousands of genes, we were able to perform a much more overall analysis of the modulated genes and to go deeper into the possible commitment of CB2 receptors on the immune functions. Our study showed that 10 genes were significantly and reproducibly modulated after activation of the CB2 receptor, and among them, 9 were up-regulated. Except the two chemokine genes mentioned above, none of the genes described here has already been reported as associated with CB2 receptor activation.

Several genes involved in cell differentiation or encoding
Normal HL-60 cells were induced to differentiate with CB2 mRNAs. The level of mRNAs was evaluated by reverse transcription-polymerase chain reaction and compared with that of the control, non-induced counterpart taken as 100%.

“markers” of cell maturation were strongly up-regulated such as junB, aldolase C, or the cytokines IL-8, MCP-1, MIP-1β, TNF-α, and the TNF-α-inducible protein A20. Most of these genes induced through activation of the CB2 receptor in the undifferentiated HL-60 cell line have been reported in the literature to be up-regulated during the differentiation process of myeloid cells. For instance, BTG2 is a major regulator of growth control and differentiation and a potent inhibitor of cell proliferation (33, 34); the expression of aldolase C as a DNA-binding protein is noticed only after exposure of HL-60 cells to differentiating inducers (32); the expression of the TNF-α-inducible protein A20 correlates with leukocyte activation and differentiation (27); the up-regulation of cytokine expression is observed during the process of differentiation in HL-60 cells (47).

In addition, we showed that the transduction signals following CB2 stimulation were in keeping with part of the events observed at the transcriptional level. Western blot studies clearly demonstrated that CB2 receptors, once activated, as demonstrated by their agonist-specific phosphorylation status, transduced the expression of activated isoforms of MAP kinases and the modulation of the IκB-α protein, a major cytoplasmic regulator of the NF-κB activity. We had already reported the major involvement of the MAP kinase network in gene induction associated with the stimulation of CB2 receptors (7). In this study, the finding of a mobilization of the NF-κB axis, observed either indirectly through the transcriptional and translational modulation of its regulator IκB or directly through the nuclear translocation of NF-κB, brings new insights in the signaling pathway and the possible functions of the peripheral cannabinoid receptor. The pleiotropic transcription factor NF-κB plays a major role in gene regulation during cell differentiation and immunoinflammatory reactions (48). NF-κB regulatory sequences have been found in promoters or enhancers of genes coding for pro-inflammatory cytokines such as TNF-α, IL-8, and MCP-1 (39). The mobilization of this pivotal transcription factor was therefore consistent with the expression of genes that encode the cytokines mentioned above and whose expression depends on the nuclear translocation of NF-κB after degradation of IκB. Regulation of NF-κB is complex, and multiple kinases are involved, but it is likely that the activation of the MAP kinase pathway observed here took part in the phosphorylation and subsequent degradation of IκB as already reported (49).

The transductional and transcriptional events observed in our model are summarized in Fig. 9, in which the central role of the NF-κB axis is highlighted by the fact that 70% of the genes shown to be up-regulated are under the control of this transcription factor. Moreover, most of them are reported in the
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literature to be associated with myeloid cell differentiation. This is the first time that an activation of a NF-κB axis is demonstrated following a specific CB2 receptor-mediated activation. This represents an important finding, considering the major role that NF-κB plays in the gene regulation during cell differentiation and immunoinflammatory reactions (48).

Taken together, the transcription program elicited by the activation of the peripheral CB receptor indicated a possible role for this receptor in the initialization of a cell maturation process. This notion was strengthened at a translational level, where the protein expression of the transcribed cytokines was effectively found also to be enhanced in the cell culture supernatants, evidence that activation via the CB2 receptor led to an enhanced expression of important effectors involved in immune and inflammatory responses. This major response was not restricted to the CB2-overexpressing cells but was also observed in normal HL-60 cells, ruling out artifactual phenomena due to transfection. Moreover, the receptor specificity of this response was demonstrated by the blockade of the induced effects by the CB2 receptor-antagonist SR 144528 (21). Intriguingly, neither anandamide nor 2-arachidonyl glycerol, two putative ligands of myelocytic cell lines undergoing differentiation (43), was effective in our model, suggesting that another high affinity CB2-restricted endogenous ligand mimicking the effect of the agonist CP 55,940 remains to be identified.

Other evidence of the initialisation of a differentiation process was the surprising CB2-mediated cell migration as a likely consequence of cytoketes reorganization, which is a feature of myelocytic cell lines undergoing differentiation (43). This is the first time that a cannabinoid ligand was shown to activate cell motility via a specific peripheral receptor-mediated process. The cell migration induced by the cannabinoid agonist CP 55,940 was a chemokinetic event and not a real directional migration evidenced by the genomic up-regulation of factors involved in cell differentiation, an enhanced synthesis of chemotactic proteins, and an increased locomotion, key events in leukocyte trafficking and the inflammatory response (52). These results suggest that CB2 receptors could induce conditions that facilitate the transition of HL-60 cells to a more mature monocyctic/granulocytic phenotype. More experiments are needed, however, in different cell lines at different stages of maturation to clarify the influence of CB2 receptors in cell differentiation and to measure their possible implications on the physiology of human hematopoietic progenitors.

Further findings obtained from models similar to the one depicted here that involve an exhaustive study of the gene expression profile of treated cells using DNA microarrays and a confirmation of the transcriptional events at different functional levels may be very useful to answer this important issue.

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